Modeling behavioral and neuropathological phenotypes of brain diseases using *in utero* electroporation

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Modeling behavioral and neuropathological phenotypes of brain diseases using *in utero* electroporation

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Sandra Sigrid Vomund

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1 Zusammenfassung

Die Analyse von Risikofaktoren sowie zugrundeliegenden Mechanismen von Gehirnerkrankungen beschränken sich hauptsächlich auf die Analyse von *post mortem* Gehirnen von Patienten sowie auf die Verwendung von Tiermodellen. Im Tier können humane Erkrankungen unter anderem durch Einbringen von Genmutationen oder durch Herunterregulieren der Expression von krankheitsassoziierten Genen modelliert werden. Diese Modelle spiegeln Aspekte der Pathologie von humanen Erkrankungen wieder und bestätigen damit die genetischen Grundlagen der jeweiligen Krankheit. Des Weiteren geben sie Aufschluss über zelluläre und molekulare Mechanismen von Krankheitsphänotypen. Die *in utero* Elektroporation ist eine Methode zur *in vivo* Manipulation von Genen in spezifischen Gehrinregionen. Diese Methode wurde in der vorliegenden kumulativen Arbeit verwendet, um Verhaltensänderungen und neuropathologische Phänotypen von Gehirnerkrankungen im Tiermodel zu generieren.

In der ersten Studie wurde zur Selektion des Elektroporationserfolges die *in vivo* Biolumineszenzbildgebung im Tier eingeführt. Hierfür wurde ein Luziferase-Gen zusammen mit dem Plasmid zur Überexpression oder Herunterregulierung der Expression des interessierenden Gens co-elektroporiert. Im geborenen Tier konnte dadurch die Lichtemission, die aus der Reaktion der Luziferase mit dem intraperitoneal injizierten Reaktionssubstrates Luziferin entsteht, zur Bestimmung des elektroporierten Areals im Gehirn genutzt werden. Des Weiteren konnte in dieser Studie gezeigt werden, dass eine Überexpression von „Disrupted-in-Schizophrenia 1 (DISC1) während der Gehirnentwicklung in der ausgewachsenen Ratte zu einer Hypersensitivität gegenüber Amphetamin führt. Diese Hypersensitivität kommt auch in Patienten mit Schizophrenie vor und deutet auf ein gestörtes dopaminerges System hin.

Um den Einfluss von zeitlich und örtlich begrenzten Neuroentwicklungsstörungen zu untersuchen wurde in der zweiten Studie die Genexpression eines für die neuronale Migration wichtigen Gens, das „*disabled-1*“ (*Dab1*) Gen, herunterreguliert und das Verhalten der adulten Ratte untersucht. Dafür wurde am Embryonaltag 16 eine „short hairpin RNA“ (shRNA) unilateral in den sich entwickelnden Kortex elektroporiert. Eine Woche nach der Geburt wurde mit Hilfe der Biolumineszenz-Bildgebung der Elektroporationserfolg in den Ratten untersucht. Daraufhin wurde im Erwachsenenalter in den erfolgreich elektroporierten Tieren Verhaltensänderungen analysiert. Die Untersuchung ergab, dass ein Migrationsdefizit

in maximal 50.000 Neuronen zu subtilen Verhaltensauffälligkeiten führt. Besonders auffällig war wiederum die Schizophrenie-assoziierte Amphetamin-Hypersensitivität.

Die in utero Elektroporation wurde bisher hauptsächlich zur Untersuchung von Neuroentwicklung und assoziierten Erkrankungen genutzt. Das Ziel der dritten Studie war daher ausgelegt zu testen, ob diese Technik auch zur Studie von neurodegenerativen Erkrankungen genutzt werden kann. Das Einbringen von Plasmid-DNA in eine begrenzte Anzahl von Neuronen könnte ein genetisches Mosaik imitieren. Ein genetisches Mosaik kann durch somatische Mutationen entstehen, welche momentan als potentielle Ursache der sporadischen Formen von neurodegenerativen Erkrankungen, wie Morbus Alzheimer, erachtet werden. Daher wurden Alzheimer-assoziierte Genmutationen in eine kleine Anzahl kortikaler Neurone von Mäusen eingebracht und ihre Expression über die Zeit beobachtet. In dieser Studie konnte gezeigt werden, dass noch in der gealterten Maus mit mindestens 1,5 Jahren die embryonal elektroporierten Gene exprimiert werden. Diese Tatsache führt zu der Vermutung, dass somatische Mutationen im Gehirn mittels *in utero* Elektroporation induziert werden können.

Zusammengenommen zeigt diese Arbeit, dass durch *in vivo* Genmanipulation im Gehirn Krankheits-assoziierte Neuropathologien sowie Verhaltensveränderungen im adulten Tier modellieren lassen. Damit eignet sich die *in utero* Elektoporation um die zugrundeliegenden Mechanismen von verschiedenen humanen Gehirnerkrankungen zu erforschen.

2 Abstract

The study of risk factors for disorders of the brain and of the underlying mechanisms of disease is mainly dependent on *post mortem* brain analysis and investigation of animal models. For this purpose, gene mutations or knockouts of candidate genes, have been introduced into animals to model human diseases. These models display aspects of human disease pathology, thereby providing confirmation of the genetic basis of a disease and helping to identify cellular and molecular mechanisms. *In utero* electroporation is a method of manipulating genes *in vivo* which is brain region specific. In this cumulative thesis, this technique was used to model behavioral and neuropathological phenotypes of brain illnesses.

In the first study, bioluminescence *in vivo* imaging was used to verify electroporation success in the born animal. Specifically, a luciferase containing plasmid was co-electroporated with the gene of interest and after intraperitoneal injection of the reaction substrate luciferin in the young animal, light emission was used to illustrate the electroporated area in the animal's brain. Furthermore, the study showed that overexpression of a mental illness associated protein, namely Disrupted-in-Schizophrenia 1 (DISC1), during brain development led to amphetamine hypersensitivity in the adult rat, which hinds toward a disturbed dopaminergic system and is also found in schizophrenic patients.

In the second study, *disabled-1* (*Dab1*), a gene important for neuronal migration was knocked down and the adult rat behavioral phenotype was investigated to determine the influence of time and spatial restricted neurodevelopmental disturbance on adult behavior. To this end, a short hairpin RNA (shRNA) was unilateral electroporated on embryonic day (E) 16 into the developing cortex. Rat pups were then selected for electroporation success at the age of 1 week and tested for behavior related to psychotic diseases upon reaching maturity. This study showed that migration deficits in up to 50000 neurons can lead to subtle behavioral deficits, and specifically again to schizophrenia related amphetamine hypersensitivity.

Thus far, *in utero* electroporation has mainly been used to study neurodevelopment associated illnesses. The aim of the third study was to establish whether this technique can also be used to study neurodegenerative diseases. The insertion of plasmid DNA in a small amount of neurons might mimic genetic mosaicism due to somatic mutations, which are considered to be a potential cause for sporadic forms of neurodegenerative diseases, such as Alzheimer's disease. The introduction of Alzheimer's disease associated mutant genes in

a subset of cortical cells and their expression up to old ages of mice was examined. It could be shown that the electroporated genes were still expressed at an age of 1.5 years in mice. Therefore, we hypothesize that *in utero* electroporation can be used as a technique to induce somatic mutations in the brain.

Together, this thesis shows that disease associated neuropathological as well as behavioral phenotypes in the adult animal can be modeled by *in vivo* gene manipulation in the brain. Therefore, *in utero* electroporation is a suitable method to study the underlying mechanisms of different human brain diseases.

3 Introduction

Worldwide, but especially in the more wealthy countries, disease burden of brain illnesses, meaning their impact on public health as measured by mortality, morbidity, and financial cost, is one of the highest when compared to other diseases (Olesen and Leonardi, 2003; Murray et al., 2012; Stein and Illes, 2015). This arises from the severe impairment of the well-being of affected people, their disability to learn and work and on their general health caused by negative symptoms, meaning the absence of thoughts, feelings, and behaviors that are normally present (Hyman, 2008).

Brain illnesses are manifold and differ for example in disease onset, severity, pathology and mortality rate. Brain diseases can be classified into different groups. This study focusses on the following disorders: 1) neurodegenerative diseases, such as Alzheimer's disease 2) neurodevelopmental diseases, such as lissencephaly and 3) chronic mental illnesses, such as schizophrenia, depressive disorder and bipolar disorder. For the first two groups, there are visible pathological changes in the brain, for example plaques and neuronal loss in Alzheimer's disease. In contrast to that, in chronic mental illnesses changes in brain anatomy are not obvious. Therefore, until today diagnosis for chronic mental illnesses depends on symptoms that are identified by clinical interviews. Single symptoms are not unique for one illness, so only a composition of different symptoms leads to a diagnosis.

The Diagnostic and Statistical Manual of Mental Disorders (DSM; current: edition 5) from the American Psychiatric Association (American Psychiatric Association, 2013) and the 5th chapter of the International Statistical Classification of Diseases and Related Health Problems (ICD; current: edition 10) published by the World Health Organization (WHO, 1992) are the most important classification and diagnostic systems for mental diseases. The DSM-5 defines a mental disorder as follows:

"A mental disorder is a syndrome characterized by clinically significant disturbance in an individual's cognition, emotion regulation, or behavior that reflects a dysfunction in the psychological, biological, or developmental processes underlying mental functioning. Mental disorders are usually associated with significant distress or disability in social, occupational, or other important activities." DSM-5 (American Psychiatric Association, 2013)

Neurodegenerative diseases, especially dementias, are often also diagnosed via clinical interview, because symptoms show up before the brain changes are visible with non-invasive imaging techniques.

It is important to understand the biological background of involved genes, pathways, and metabolism of mental disorders for two reasons: Firstly, to find objective biomarkers that support diagnosis to better distinguish between diseases and especially to detect them at an earlier time point in disease progression (Quinones and Kaddurah-Daouk, 2009). Because one major problem with brain illnesses still is the late diagnosis when patients already show severe symptoms, for example in Alzheimer's disease ("Alzheimer's Association," 2011). This prevents treatment of mental disorders in early stages when treatment would be particularly beneficial. Secondly, understanding the specific mechanisms underlying the symptoms would make it easier to generate drugs targeting or overcoming the disrupted pathway. Thus, for the neurodegenerative diseases, like Parkinson's and Alzheimer's disease, biological studies of pathological brain situations have led to identification of disturbed pathways, changed metabolism of specific proteins and identification of genes. This information is now used to develop drugs and therapies to at least reduce the disease symptoms, like L-DOPA administration and deep brain stimulation for Parkinson patients (Lloyd et al., 1975; Weaver et al., 2009).

Although the exact underlying mechanisms are not known yet, it is well established that most mental illnesses either arise from neurodevelopmental disturbances or have a neurodegenerative character. In the following chapters neurodevelopment and associated diseases, chronic mental illnesses, neurodegenerative diseases, as well as the possibilities to study brain illnesses in general are introduced.

3.1 Neurodevelopment and associated disorders

3.1.1 Brain development

Due to its complexity, the brain needs a long time to develop. The brain mainly develops until late adolescence (Insel, 2010). Thereafter it is still flexible for connection changes due to learning and memory formation, although the main number of neurons is already placed at the time of birth. Only in specific areas of the brain, such as the dentate gyrus of the hippocampus, progenitor cells still exist in adults (Altman and Das, 1965). Therefore, damage and loss of neurons in the central nervous system (CNS) is irreparable and can result in neurodegenerative diseases (see chapter 3.3).

Prenatal development of the brain involves mainly cell proliferation and migration.

All CNS neurons arise from progenitor cells of the neural tube, which enlarges on the rostral end to build the three primary vesicles of the brain. The cell layer that encloses the ventricles (ventricular zone) and the adjacent layer (subventricular zone) are the areas of brain cell proliferation.

Proliferation of progenitor cells (radial glia) in the early neural tube is mainly symmetric to produce more progenitor cells during early development, while later on more and more asymmetric cell divisions lead to one progenitor and one postmitotic neuron or glia apiece (Figure *3.1* A, step 1). The postmitotic cells need to differentiate further, this happens outside of the ventricular zone in the marginal zone.

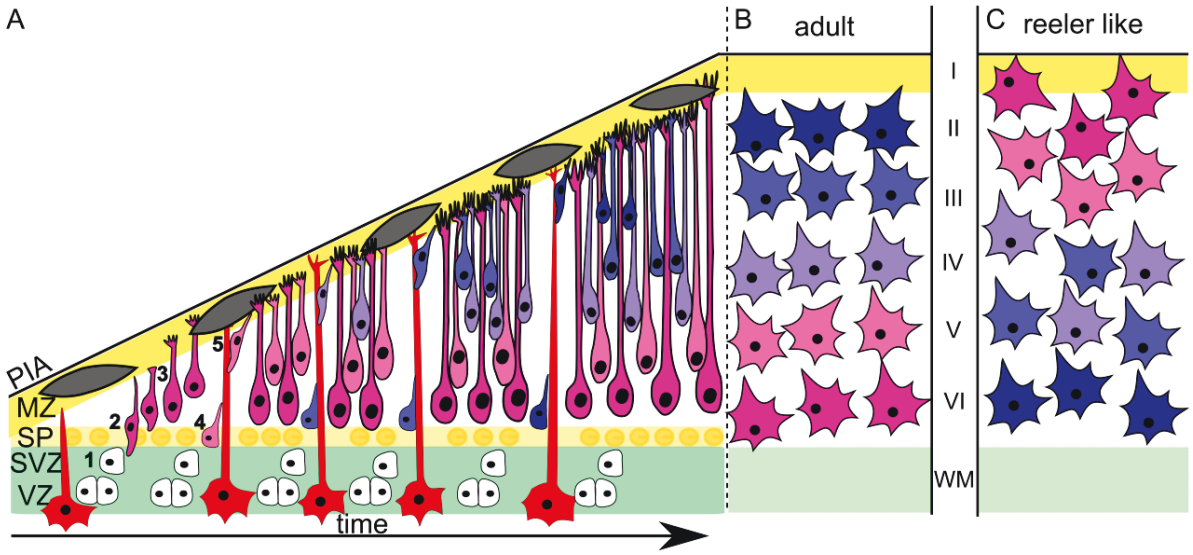


Figure 3.1 Timeline and mechanism of cortical plate development and layering in adult wildtype and reeler like cortex

A) Formation of the cortical plate during late prenatal development showing proliferation (1), somal translocation (2,5), anchoring and branching (3) and glia-guided locomotion (4) of neurons.

B) Cortical layering in the healthy adult brain. C) Cortical layering in diseased adult brain with reeler like phenotype.

Abbreviations: MZ = marginal zone; PIA = pia mater; SP = subplate; SVZ = subventricular zone; VZ = ventricular zone; WM = white matter

In *Drosophila* and nematodes it has been shown, that each nerve cell has a defined position (Sulston et al., 1983; Klämbt et al., 1991), that is reached by directed migration to build up the nervous system. Also, in higher vertebrates and mammals, positioning of neurons results from coordinated migration.

Migration of postmitotic cells from the ventricular zone to their final destination in mammals is best explained by the development of the cerebral cortex, because the cortex architecture is composed of six specific layers which are built up one after another.

First the preplate is built which is split into the marginal zone and the subplate. In the rat the marginal zone emerges at embryonic day 14 (E14) and contains large horizontal cells called Cajal-Retzius cells (Bayer and Altman, 1990). Later on, it is called layer I. The subplate is a deep layer also called layer VIb or VII and disappears in adult stages. Within these two layers, the cortical plate is built. In a first step the earliest born cortical plate neurons split the preplate by migration through somal translocation. Somal translocation is a fast motion of the cells in which the leading process extends into the marginal zone, branches and appears to be anchored there. Thereupon the leading process shortens again, so that the cell soma moves rapidly upwards (Figure *3.1* A, steps 2-3). Later born cortical neurons migrate via glia dependent locomotion (Figure *3.1* A, step 4). This is a saltatory movement of repeated cycles of attachment to the glia fiber, extension of the leading process, forward movement of the nucleus, and retraction of the rear. The moving cell passes by its predecessors and as soon as it reaches the marginal zone, it detaches from the glia and finds its final destination via somal translocation (Figure *3.1* A, step 5) (Valiente and Marin, 2010).

Therefore, the neocortex shows a typical ‘inside-out’ layering, with firstborn neurons in the deepest layer and later born neurons more superficial is seen in the healthy adult brain (Figure 3.1 B). In the cortex with disturbed migration signaling pathway new born neurons are not able to pass by their predecessors so that the oldest cells are found in layer II/III, sometimes also invading layer I, and younger cells are sticking in the underneath layers as seen in the reeler like phenotype in Figure 3.1 C. A key pathway for neuronal migration seems to be the Reelin – Dab1 pathway (Jossin et al., 2003).

3.1.2 Reelin - Dab1 pathway

Reelin is an extracellular glycoprotein that is released during brain development mainly from Cajal-Retzius cells in the marginal zones of the neocortex and the hippocampus. Reelin has been found nonfunctionally mutated in the reeler mouse. This mouse was named because of its reeling ataxia of gait which was first described by Falconer as a spontaneous mutation in inbred mice siblings (Falconer, 1951). Later studies on these mice showed that homozygous mice exhibit a severely disrupted lamination of the brain, for example in the neocortex, where the usual ‘inside-out’ layering of the cortical plate is altered (Hamburgh, 1963; Caviness and Sidman, 1973). In the reeler cortex first born neurons are situated more superficially than

their succeeding cells, whereas in the normal brain the later born neurons form the upper cortical layers (Figure 3.1 B+C).

Frotscher (Frotscher, 1997) postulated that the secreted Reelin from the Cajal-Retzius cells provides a stop signal for migrating neurons, which then start to differentiate and build up dendrites that lead to the marginal zone. Therefore, in between the earlier born cells and the marginal zone some space is occurring in which the later neurons are able to migrate until they are also stopped by the Reelin signal and start to differentiate. The missing stop signal in

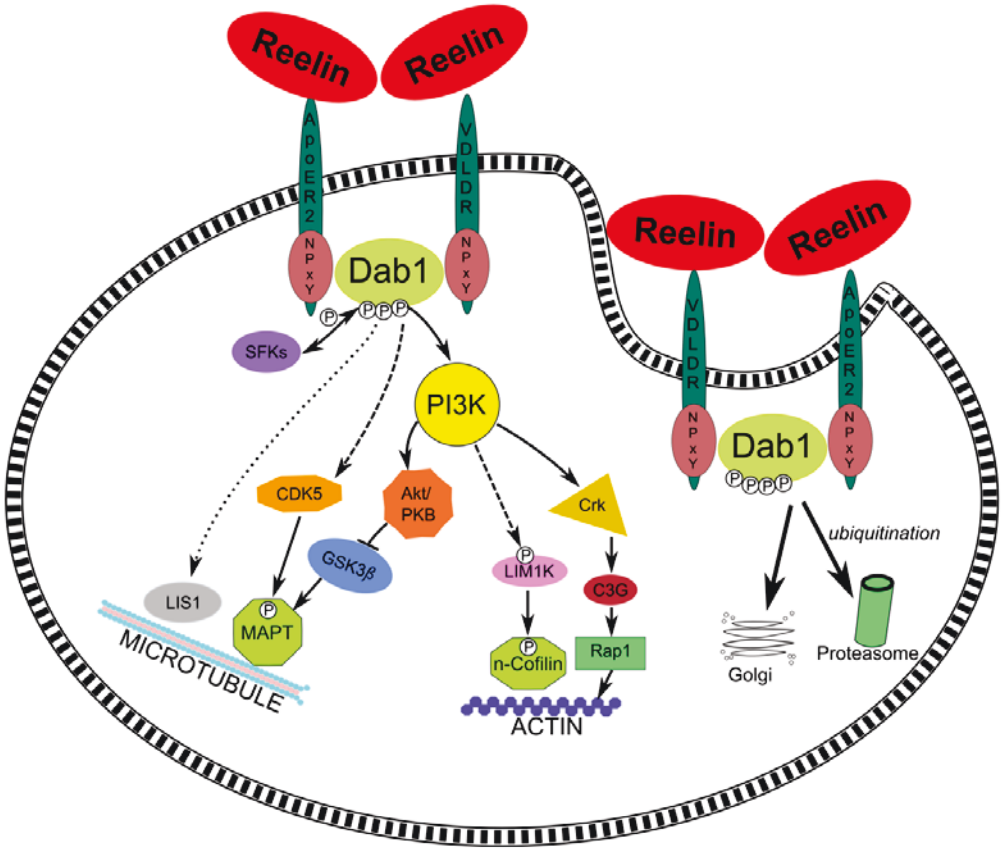


Figure 3.2 Reelin-Dab1 pathway

Overview of the main intracellular signaling cascades induced by reelin. Reelin binds to VDLDR or ApoER2 receptors. Then the intracellular adapter protein Dab1 is phosphorylated by Src family kineases and induces reactions at the microtubule, actin filament or lead to internalization and degradation of the protein complex via the shown pathways.

Abbreviations: Akt/PKB = serine/threonine-protein kinase; C3G = guanine nucleotide-releasing protein; ApoER2 = Apolipoprotein-E Receptor Type 2; CDK5 = Cell division protein kinase 5; Crk = proto-oncogene c-Crk or p38; Dab1 = Disabled-1; GSK3β = Glycogen synthase kinase 3 β; LIM1K = LIM (Lin11, Islet-1 and Mec-3) domain kinase 1; LIS1 = lissencephaly 1; MAPT = microtubule associated protein tau; PI3K = Phosphoinositid-3-Kinase; Rap1 = Ras-proximate-1; SFKs = Src family kineases; VDLDR = Very Low Density Lipoprotein Receptor

the Reelin deficient reeler mice leads to a complete migration of early born cells towards the towards the marginal zone/preplate, where they stuck so that the later arriving neurons are not able to pass by. Moreover, in the reeler brain the cortical plate does not split the preplate into the marginal zone and the subplate. Instead, the cortical plate cells accumulate underneath the whole preplate and invade the preplate just partially (Caviness, 1982; Sheppard and Pearlman, 1997). Meanwhile it has been shown, that Reelin not only functions as a stop signal, but also acts in the detaching of neurons from glia guidance ("detach and stop" model) and also promotes movement ("detach and go" model) (Cooper, 2008). In 2010 Förster (Förster et al., 2010) hypothesized, that the low concentration of Reelin in the subventricular zone first attracts the neurons to migrate by glia dependent locomotion, after reaching of the marginal zone with its high Reelin concentration detaching of neurons from the glia cells is induced. Then the cells start to reach their final destination by somal translocation.

The mechanism with which Reelin alters the behavior of the migrating cells starts with the binding of Reelin to receptors of the migrating neurons, namely Very Low Density Lipoprotein Receptor (VLDLR) and Apolipoprotein-E Receptor Type 2 (ApoER2) (Hiesberger et al., 1999). Mice lacking both of these receptors display the same phenotype as reeler mice (Hiesberger, et al., 1999). The binding of Reelin to VDLDR and ApoER2 leads to membrane recruitment of the intracellular adapter protein Disabled-1 (Dab1) which interacts by its phosphotyrosine binding (PTB) domain with the NPXY sequences of the receptors and other membrane bound proteins. Dab1 is tyrosyl phosphorylated by Src family kineases (SFKs), such as Fyn and Src, first on the tyrosins (Tyr) 185 and 198. After transphosphorylation on Tyr 220 and Tyr 232 the recruitment and activation of non-receptor kinases induces cytosolic kinase cascades which in the end lead to alteration of microtubule-stability (Beffert et al., 2002; Beffert et al., 2004), regulation of actin cytoskeleton (Chai et al., 2009), internalization/ endocytosis of the entire Reelin-signaling complex, and ubiquitination and degradation of phosphorylated Dab1 (Bock et al., 2004; Suetsugu et al., 2004; Morimura et al., 2005). See Figure *3.2* for an overview of the single steps of the pathway. Not all these different intracellular signaling pathways happen at the same time or in the same membrane compartment. Different protein expression patterns (Hack et al., 2007) and different splice variants, e.g. for *Dab1*, have been shown to act on different parts or at different time points of the pathway (Gao and Godbout, 2013).

All these ways and actions implicate different points where dysfunction of the pathway interferes with proper migration, but also show that the Reelin/Dab1 signaling pathway has a role apart from controlling the layering structures of the brain, for example in synaptic

plasticity (Weeber et al., 2002), proper neurite outgrowth (Chameau et al., 2009; Hoe et al., 2009), and in dendrite and spine formation (Jossin and Goffinet, 2007; Niu et al., 2008). These functions are mainly occurring in the postnatal brain, including the adult.

3.1.3 Neurodevelopmental disorders

As the name implies neurodevelopmental disorders are illnesses in which the development of the central nervous system is disrupted. A very serious neurodevelopmental illness is lissencephaly. This general term literally meaning 'smooth brain' encodes for neuronal migration diseases of different severities, phenotypes, pathologies and genetic backgrounds (Dobyns and Truwit, 1995; Forman et al., 2005). Patients suffering from this disease show significant developmental delays, mental retardation and behavior problems and have seizures starting to occur often in the first year of life. Most lissencephaly patients die during childhood due to seizures or aspiration and respiratory disease. The brain malformation is characterized by the absence or reduction of gyri, diminished cerebral white matter, widened cortical band, enlarged ventricles and subcortical band heterotopia (SBH), that can be diagnosed by ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI). Most often mutations in one of the genes 'Platelet-activating factor acetylhydrolase IB subunit alpha' (*PAFAH1B1,* often referred to as *LIS1*), doublecortin (*DCX)*, *Reelin*, and 'Aristaless related homeobox' (*ARX*) is the cause of lissencephaly (Forman, et al., 2005).

Other, less lethal illnesses with neurodevelopmental disturbances can have a genetic background, such as trisomy 21 (Down syndrome) or might be due to alcohol or other drug consumption during pregnancy, like Fetal Alcohol Spectrum disorders. Patients suffering from these illnesses have longer live expectancies than lissencephaly patients and reach adulthood. Nevertheless, they have serious mental retardation, often with a low I.Q., and learning problems (Määttä et al., 2006; Williams et al., 2015).

3.2 Chronic mental illnesses

The term chronic mental illnesses are mainly used to describe long lasting and recurring psychotic disorders, such as schizophrenia (SCZ), or the recurrent affective disorders (depression, bipolar) (Bachrach, 1988).

Unfortunately, the diagnosis of chronic mental illnesses is mostly dependent on clinical interviews and are based on the identification of clusters of symptoms and scales due to the absence of objective biological markers (Quinones and Kaddurah-Daouk, 2009; Korth, 2012).

This is because the underlying mechanisms of higher brain functions, which are disturbed in mental diseases, are still not completely understood. The reasons for that are manifold. For example, studying of the human brain is limited to non-invasive or *post mortem* methods due to ethical and practical reasons. But also because although a lot of genes and risk factors have been identified for different illnesses their composition remains rather complex. In one mental illness often a whole system of gene regulating networks, proteins and metabolic alternations seems to be dysregulated (Ramocki and Zoghbi, 2008; Cristino et al., 2014). In addition, some genetic risk factors seem to play a role in different illnesses (e.g. *Disrupted-in-schizophrenia 1*; (Hennah et al., 2006)). Another reason for the slow progress in disease understanding is that animal models for reproduction of human cognitive, social, and emotional function or dysfunction are reductionist models that do not reflect the whole spectrum of the human disorder (Hyman, 2008; Quinones and Kaddurah-Daouk, 2009).

In most cases these illnesses manifests early in life during childhood, like autism spectrum disorder (ASD) or attention deficit hyperactivity disorder (ADHD). But there are also diseases, such as schizophrenia, that have onset, respectively start showing symptoms during young adulthood (with the age of 20 – 30 years), although the trigger seems to be during brain development (Murray and Lewis, 1987; Weinberger, 1987). Most of these diseases show high heritability (see Table *3.1*) even though affected individuals harbor different risk alleles in a heterogeneous genetic background (Cristino, et al., 2014).

Table 3.1 Overview of mental illnesses with supposed neurodevelopmental disturbance showing prevalence, disease onset and heritability

disease	prevalence in population	approximate disease onset	twin study heritability	references
autism spectrum disorder	1 %	~ 1 -2 years	~ 90 %	(Bailey et al., 1995)
attention deficit hyperactivity disorder	5.9-7.1 % in children/ adolescents	~ 7 years	75-91°%	(Levy et al., 1997; Willcutt, 2012)
schizophrenia	~0.6 %	16 – 30 years	~ 50°%	(Gottesman and Erlenmeyer-Kimling, 2001; Saha et al., 2005)
major depression	11.1 – 14.6°%	20 – 30 years	31 - 42°%	(Sullivan et al., 2000; Kessler and Bromet, 2013)
mood disorder	~4.4°% (US population)	~ 20 years	59 – 87°%	(Smoller and Finn, 2003; Merikangas et al., 2007)

3.2.1 Schizophrenia

Schizophrenia, is a disabling mental illness in young adults characterized by positive symptoms like hallucinations and delusions, negative symptoms like anxiety, low mood, and social withdrawal, and cognitive impairment still needs to be diagnosed by clinical interview. Although schizophrenia has been described more than 100 years ago by the psychiatrist Emil Kraepelin as “dementia praecox” (Kraepelin, 1899) most treatments mainly target the psychotic part of the disease and all present anti psychotics essentially use the same mechanism as that for drugs discovered in the 1950s (Howes et al., 2009). At least, in the last decades, progress in understanding the underlying mechanisms of symptoms could be achieved. Disturbed dopamine metabolism seems to be the major cause of the psychotic part of schizophrenia (dopamine hypothesis reviewed in (Howes and Kapur, 2009)), but the dopamine dependent reward circuitry of the brain also plays a role in other psychotic and neurodevelopmental diseases, such as obsessive-compulsive disorder (OCD), ADHD, autism, Tourette’s syndrome (TS) and many more (Dichter et al., 2012).

In 1987 the neurodevelopmental hypothesis of schizophrenia was postulated based on associations between SCZ patients and pre- and perinatal complications, markers of developmental deviance in children which later developed schizophrenia, as well as small structural abnormalities in brain imaging studies like enlarged ventricles, without displaying any *post mortem* neurodegeneration (Murray and Lewis, 1987; Weinberger, 1987). This hypothesis indicates that exposure to hazards or viral infections during the prenatal phase leads to disturbances of neurodevelopment through interference of for example cytokines on developmental processes (Vuillermot et al., 2010). Along with that, the high heritability of SCZ of ~ 40- 80 % indicates that genetic risk factors are associated with neurodevelopmental processes, for example *Neuregulin-1* (*NRG1*) or *Disrupted-in-schizophrenia 1* (*DISC1*) (Cannon et al., 1998; Jaaro-Peled et al., 2009).

DISC1 was first described in a Scottish pedigree, in which 37 members carry a balanced chromosome translocation (t(1;11) (q42.1;q14.3)), leading to the disruption of the *DISC1* gene in intron 8 and a non-coding gene on the antisense strand named *DISC2*. Out of these 18 were diagnosed with mental illnesses (seven schizophrenics, one bipolar and ten people with major recurrent depression) (St Clair et al., 1990; Millar et al., 2000; Blackwood et al., 2001). An independent American pedigree, associated to mental illnesses, also has a C-terminal but much shorter mutation and deletion in DISC1 (Sachs et al., 2005). Induced pluripotent stem cells (iPSCs) received from members of this family have recently been

produced and may provide a new opportunity to study disease mechanism and to pursue the discovery of drugs (Wen et al., 2016).

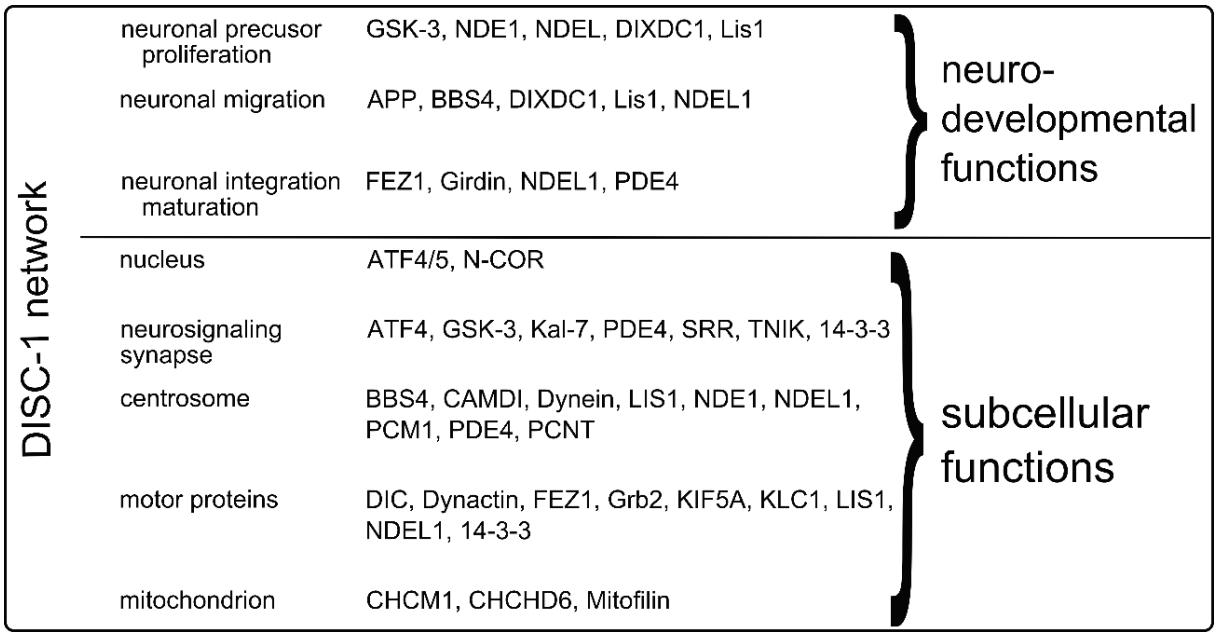


Figure 3.3 Selective DISC1 interactome

Selection of DISC1 interaction partners out of the 158 protein-protein network with functional grouping (Lipina and Roder, 2014). DISC1 and its interaction partners have functions in neurodevelopment, including proliferation, migration and maturation of neurons, as well as subcellular function within the nucleus, at the centrosome, at the synapse, at the cellular motor complex, and at the mitochondria. *Abbreviations*: 14-3-3: proteins of the 14-3-3 family, APP = amyloid precursor protein; ATF4/5 = activating transcription factor4/5; BBS4 = Bardet-Biedl syndrome 4; CAMDI = coiled-coil protein associated with myosin II and DISC1; CHCM1 = coiled-coil helix cristae morphology 1; CHCHD6 = coiled-coil-helix-coiled-coil-helix domain containing 6; DIC = dicarboxylate ion carrier; DIXDC1 = DIX domain containing 1; FEZ1 = fasciculation and elongation protein zeta 1; Grb2 = growth factor receptor-bound protein 2; GSK-3 = glycogen synthase kinase 3; KIF5A = kinesin family member 5A; KLC1 = kinesin light chain 1; LIS1(PAFAH1B1) = lissencephaly-1; NDE1 = nudE nuclear distribution E homolog 1; NDEL1 = nudE nuclear distribution E homolog 1-like 1; PDE4 = phosphodiesterase 4B or 4D; N-CoR = nuclear receptor co-repressor; Kal-7 = kalirin 7; TNIK = TRAF2 and NCK interacting kinase; SRR = serine racemase; PCM1 = pericentriolar material 1; PCNT = pericentrin

The importance of the DISC1 protein in neurodevelopment is due to its influence on neuronal proliferation, migration as well as on intracellular signaling pathways (Kamiya et al., 2005; Mao et al., 2009; Carlyle et al., 2011; Ishizuka et al., 2011). Neuronal migration and axonal formation are regulated by the microtubule-based dynein motor complex in which DISC1 acts as a key component (Kamiya, et al., 2005). Ishizuka and colleagues claimed that by posttranslational modification of the protein, DISC1 acts as a switch between cell proliferation

and neuronal migration, the key components of cortical development (Ishizuka, et al., 2011). They showed that phosphorylation at residue S710 increases binding of dynein motor-related proteins to the centrosome, like Bardet–Biedl syndrome proteins (BBS1 and 4). This binding might titrate DISC1 away from proteins of the canonical Wnt pathway, which is a key regulator of progenitor cell proliferations. However, DISC1 seems to have multiple functions during development and adulthood as indicated by its multiple interaction partners called 'DISC1 interactome' (Camargo et al., 2007) (Figure *3.3*).

Another function of DISC1 linking it to schizophrenia phenotypes, is the involvement in the regulation of the dopaminergic system. Several studies including cellular and animal models found interaction of DISC1 with dopamine function. Especially an amphetamine sensitization, which is also observed in SCZ and is considered as a proof for a disturbed dopaminergic system, has been reported (Lipina et al., 2010; Niwa et al., 2010; Jaaro-Peled et al., 2013; Trossbach et al., 2016). A study on *post mortem* brain tissue of SCZ patients as well as in a genetically altered (tg) Disc1 mouse line showed formation of dopamine 2 receptor (D2R) - DISC1 complex which supports GSK-3 signaling and inhibits D2R internalization (Su et al., 2014). At the moment D2Rs are the primary target of antipsychotic drugs, which have serious side effects, including extrapyramidal symptoms (EPS), weight gain, and a sedating effect (Leucht et al., 2009). Therefore, the identification of this mechanism could improve the production of antipsychotic drugs.

Recently, Troßbach, Bader and colleagues revealed significant functions of DISC1 in D2R affinity and dopamine transporter (DAT) activity, which are two major regulators of dopamine homeostasis (Trossbach, et al., 2016). They demonstrated a reciprocal relationship of DISC1 misassembly and dopamine homeostasis. Their nonmutant human DISC1 overexpressing rat model showed amphetamine supersensitivity as well as hyperexploratory behavior and rotarod deficits, which can be linked to alternations in the striatal dopaminergic system. Another DISC1 animal model also show impaired behaviors, e.g. in prepulse inhibition (PPI) and working memory tasks (Niwa, et al., 2010) (see section 3.4). The pathology of these mice, generated by cortical knock down of DISC1 via *in utero* electroporation (see 3.4.2), also showed decreased neurite outgrowth and disturbed neuronal migration. This again hints towards a neurodevelopmental origin of DISC1 related disorders, so called DISC1opathies such as SCZ (Korth, 2009; Korth, 2012).

3.3 Neurodegenerative disorders

Neurodegenerative disorders are characterized by loss of function, atrophy and cell death of cells in the mature nervous system. A common feature in neurodegenerative diseases is the aggregation and deposition of proteins, both intra- and extracellular. Often these protein assemblies occur as a result of misfolded proteins (Taylor et al., 2002). This is the case for example with Prion protein, that misfolds and aggregates in Prion diseases like Creutzfeldt-Jakob disease (Prusiner, 1991). In Parkinson's disease, α-synuclein forms lewy bodies (Polymeropoulos et al., 1997) and in Alzheimer's disease extracellular plaques of aggregated Aβ protein (Glenner and Wong, 1984) and intracellular tau-tangles are found (Grundke-Iqbal et al., 1986).

Neurodegeneration most often results in problems with movement (ataxia), mental function (dementia), or both. Dementias are accountable for the greatest burden of disease, and an estimated number of 46.8 million people worldwide are living with dementia in 2015 (World Alzheimer Report, 2015). The World Alzheimer Report also presented that the prevalence of dementia increases exponentially with age, approximately doubling the risk with every 5-7 years (depending on the region). With an increase of age expectation and no known cure present, they estimated an increase in dementia to affect 131.5 million people in 2050. Although most of the illness cases begin at an age of 60 years and older, there are younger patients. These are most often familiar, inherited cases with known gene mutations unlike the mostly sporadic occurring age dependent dementias. Fortunately, younger onset dementia is a rare condition, accounting only for approximately 2-8 % of all cases (Dementia: a public health priority, 2012).

To date there is no cure for neurodegenerative diseases. One of the main problems for treatment is that at the time point of diagnosis, when patients exhibit first symptoms, already 50 – 70 % cell loss of neurons in specific areas has occurred, for example nigral neurons in Parkinson's disease, or layer II neurons of the entorhinal cortex in the mildest clinically detectable dementia (Fearnley and Lees, 1991; Gomez-Isla et al., 1996). Unfortunately, dead or dying neurons are not replaceable due to network complexity that was built up during development. At least there have been efforts to decelerate disease progression and improve disease symptoms. For example, in Parkinson's disease the administration of substances of the dopaminergic pathway (L-DOPA) as well as deep brain stimulation with an implanted neurostimulator are used to improve the symptoms (Bronstein et al., 2011; Kakkar and Dahiya, 2015). In hope to repair neuronal loss, transplantations of stem cells in different diseases, like stroke, spinal cord injury, amyotrophic lateral sclerosis, neuronal ceroid

lipofuscinosis, and Parkinson's disease, are currently studied in clinical studies of phase 1-2 (Akesson and Sundström, 2015).

Although these treatments alleviate symptoms, they mostly have side effects (like dyskinesia for L-DOPA treatment in Parkinson's disease) or lose their symptom rescue ability over time. Therefore, studying the exact mechanisms of the disease to find biomarkers for earlier diagnosis and to develop disease specific targets for treatment is still necessary.

3.3.1 Alzheimer's disease

Alzheimer's disease, the most common form of dementia, got its name from Emil Kraeplin in 1910 due to a description of a patient by Alois Alzheimer in 1907 (Alzheimer, 1907; Alzheimer et al., 1995). With the age of 51 years this patient showed severe change of personality, disorientation to time and place, and confusion. Until she died five years later her symptoms worsened until she was totally apathetic. The examination of her brain revealed fibrils (= tau-tangles) and miliary foci (= amyloid plaques) as well as a massive loss of neurons. At the end of 19th to the beginning of 20th century, tangles and plaques were reported from different groups in epileptic and senile dementia patients (for review see (Berrios, 1990; Cipriani et al., 2011). It took until the end of 20th century to reveal the molecular basis of these aggregates (Glenner and Wong, 1984; Grundke-Iqbal, et al., 1986), but although a lot is known by now, even until today, the toxic species leading to cell death are discussed. Amyloid plaques mainly consist of amyloid β peptides (Aβ). These peptides are degradation products of the amyloid precursor protein (APP).

APP is a type-I-transmembrane protein protein which spans the membrane once near the intracellular C-terminus and exhibits a large extracellular N-terminal domain (Kang et al., 1987). This extracellular domain has functions in cell-cell and synaptic adhesion and has been shown to interact with several extracellular matrix components such as laminin, collagen, heparin, and reelin (Kibbey et al., 1993; Beher et al., 1996; Clarris et al., 1997; Hoe, et al., 2009). These interactions of APP with the extracellular matrix have effects on cell movement. APP's localization at the growth cone (Sabo et al., 2003) and results from *in vivo* knock-out and knock-down experiments suggest roles in neurite outgrowth and neuronal migration (Herms et al., 2004; Young-Pearse et al., 2007; Hoe, et al., 2009). The mechanism behind this is not completely understood, but colocalization of APP with integrins, interactions with the intracellular signaling via Dab1 / Abl (=Abelson murine leukemia viral oncogene homolog 1), which is involved in actin polymerization, and the interaction with FE65

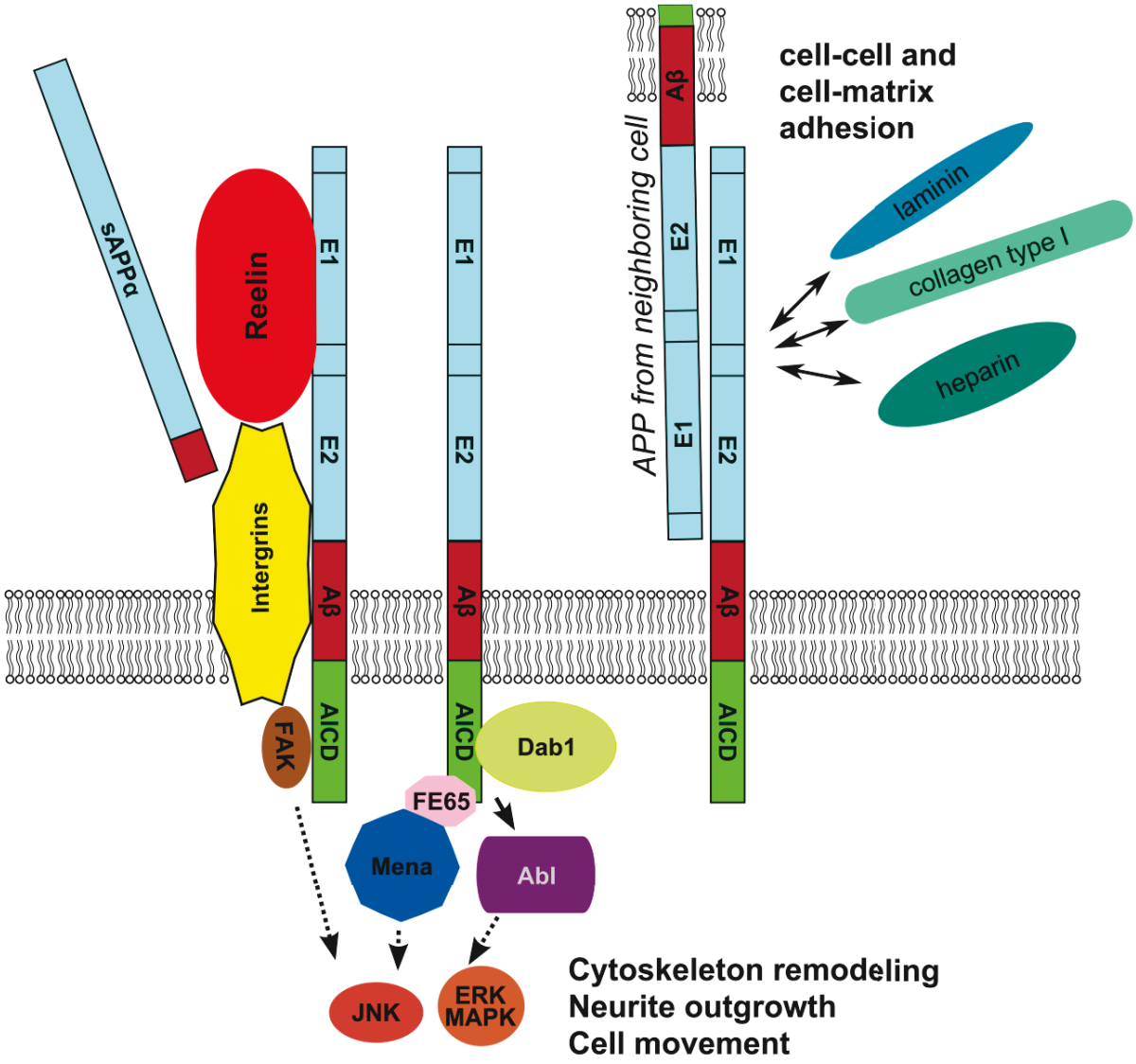


Figure 3.4 APP interaction partners

Known interaction partners of APP protein and intracellular pathways suggest APP functions in cytoskeleton remodeling, neurite outgrowth and cell movement (Muller and Zheng, 2012; Soldano and Hassan, 2014)

Abbreviations: Abl = Abelson murine leukemia viral oncogene homolog 1 (tyrosinkinase), AICD = APP intracellular domain, Dab1 = disabled-1; ERK/MAPK = Extracellular-signal Regulated Kinase or other mitogen-activated protein kinase, FAK = focal adhesion kinase, FE65 = multidomain adaptor protein, JNK = Jun N-terminal Kinase, Mena = actin-associated protein, sAPPα = extracellular α-secretase cleveage product, sAPPβ = extracellular β-secretase cleveage product; dotted lines = direct or indirect interaction

(Amyloid beta A4 precursor protein-binding family B member 1, APBB1) indicate involvement in cytoskeleton rearrangement (Sabo et al., 2001; Young-Pearse, et al., 2007; Young-Pearse et al., 2008; Song et al., 2010) (indicated in Figure 3.4).

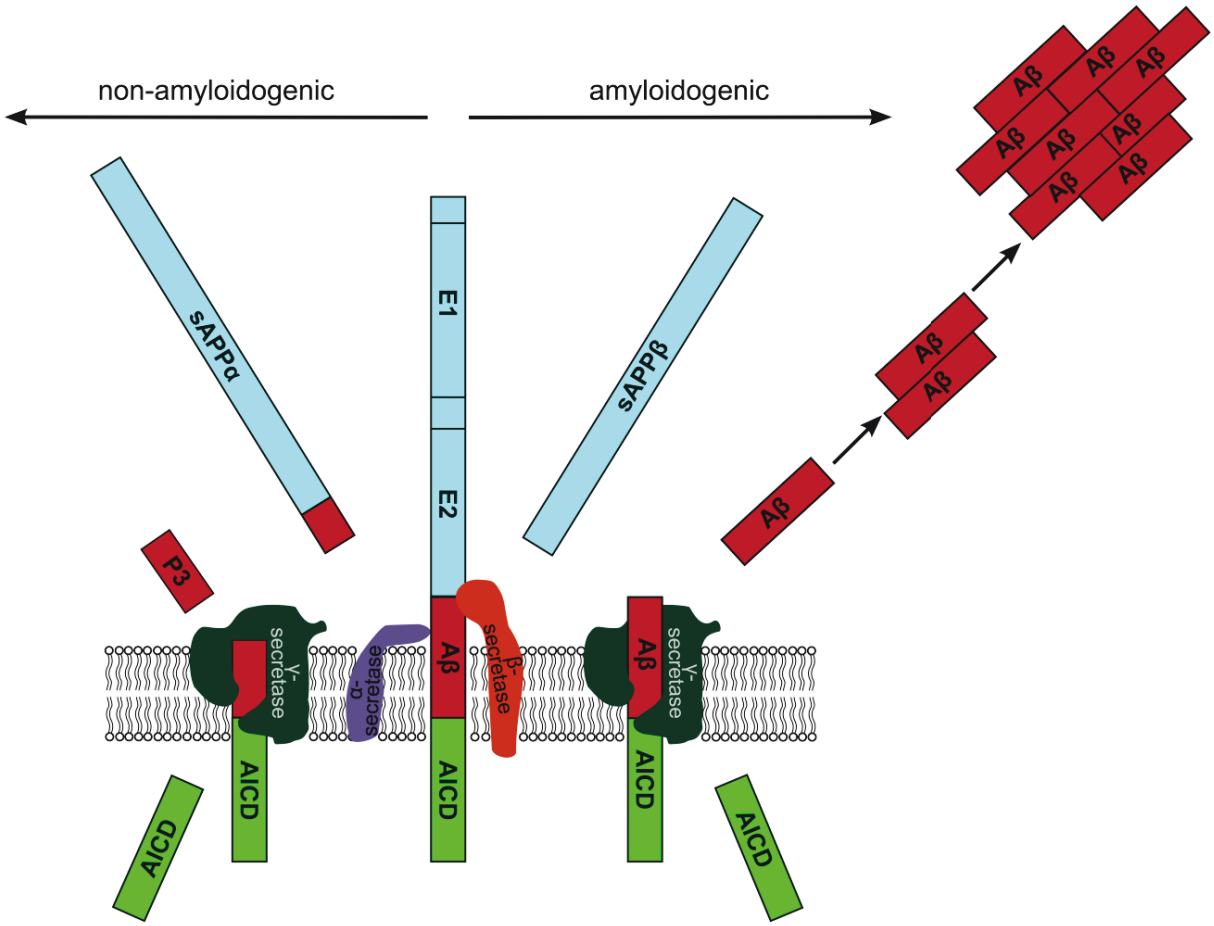


Figure 3.5 APP cleavage mechanisms

APP cleavage mechanisms for the non-amyloidogenic and the amyloidogenic way. The non-amyloidogenic cleavage by α-secretase and γ-secretase results in the formation of sAPPα, P3 fragment and intracellular AICD. Aβ is produced in the amyloidogenic pathway by β-secretase and γ-secretase cleavage of APP. Aβ, especially the 42 amino acid long form, is prone to dimerize and oligomerize and is able to build plaques.

Moreover, impairment of long-term potentiation (LTP) has been found in APP deficient neurons that correlates with decreases of dendritic spine numbers in these cells (Tyan et al., 2012). This phenotype can be rescued by sAPPα, a secreted form of APP (Ishida et al., 1997). sAPPα is generated under physiological conditions by cleavage of APP with α-secretase near the membrane at the cell surface, which is the beginning of the so-called non-amyloidogenic processing (Esch et al., 1990; Parvathy et al., 1999; Zhang et al., 2012). Afterwards, the membrane bound and C-terminal part is cleaved by γ-secretase to release fastly degraded APP intracellular domain (AICD) and P3 peptide (see Figure *3.5*). In contrast, the amyloidogenic processing, occurring mostly within intracellular compartments first by β-secretase (β-site APP cleaving enzyme 1; BACE1) followed by γ-secretase cleavage,

produces Aβ peptides and AICD (Schenk et al., 1995; Vassar et al., 1999; Zhang, et al., 2012) (see Figure *3.5*).

Aβ peptides can vary in their amino acid numbers from 38 to 43 amino acids. This is due to γ-secretase cleavage, which first cleaves the C99 fragment (AICD + Aβ) at residue 49 or 48 (ε-cleavage site) and generates AICD 49 or AICD 50, before subsequently cleaving of the two fragments to different smaller Aβ fragments (Sato et al., 2003; Funamoto et al., 2004; Kakuda et al., 2006; Selkoe and Wolfe, 2007).

The most common Aβ peptides are $A\beta_{40}$ and $A\beta_{42}$. Under physiological conditions, as major species, $A\beta_{40}$ is produced to approximately 90 % of all Aβ peptides, followed by $A\beta_{42}$ (Amemori et al., 2015). $A\beta_{42}$ is more prone to oligomerization and aggregation than other Aβ species and is therefore the main core component of amyloid plaques and cerebrovascular deposits (Roher et al., 1993; Iwatsubo et al., 1994). Therefore, an increase in the $A\beta_{42}/A\beta_{40}$ ratio is assumed to be a cause of Alzheimer's disease progression (Snyder et al., 1994; Kuperstein et al., 2010). Ratio changes can be caused by mutations in APP, as well as in γ-secretase forming proteins. Both have been found to be mutated in Alzheimer's disease, especially in early onset familiar form (Murrell et al., 1991; Mullan et al., 1992; Sherrington et al., 1995; Tomita et al., 1997). The γ-secretase is a multi-protein complex consisting of four major components, namely presenilin (PSEN; 1 or 2), nicastrin, Pen-2 (presenilin enhancer 2), and Aph-1 (anterior pharynx-defective 1) (Yu et al., 2000; Kimberly et al., 2003)

Presenilins build the main catalytic subunits of γ-secretase (Li et al., 2000). In humans two presenilin genes have been identified, *PSEN1* on chromosome 14 and *PSEN2* on chromosome 1 (St George-Hyslop et al., 1992; Levy-Lahad et al., 1995). Presenilin proteins consist of several transmembrane domains, PSEN1 for example is composed of ten hydrophobic regions which results in nine transmembrane domains (Spasic et al., 2006). In the active site, two aspartates on adjacent transmembrane domains were found, suggesting presenilins to be membrane-embedded aspartyl proteases (Wolfe et al., 1999). Apart from cleaving Aβ, presenilins, respectively γ-secretases, cleave several other type-I-transmembrane-domain proteins, like Notch receptors, ERBB4 (Receptor tyrosine-protein kinase) or cadherins (for review see (Kopan and Ilagan, 2004)). However, the most prominent function still is the cleavage of APP to Aβ species and therefore the involvement in Alzheimer's disease progression. Several mutations in *PSEN1* and *PSEN2* have been linked to familiar Alzheimer's disease, for example on position 166 in *PSEN1* (Van Broeckhoven, 1995; Moehlmann et al., 2002). Therefore, presenilins and their cleaving mechanism have been in the focus for drug development in Alzheimer's disease. Unfortunately, due to its manifold cleavage substrates, unspecific inhibition of presenilin or the γ-secretase complex,

has severe side effects. For example, a trial III study using a γ-secretase inhibitor showed a worsening of functional ability, like cognitive scores, and an increased risk for skin cancer and infections (Doody et al., 2013). In addition, a complete knockout of PSEN1 in an animal model resulted in skeleton deformation, impaired neurogenesis and ultimately in death shortly after birth (Shen et al., 1997). Therefore, recent research is focusing on γ-secretase modulators, which target γ-secretase activity to lower $A\beta_{42}$ production without blocking the overall processing of γ-secretase substrates (Crump et al., 2013).

Microtubule-associated protein tau (MAPT or tau) is another aggregated protein, which has been found to be aggregated in Alzheimer's pathology apart from Aβ. Tau is the main part of neurofibrillary tangles, which are not only found in Alzheimer's disease, but also in diseases summarized as tauopathies, such as Down syndrome, corticobasal degradation, and frontotemporal dementia with parkinsonism-17 (Delacourte, 2005).

Tau has been discovered as a microtubule associated protein that assembles and stabilizes microtubules (Weingarten et al., 1975). Human tau is encoded by the *MAPT* gene on chromosome 17q21 and comprises 16 exons, from which six tau isoforms via alternative splicing can be generated. Native tau protein is unfolded and mainly found in axons of neurons where it also seems to have function in axonal elongation and maturation (Caceres and Kosik, 1990). Beside this tau may have a regulatory function in axonal transport (Stamer et al., 2002). Tau is subject to posttranslational modifications (Martin et al., 2011). Especially phosphorylation has been associated with initialization or increase of aggregation of the natively hydrophilic protein, which only has little tendencies to aggregate. Tau phosphorylation reduces its binding affinity to microtubules (Drewes et al., 1995) which is a crucial part for the regulation of its physiological function especially during development (Kanemaru et al., 1992). Nevertheless, hyperphosphorylation seems to induce disease pathology through tau dissociation from the axons to the somatodentric compartment, altered tau degradation, enhanced aggregation and changed association to interaction partners (Grundke-Iqbal, et al., 1986; Bhaskar et al., 2005; Guillozet-Bongaarts et al., 2006; Thies and Mandelkow, 2007; Ittner et al., 2009). Other factors for elevated aggregation are mutations, especially within the microtubule binding domain, like the P301L mutation, which has been found in patients with dementia (Hong et al., 1998; Dayanandan et al., 1999).

While Aβ plaque formation does not correlate with Alzheimer's disease progression, neurofibrillary tangle formation and distribution led Braak to formulate the neuropathological staging of Alzheimer (Braak and Braak, 1991). The toxic species of Alzheimer's disease is still discussed, but more and more evidence has been gained that an interplay between Aβ and tau to cause the toxicity (Ittner and Gotz, 2011).

Moreover, apart from the inherited familial forms of Alzheimer's disease, where genetic mutations have been found, the starting point for protein aggregation is not clear. Recently, more and more evidence has been found for somatic mutations as a cause of sporadic forms of the illness (Bushman et al., 2015; Sala Frigerio et al., 2015). This might lead to genetic mosaicism in the brain with spacial located cells containing mutated and overexpressed genes, as starting points for disease progression.

3.4 Animal models of human brain diseases

Possibilities for human brain disease investigations

For studying brain disorders, animal models still are indispensable, due to practical and ethical difficulties of examining the living human brain. Although there has been progress in studying human brain structures and functions by noninvasive technologies, like magnetic resonance imaging (MRI), computed tomography (CT), single-photon emission computed tomography (SPECT), and positron emission tomography (PET). However, the ability to investigate physiological and molecular details of the human brain is still limited. Likewise, at the moment there are only limited knowledge gain from cell-based *in vitro* studies. In cell cultures it is possible to study molecular and morphological changes in single cells or simple networks. But the influences within a complex network structure as shown in mammal brains or even the impact on phenotype expression cannot be investigated. At least, stem cell technology and especially studies on human induced pluripotent stem cells (hiPSC) enabled the screening and identification of novel drugs and subsequently, the testing of their specific efficacy and toxicity in human cells (Doege and Abeliovich, 2014). Nevertheless, the influences of gene mutations, disrupted development, or disturbed connectivity of brain network and phenotype outcome, as well as disease progression, can only be studied in animal models.

Simple animal models

Animal models with invertebrates, for example flies (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*), exhibit simple and clearly arranged neuronal networks (Simpson, 2009; Gama Sosa et al., 2012). These are reproducible in great numbers and due to the limited number of nerve cells, single cell fate and network development can be studied. Hence, outcome and phenotypes of manipulated genes can easily be identified. For example, a lot of genes influencing brain development have first been identified and named in *Drosophila*, like *disabled* (*dab*) (Gertler et al., 1989). Zebrafish (*Danio rerio*) have also been

used as animal models (Lieschke and Currie, 2007). As simple vertebrates, their evolutionary relationship to mammals is closer than with invertebrate models and their overall brain organization shows many similarities to human. But they also exhibit beneficial features of invertebrates, like high reproducibility and the power of forward and reverse genetic screens, meaning the identification of genes responsible for a phenotype as well as phenotype analysis of an disrupted gene function. Furthermore, due to its external development and the transparency of the body, zebrafish can easily been used for imaging. With their ability to also show higher order behaviors like memory, conditioned responses, and social behavior, they have been shown to be useful as experimental organism.

Rodents as models

However, still the most often used animals to model diseases are rodents, especially mice and rat. This might in part be because in some diseases it is regarded as a necessary step for therapeutic development to do preclinical testing with rodents before starting clinical trials in human. Mice are suitable for that due to their similarity in the general outline of development and the high gene homologies to the human genome (Waterston et al., 2002). Technologies for genome manipulation are well developed for mice, which allows introduction of human disease-associated genetic alternation into the mouse. Since transgenic technology and gene targeting became widely available in mice, they have been used as models relevant for several diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, schizophrenia, depression and much more (Gama Sosa, et al., 2012). Gene mutations, which have been found in *post mortem* brain material of patients (mostly exhibiting familiar forms of the disease), or knockouts of suspicious genes, have been introduced in animals to model human diseases. In most cases this leads to models of pathogenesis, with the models displaying aspects of human disease pathology thereby providing confirmation of the genetic basis of disease and helping to identify cellular and molecular mechanisms of disease phenotypes (Gama Sosa, et al., 2012).

Until today, it has not been possible to generate an animal model that displays the whole spectrum of the studied disease. The reasons for that are manifold. One major point is the potential different function and interaction of the relevant protein or the different splicing of the gene in rodent and human brain. Another reason is that especially in neuropsychiatric diseases pathological markers are not as apparent as in neurodegenerative diseases (Arguello and Gogos, 2006). Nevertheless, also most neurodegenerative diseases have a sporadic character. Identification of genetic risk factors came from the rare cases of familial/inherited forms of the diseases, such as familial Alzheimer's disease. So, using

transgenic animals to model the disease may not display the exact mechanism of the most common sporadic diseases. But due to the similar phenotypic outcome, a common targeted pathway is likely and insights into these sporadic forms might be achieved by genetic manipulation. Nevertheless, even in Alzheimer's disease, with its well-defined neuropathology in humans, pathological outcome in mice is often only achieved in overexpression models and models targeting mutations in different genes. This does not exactly mimic the human condition. For example, Alzheimer's disease mouse models that exhibit extensive amyloid pathology fail to show the neuronal loss that is seen in humans (Irizarry et al., 1997; Irizarry et al., 1997). Hence, in order to better approximate the full disease spectrum multi-genetic approaches are necessary, like in the 5XFAD mouse overexpressing three mutations in *APP* and two mutations in *PSEN1* (Oakley et al., 2006). But although this is not comparable with the genetic situation in humans, the induced pathology is useful to better study disease progression and effects of drug treatment.

As mentioned, mental disorders are mainly diagnosed by phenomenology and symptoms examined in a clinical interview. For neuropsychiatric disorders, single symptoms are not solely shown in one specific illness, but rather contribute to several ones and only the accumulation of different symptoms lead to a specific diagnosis. And even within a diagnosis variation of symptoms is possible. Another problem is that some symptoms seem to be uniquely human, like hallucinations, delusions, sadness, and depression, and therefore cannot be convincingly mimicked in animals (Nestler and Hyman, 2010). However, there are possibilities to model symptoms of neuropsychiatric diseases, like attention, working memory, executive function or cognitive deficits, as well as hyperactivity in response to stress and novelty, and hypersensitivity to psychostimulants as correlates to positive syndromes (Arguello and Gogos, 2006).

The differences in size between human cerebrum and most animal models, and therefore the associated differences in functions, are the biggest problems for studying human brain diseases in animal models. The key difference of mammal brains compared to other vertebrates, and even within the mammalian family, is brain size and the proportions of the different parts. Especially the cerebral cortex, belonging to the cerebrum, has been enlarged throughout evolution (Figure *3.6*). Functionally the cerebrum contains the higher brain functions, like speech, memory, emotions, motivation and so on, which have been considered to be unique or specially developed in humans. Often these functions are disrupted in mental illnesses. For example, memory and speech loss in Alzheimer's disease through neuronal

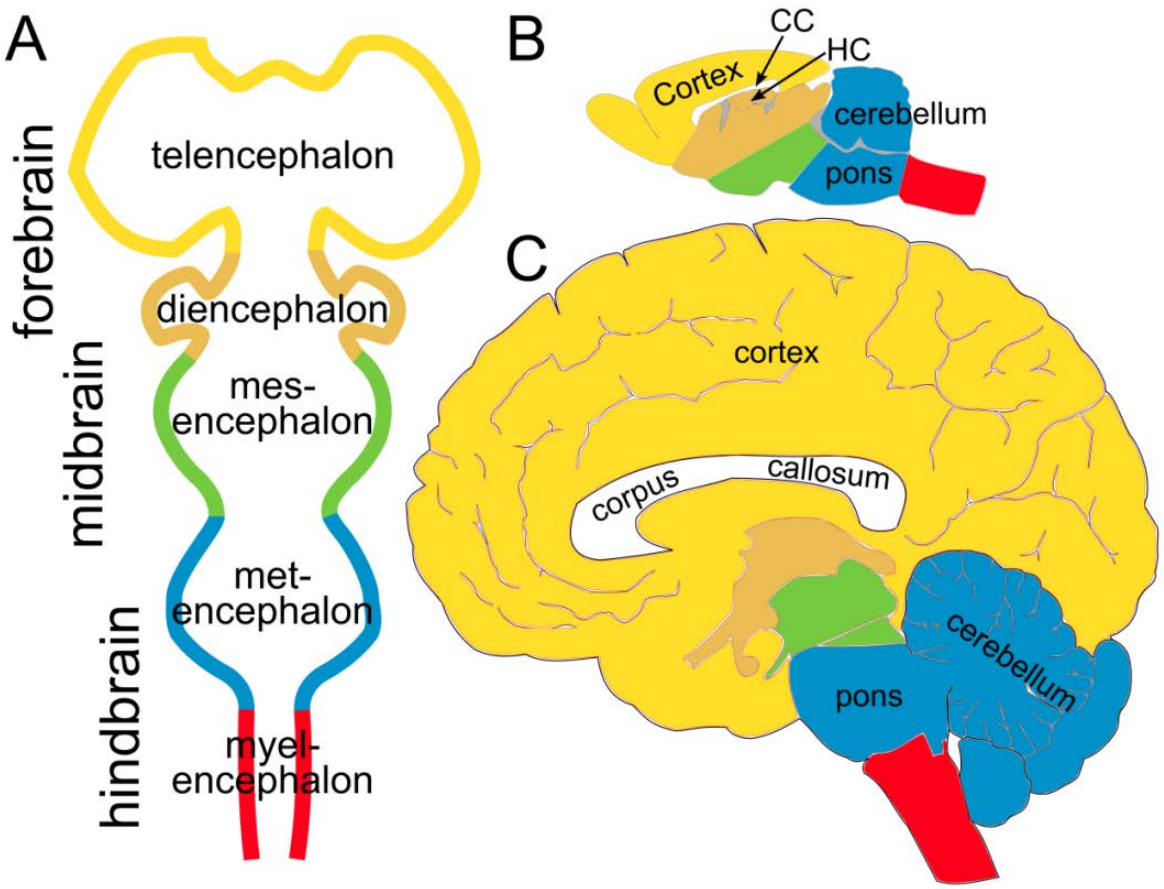


Figure 3.6 Brain structure in rodent and human

The composition of brain parts in A) vertebrate embryonic state, B) rat brain, C) human brain; shows evolutionary expansion especially of the cortex.
Abbreviations: CC = corpus callosum; HC = hippocampus

loss in the cerebrum (Whitehouse et al., 1982). Or the dysregulation of neurotransmitters, like dopamine, within the cerebrum which lead to the positive (psychotic) symptoms for example in schizophrenic patients (Howes and Kapur, 2009).

Criteria for evaluating animal models for human disease

Altogether, an animal model for a disease generates a specific phenotype and can mimic some aspects of disease symptomatology. This phenotype has to be evaluated with different specific behavioral tests to get a readout on which conclusions can be drawn (Razafsha et al., 2013). Three criteria have to be considered to validate an animal model: 1) construct validity, meaning a conceptual analogy to the cause of the human disease; 2) face validity, which reflects the extent of human disease symptom incorporation by the animal model; and 3) predictive validity that compares obtained results with previous results from other models

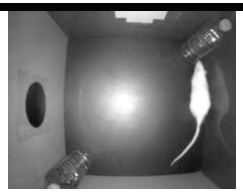
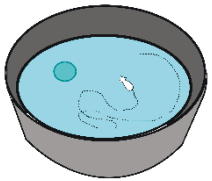
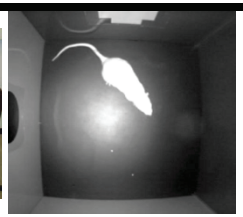
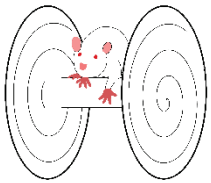
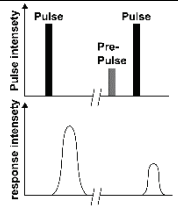
or specificity of responses to human treatments, preferably to a “Gold Standard” (Chadman et al., 2009; Razafsha, et al., 2013). Therefore, behavioral test and their combination have to be chosen with care to be able to make statements concerning illness comparability between animal model and human disease.

There are several established behavioral tests for rats and mice to study specific brain functions. Some with a high comparability to human function/human disease like the prepulse inhibition (PPI) test, which is performed especially with schizophrenic patients and can be used in the same manner in animal models of impaired sensory-motor gating (Braff et al., 1978; Kohl et al., 2013). An overview of behavioral tests used to study disease dependent phenotypes is shown in Table *3.2*. These tests can also be used to measure behavioral changes due to drug treatment. For example, a simple open field paradigm for studying the animal behavior like locomotion, rearing, and grooming in an empty box, is used to examine hyperactivity induction after amphetamine treatment (Pum et al., 2007).

In general, rats compared to mice exhibit a more robust capacity for learning in behavioral tests and some aspects of physiology and pharmacology are easier to observe and might be more relevant to humans. But because transgenic technologies were first developed for mice rather than rats, mice have been preferred as genetic animal models in the past (Gama Sosa, et al., 2012).

Depending on the kind of required effect, animal models can be generated using various strategies. Generally, four types of different approaches can be used, namely the pharmacological approach, the environmental approach, the stimulating or lesioning approach, and the genetic approach (Nestler and Hyman, 2010). Pharmacological approaches using administration of neurotransmitter agonists or antagonists, lead to a temporal and spatial control of the neurotransmitter system of interest. Environmental approaches, mostly using either social or physical stress are used to model disease relevant conditions, especially for depression studies or to enhance genetic phenotypes. Brain stimulation by electric stimulus or optogenetic approaches as well as anatomical lesions, act on the level of neuronal circuit function. Genetic approaches are the mostly used and are sometimes combined with one of the other approaches to enhance or counteract the phenotype.

Table 3.2 Examples of behavioral test used to study disease dependent phenotypes in mice and rats

test	 Novelty preference task	 Morris water maze	 T-Maze alternation tasks	 Open field habituation	 Rotarod	 Prepuls inhibition	 Forced swim test
procedure	Presentation of objects the subjects can explore. After a delay one object changes in shape or place or both. Exploration of novel object is recorded	Subject is placed in a water-filed round pool and need to find the hidden platform. Learning ability is recorded regularly over days.	Subject is either free to visit all arms of the maze and spontaneous alternations are recorded over time or in a two trial test, first one arm is blocked and subject is forced to enter the open arm, the free choice in the 2. trial is recorded	Subject is placed in an empty box, locomotion and environmental exploration, including rearing and grooming is recorded. Repeated testing shows habituation behavior	Subject is placed on a rotating wheel with constant or accelerating speed. Time until subject falls of the wheel is recorded	A pulse is presented to the subject, which responds with a startle reflex, recording of startle reflex inhibition through a weaker prepulse is recorded.	Subject is placed in a water filled cylinder without any chance to escape. Time the animal spends immobile is recorded.
modeled phenotype/ disease relevance	Working memory / relevant for diseases with cognitive impairment	Long term memory / relevant for diseases with cognitive impairment	Working memory / relevant for diseases with cognitive impairment	Unconditioned fear and anxiety, locomotion / e.g. anxiety test for depression	Motoric ability / exclusion of motoric disabilities	Impaired sensory-motor gating / e.g. in schizophrenia	Learned helplessness / Stress response, used to test anti depression treatment
Reference	(Ennaceur and Aggleton, 1997	(Morris, 1981; D'Hooge and De Deyn, 2001)	(Olton, 1979; Dudchenko, 2001)	(Denenberg, 1969)	(Hamm et al., 1994)	(Ison et al., 1973; Graham, 1975)	(Petit-Demouliere et al., 2005)

3.4.1 Generation of germline transgenic rodents

The most common strategies to produce transgenic mice is to induce DNA cassettes into pronuclei of fertilized mouse eggs or infect germ cells with viral vectors, which are then implanted into a pseudopregnant mouse. Another conventional method to introduce gene-manipulation in mice is to genetically modify pluripotent embryonic stem cells, which are then injected in early mouse embryos (Bockamp et al., 2008). By varying parts of DNA cassettes, it is possible to target different cell populations or cell types, as well as to change the expression strength and expression or silencing time point of the gene of interest. One major decision lies in the chosen promoter. Depending on the selected promoter transgene expression restricted to neurons can be achieved just as regionally restricted expression for example in the forebrain with D6 promoter/enhancer or the CAMKIIα promoter (Mayford et al., 1996; Machon et al., 2002). To express a gene of interest for a specific time frame, an inducible system can be used, like the tet system in which gene expression is induced (tet-on) or suppressed (tet-off) by treatment with tetracycline or derivates like doxycycline (Furth et al., 1994; Gossen et al., 1995). The introduction of the so-called conditional mouse models has been an important improvement to overcome misinterpretation of adult gene and protein functions. The adult phenotype in gene manipulated mice might also be due to disturbed development, caused by the lack or malformation of the targeted gene/protein, or the up-/down regulation of compensatory genes/pathways to overcome the dysfunction of the manipulated gene (Bockamp, et al., 2008). If the goal of the gene manipulation in the animal is to knockout, insert point mutations, or to integrate the gene of interest in a specific chromosomal site, systems using site-directed recombination should be considered. For example, the Cre/loxP recombination system from P1 bacteriophage is widely used. Hereby, Cre recombinases recognize and bind to their recognition sequences and catalyze recombination between LoxP sites (Sauer and Henderson, 1988).

A recently developed technology for genomic editing is the CRISPR/Cas9 system (Jinek et al., 2012; Doudna and Charpentier, 2014). Thereby, specific double-strand breaks by bacterial type II clustered regularly interspaced short palindromic repeats (CRISPR) and the nuclease CRISPR-associated protein 9 (Cas9) are used to achieve genome editing. This method can be used to manipulate large genomic DNA fragment deletion, insertion and exchange with high efficiency (Zhang et al., 2015). Unfortunately, all these techniques also have their limitations, like background gene expression in the tet-on system also without tetracycline or the promoter activity dependent expression of the Cre recombinase in the Cre/loxP system. Therefore, combinations of different systems can be used, like controlling of

tet-on regulated Cre recombinase expression through tetracycline induction (Schönig et al., 2002).

All these conveniently generated animal models described above need breeding and genotyping approaches to manifest and ensure the changed genotype. This is time and animal consuming. But nowadays an ultimate goal is to minimize animal numbers used for research and treatment testings. Therefore, methods to reduce the amount of animals are appreciated. This can for example be reached by direct manipulation of the genetic background of cells within the tissue of interest in the living animal and subsequent observation of the induced changes on molecular, cellular, neuronal circuit and behavioral level.

3.4.2 *In utero* electroporation

In utero electroporation (IUE) is a technique to study gene overexpression or suppression in a specific brain region *in vivo* especially during development. IUE was originally supposed to be useful to study gene function in developmental pathways and as gain-of-function analysis of various genes involved in developmental events of the brain (Tabata and Nakajima, 2001).

In utero electroporation, a plasmid targeting the gene of interest is injected into the ventricle of the rodent embryonic brain and then introduced into cells of specific brain regions by electric pulses (schematically shown in Figure *3.7*). During the whole procedure, the embryo is located within the uterus and is allowed to develop further in its mother thereafter. The technic hereby uses different circumstances: 1) DNA is negatively charged and moves toward the anode, so cells or areas targeted by the electroporation can be determined by placement of the electrodes. 2) During brain development cells are proliferating in the ventricular zone directly next to the ventricles, from where they start to migrate to their final position. Therefore, access of desired cell populations for the ventricular located plasmids is easily achieved. 3) Not only by electrode positioning, but also by choosing the injection time point during development different cell populations can be targeted. For example, cortical *in utero* electroporation in mice at embryonic day (E) 14.5 results in targeting neurons of cortical layer II/III, while astrocytes can be targeted in late embryonic stages, like E18 in the rat (Tabata and Nakajima, 2001; LoTurco et al., 2009).

In utero electroporation has been quite useful to study brain development. Studies using plasmids encoding for fluorescence proteins, like green fluorescence protein (GFP), reveal the fate of electroporated cells, showing proliferation and migration patterns (Tabata and Nakajima, 2001; Tabata and Nakajima, 2003; Wang et al., 2007; Rice et al., 2010). By

targeting genes connected with developmental mechanisms the impact of these genes on proliferation, migration, neurite

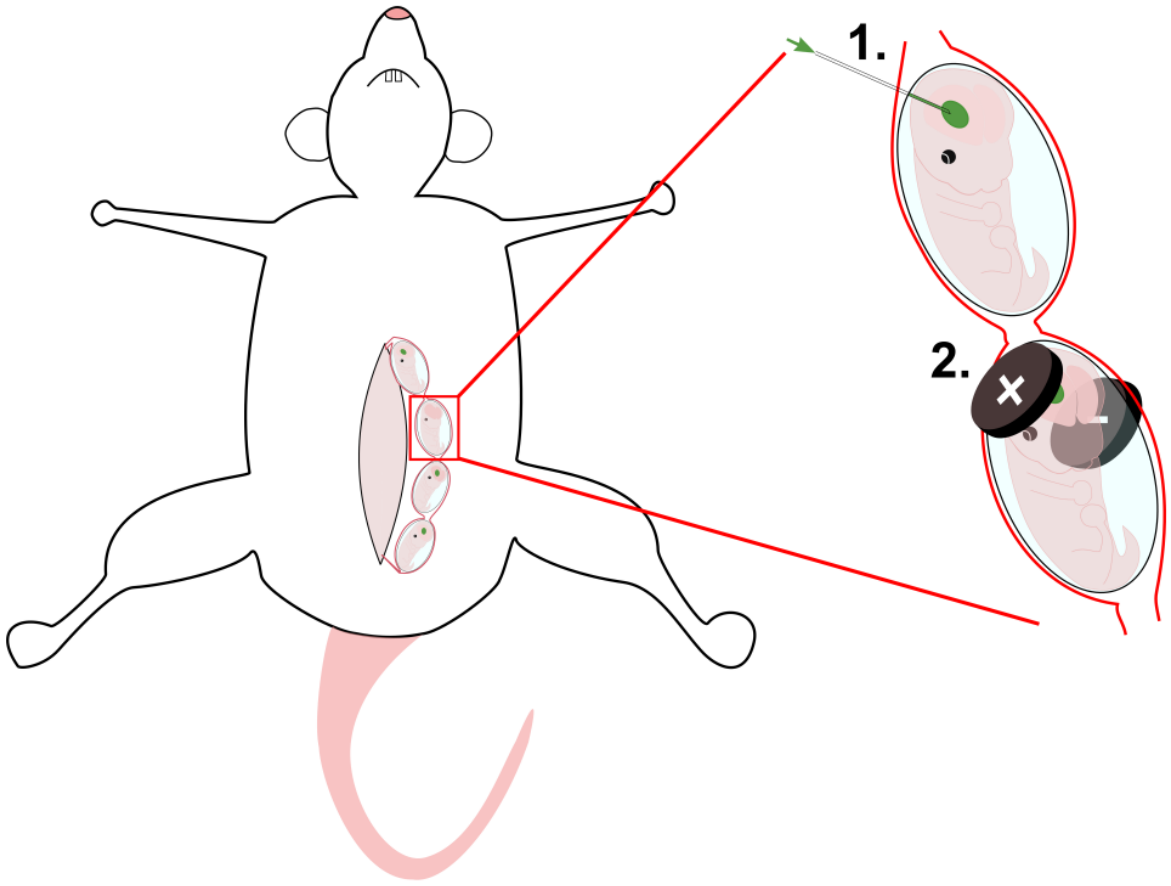


Figure 3.7 *In utero* electroporation scheme
An anesthetized pregnant mouse or rat gets an abdominal incision and the uterus horns are exposed, which need to be kept wet with PBS during the procedure. As a first step, (1) the injection of DNA-mixture containing 'Fast Green Dye' though the uterus wall into the ventricle (here the lateral ventricle) with a pulled glass capillary is performed. Thereafter, (2) using a tweezer electrode the electrode poles are positioned around the embryos head and electroporation with 5 pulses of 50 ms is applied. The position is chosen depending on the desired targeted area, here for example to target the cortex. Afterwards the uterus horn is placed back into the abdominal cavity of the mother and abdominal wall and skin are sutured, so the embryos can develop further. (figure adopted and changed from (Vomund et al., 2013))

outgrowth and cortex patterning can be determined (Fukuchi-Shimogori and Grove, 2001; Kamiya, et al., 2005; Tsai et al., 2005; Ramos et al., 2006; Sapir et al., 2008; Pacary et al., 2012). Often gene expression is knocked down in experiments IUE to induce a loss of function of the gene of interest within the targeted cells. This is achieved by usage of plasmids encoding small interfering RNAs (siRNAs; e.g. small hairpin RNA = shRNA), these interfere via base-pairing interaction with targeted mRNAs (Brummelkamp et al., 2002; Paddison et al., 2002). The underlying mechanism of this method is based on double-

stranded RNA (dsRNA) interference, which occurs in many organisms and might be a general mechanism for gene regulation. Here RNA is transcribed by polymerase III or II, processed by Dicer (an endonuclease that is part of the RNAse III family) and loaded into RNA-induced silencing complex (RISC) to target the desired mRNA and either represses the translation of or even cleaves the mRNA (Sharp, 1999; Bernstein et al., 2001). Therefore, no changes are introduced into the genome, but rather the gene translation from gene to protein is suppressed. By constructing a shRNA complementary to 18 - 20 nucleotides of a gene of interest theoretically the expression of all genes can be suppressed.

Table 3.3 Comparison of IUE and germ line genetically engineered transgenic rodents

	Advantage	**Disadvantage**
Germ line genetically engineered	Low variability in progeny of single founder lines Targeting of cell types in the whole brain is possible Stable genetic changes throughout the animals live	Time and cost intensive Possible compensatory mechanisms during early development
IUE	Rapid and resource saving No necessity of establishing full lines Unilateral targeting possible	Variability due to different number of transfected neurons Only subpopulations of cells in a specific area are targeted

However, not only gene suppression, but also overexpression and inducible as well as cell type specific gene targeting systems (like Cre/loxP systems and others explained in 3.4) can be introduced into the rodent brain via IUE (Matsuda and Cepko, 2007; Manent et al., 2009). For overexpression or rescuing studies in which the effect of a shRNA is complemented to test if the discovered phenotype was induced by the shRNA, a pCAGGS vector can be used (Liu et al., 2014). This vector contains a chicken Ac promoter, that is ubiquitously and strongly expressed in all mammalian cells, combined with a cytomegalovirus-immediate early (CMV-IE) enhancer, which together exhibit high level expression of transient genes (Niwa, et al., 2010).

Due to the efficacy of IUE in manipulating gene expression transiently in specific brain areas during development, this method has raised interest for usage in studying neurodevelopmental disorders quite early after being established. As reviewed byTaniguchi, Young-Pearse, Sawa and Kamiya, IUE has proven to be useful for studying the function and phenotypic outcome of manipulation of genetic risk factors of psychiatric disorders (Taniguchi et al., 2012). For example, in human patients with X-linked lissencephaly or double-cortex syndrome (also known as subcortical band heterotopia) mutations in the doublecortin gene

(*DCX*) have been found (Gleeson et al., 1999). A subcortical band heterotopia could not be achieved in transgenic mice with loss of function mutation in the *DCX* gene, although they showed hippocampal malformation (Corbo et al., 2002), whereas IUE with RNAi against *DCX* in the rat cortex could reproduce the phenotype seen in human patients (Bai et al., 2003; Bai et al., 2008). Although the same group found out that IUE with *DCX* RNAi in mice only led to migrational deficits but not to subcortical band heterotopia (Ramos, et al., 2006). This example shows that not only targeting gene and brain region should be carefully chosen, but also differences between species should be considered. Probably rats could be the more appropriate animal model, especially if the study includes behavioral phenotyping in which rats are more consistent. Nonetheless, also studies combining IUE in mice with behavioral examination have been successful. For example, knockdown of DISC 1 via IUE in the mouse cortex has been shown to induce behavioral abnormalities in the adult mouse, like decreased PPI and amphetamine hypersensitivity (Niwa, et al., 2010). These phenotypes directly link to schizophrenia patients for which DISC1 is a suspicious risk factor. This study indicates the feasibility of IUE for studying psychiatric disorders.

3.5 Aims of this work

As described in the sections before, investigation and treatment of mental diseases suffers by a lack of suitable diagnostic tools. To study complex disease underlying mechanisms animal models are the most significant study object, but most of the tools to create animal models are time and animal consuming. Therefore, the following aims were addressed in this study

1) Establishment of *in utero* electroporation to study behavioral or neuropathological consequences in the adult animal, as a time- and animal number saving method to investigate mental illnesses.

2) Create a method combinable with IUE to select born animals for electroporation success and reduce the variability of targeted area, in order to decrease the number of animals for behavioral and long-term investigations.

3) To investigate suitability of IUE for modeling somatic mutations of chronic brain disease genes, to study basic mechanisms of neurodegenerative diseases.

4 Compendium of studies

Study I:

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Video Article

Generation of Topically Transgenic Rats by *In utero* Electroporation and *In vivo* Bioluminescence Screening

Study II:

OXFORD

Cerebral Cortex, 2016, 1–12

doi: 10.1093/cercor/bhw060
Original Article

ORIGINAL ARTICLE

Behavioral Resilience and Sensitivity to Locally Restricted Cortical Migration Deficits Induced by In Utero Knockdown of Disabled-1 in the Adult Rat

Study III:

Efficient clearance of amyloid-β in a novel mouse model displaying somatic mutations for Alzheimer´s disease in single neurons

4.1 Study I:

Title	**Generation of Topically Transgenic Rats by *in utero* Electroporation and *in vivo* Bioluminescence Screening**
Authors	Sandra Vomund[1], Tamar Sapir[2], Orly Reiner[2], Maria A. de Souza Silva[3], Carsten Korth[1]
Published in	Journal of Visual Experiments 2013 Sep 24;(79):e50146.
doi	10.3791/50146.
Impact factor	1.325
Author's contribution (~90 %)	design of experimental setup *in utero* electroporation live imaging behavioral experiments intracardiac perfusion & brain dissection brain sectioning immunohistochemistry microscopy data analysis writing the manuscript video performance

The video is open access and can be watch at

http://www.jove.com/video/50146/generation-topically-transgenic-rats-utero-electroporation-vivo

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Video Article

Generation of Topically Transgenic Rats by *In utero* Electroporation and *In vivo* Bioluminescence Screening

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URL: http://www.jove.com/video/50146
DOI: doi:10.3791/50146

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Abstract

In utero electroporation (IUE) is a technique which allows genetic modification of cells in the brain for investigating neuronal development. So far, the use of IUE for investigating behavior or neuropathology in the adult brain has been limited by insufficient methods for monitoring of IUE transfection success by non-invasive techniques in postnatal animals.

For the present study, E16 rats were used for IUE. After intraventricular injection of the nucleic acids into the embryos, positioning of the tweezer electrodes was critical for targeting either the developing cortex or the hippocampus.

Ventricular co-injection and electroporation of a luciferase gene allowed monitoring of the transfected cells postnatally after intraperitoneal luciferin injection in the anesthetized live P7 pup by *in vivo* bioluminescence, using an IVIS Spectrum device with 3D quantification software.

Area definition by bioluminescence could clearly differentiate between cortical and hippocampal electroporations and detect a signal longitudinally over time up to 5 weeks after birth. This imaging technique allowed us to select pups with a sufficient number of transfected cells assumed necessary for triggering biological effects and, subsequently, to perform behavioral investigations at 3 month of age. As an example, this study demonstrates that IUE with the human full length *DISC1* gene into the rat cortex led to amphetamine hypersensitivity. Co-transfected GFP could be detected in neurons by *post mortem* fluorescence microscopy in cryosections indicating gene expression present at ≥6 months after birth.

We conclude that postnatal bioluminescence imaging allows evaluating the success of transient transfections with IUE in rats. Investigations on the influence of topical gene manipulations during neurodevelopment on the adult brain and its connectivity are greatly facilitated. For many scientific questions, this technique can supplement or even replace the use of transgenic rats and provide a novel technology for behavioral neuroscience.

Video Link

The video component of this article can be found at http://www.jove.com/video/50146/

Introduction

The development of the *in utero* electroporation (IUE) method which allows a modulation of gene expression in the developing brain, has been a break-through since it enabled studying neurodevelopment with relative ease.[1-7] Changes in expression levels of a target gene in a specific brain region during embryonic and/or perinatal development in rodents were demonstrated to critically influence neuronal proliferation, migration, arborization, and connectivity.[8-10]

Schizophrenia is a complex mental illness with acute and chronic symptoms that has been related to neurodevelopmental abnormalities[11, 12] and therefore many of the identified candidate genes for schizophrenia are investigated for potential modulating effects on neurodevelopment, like for example for the *disrupted-in-schizophrenia-1* (*DISC1*) gene[13-15].

Brain development is regulated by genetic factors and their interactions with environment which play roles in pre-, peri- and postnatal developmental periods. One major genetic risk factor for various behavioral disorders is the *DISC1* [16] gene. *DISC1* knockdown leads to migration defects in mice [13, 17], and manipulation of *DISC1* expression in the developing cortex by IUE has been shown to impact behavior of adult mice[18].

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Manipulating brain gene expression by IUE has several advantages[19] over the generation of transgenic animal lines. First, gene expression within areas of interest is achieved within weeks to months rather than several generations of breeding transgenic rodent lines. Second, compensatory mechanisms during early development that may shield phenotypes in germline-engineered animals [20] are avoided. Third, through targeting only a specific cell population or specific area of the brain, migration or proliferation differences can be directly compared with the non-mutant or control contralateral side if unilateral electroporations are chosen. On the other hand, IUE does not have the accuracy of promoter-driven cre/lox-induced timing of expression and only a subpopulation of the cells in a certain area is targeted leading to a mosaic kind of gene expression pattern.

For many experimental applications in adult rodents, a transient transfection of a limited number of cells in a brain region may be sufficient, or even desired, so that the major advantage of stable, germline-transgenic rodents is negligible. In fact, IUE is useful to investigate whether some abnormally developed cells may affect a whole network of cells or circuitry. Another advantage may be the ability to demonstrate non cell-autonomous effects of a gene due to the mosaic nature of the hit. Furthermore, the generation of transgenic and knockout rats is still in its infancy and the use of IUE in this species for studying aberrant brain development consequences is of high interest.

So far, a major obstacle of using IUE for investigating the intervention consequences in those animals as adults is the lack of monitoring electroporation success. So far, GFP-co-transfected fluorescent neurons in the live newborn rat pups could not be detected under a suitable binocular fluorescence microscope or with the fluorescence imaging of the IVIS Spectrum.

To overcome this obstacle, we co-transfected a luciferase reporter gene and performed bioluminescence live imaging of pups by 3-dimensional (3D) quantitation of the IUE brain area.

As an example for demonstrating the applicability of this method in a later functional assay testing the neurodevelopmental genetic manipulation, a co-injection of plasmids containing human DISC1, luciferase, and GFP into the lateral ventricle of rat embryos[3] followed by electroporation with a tweezer electrode was performed. While fluorescence signals could not be detected in postnatal stages *in vivo*, a solid bioluminescence signal derived from luciferin metabolism by co-transfected luciferase gene was detected up to five weeks after birth. 3D-measurements of the electroporated brain area allowed quantification whereby pups with insufficient or misplaced electroporation were identified from the outset, thus, enabling the assignment of IUE animals (gene of interest and scrambled control) to experimental groups with matched electroporated brain areas of low variability. The use of adult IUE rats in behavioral paradigms was demonstrated as an example of the usefulness of this protocol.

Protocol

All animal experiments were authorized by the responsible Landesministerium für Natur, Umwelt und Verbraucherschutz (LANUV NRW; 87-51.05.2010.A301) in accordance with National and European legislation.

1. *In utero* Electroporation

This method has been described in detail in JoVE for the rat by Walantus *et al.*[3], as well as Rice *et al.*[4] and is here summarized only briefly. A litter size of 6-8 pups yields a good outcome. There should be at least two non-electroporated embryos in order to increase the overall survival rate (see below).

1. Prepare DNA-Mixture that contains 1.5 µg/µl of target-Plasmid (shRNA: pENTR-U6; Invitrogen, Eugene, OR / DISC1 overexpression: pCAX[21]), 0.5 µg/µl Luciferase containing Plasmid (pCAX), 0.5 µg/µl GFP containing plasmid (pCAGGS[22]) in 1x PBS solution stained light blue with Fast Green Dye.
2. Prepare injection needles out of glass capillaries with a Needle Pipette Puller. And sterilize surgical instruments either by autoclaving or incubation with an alcohol-based disinfectant (kodan Tinktur forte).
3. Administer a pre-operative dose of buprenorphine (0.05 mg/kg) 15 min before surgery to a pregnant rat 16 days after fertilization (E16). Then anesthetize the animal in an isoflurane chamber.
    1. Upon anesthesia, place the rat in a supine position on a 37 °C-warmed operation table with breathing mask connected to the anesthesia device, using oxygen setting at 0.4 L/min and isoflurane at 1.8%.
    2. After shaving the abdomen, disinfect the shaved area three times with kodan Tinktur Forte (an alcohol-based disinfectant).
    3. Cover the rat with sterile cloths, exposing only the shaved operation field.
4. Perform *in utero* electroporation.
    1. Cut the abdomen with a scissor along the *linea alba* (~2 cm).
    2. Expose the uterine horns carefully with a ring forceps.
    3. Take care to keep the uterus wall wet with warmed sterile PBS during the whole surgery.
    4. Inject DNA solution with a thin glass needle into one of the lateral ventricle of the embryos.
    5. Place the 7 mm electrode around the head of the embryo. To hit a cell population of upper cortical layers, perform the IUE at E16[23] and position the positive electrode on the hemisphere above the injected ventricle with a slight dorsal/lateral tendency. To target hippocampal cells change the positive electrode placement to the opposite side than the injected ventricle with lateral to slightly dorsal direction (**Figure 1**).
    6. Perform electroporation by five 50 msec pulses at 55 V with 950 msec breaks with a square wave pulse electroporator.
    7. Spare the first embryo at the vaginal end of each uterus horn in order to increase the chances of survival of all embryos.
    8. Put the uterus horns back into the mother rat.
    9. Stich the abdominal wall up with an absorbable Vicryl surgical suture material.
    10. Close the skin with the Vicryl suture material or with suture clips.
    11. Place the mother rat back into the home cage and keep it warm for 2-3 hr.
    12. Hold the rats alone in their home cage in the animal facility room and feed *ad libitum*. They give birth between E22-24.

13. Keep the rat pups with their mother for three weeks and separate them afterwards by gender.

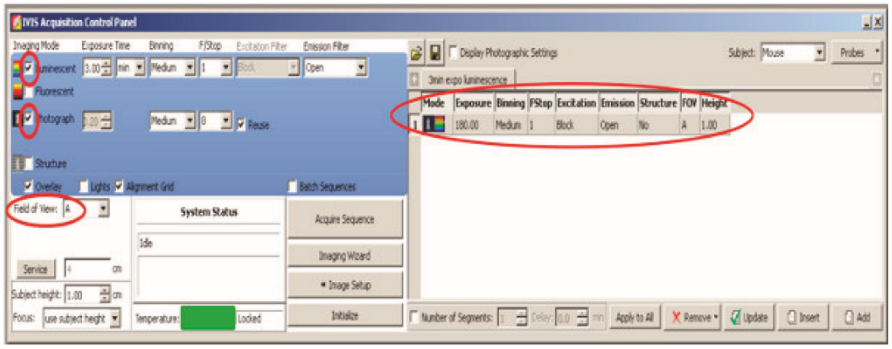
2. Bioluminescence Live Imaging of the Enzymatic Luciferase Reaction

This method is used to analyze the position of the *in utero* transfected cells. Co-electroporated firefly luciferase cDNA is translated into active luciferase, which upon metabolizing D-luciferin to oxyluciferin, emits a photon (**Figure 2**). The resulting luminescence can be detected in the brains of positive electroporated young rats in an IVIS Spectrum up to the age of around 35 days postnatally (**Figure 4**).

In the present study, the luciferase assay and bioluminescence imaging was performed starting at P7. This time point was chosen to allow mother and pups to recover from birth stress. When initially working with the pups at P0, pup survival was severely affected in that the pups were found dead or eaten by the mother.

Initially, rat pups with successful electroporation are identified by a 2D-bioluminescence picture with an exposure time of three minutes. Subsequently, positive pups are used for creating 3D images in order to specify the location of the electroporated area.

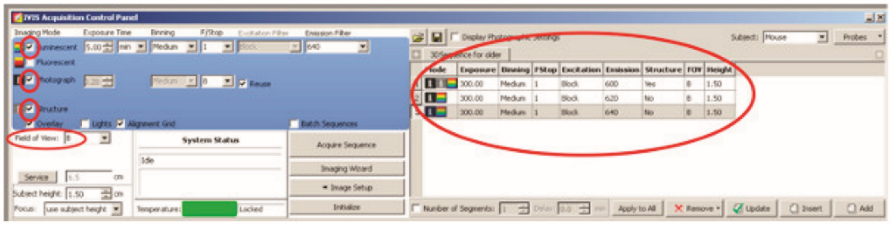
1. Dilute D-luciferin sodium salt in PBS to a concentration of 15 mg/ml and sterilize it by filtration through a sterile syringe filter.
2. Weigh the pups.
3. Take the pup in one hand with the abdomen on top and stretch the abdomen slightly. Inject 10 µl/g of body weight of luciferin solution intraperitoneal. For older and more agile ones, pre-anaesthetize the pups with isoflurane in the induction chamber before injecting the luciferin.
4. Turn on the isoflurane influx of the XGI-8 Gas Anesthesia System within the IVIS Spectrum with 3% isoflurane.
5. Put the snout of the animal into the glass nose cones of the Anesthesia System.
6. Hold the animal in a prone position until it is in deep anesthesia (2-3 min). Then reduce the isoflurane influx to 1.5%.
7. Choose a 2D-bioluminescence measurement to select positive pups from the whole litter. Use the following settings



Click here to view larger figure.

1. Set a checkmark on Photograph with medium binning and F/Stop at 8, the camera takes a photo from above after starting the measurement.
2. Set Excitation filter: block.
3. Set Emission filter: open.
4. Set Binning to medium.
5. Set F/Stop at 1.
6. Set Stage level A.
7. Set luminescence exposure time to 180 sec.

8. For creation of 3D pictures in order to better quantify the electroporated area from the positive pups, use the following DLIT settings for firefly luciferase.



Click here to view larger figure.

1. Set a checkmark on Photograph; the camera takes a photo from above after starting the measurement.

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2. Set a checkmark on Structure, the surface of the animal is scanned by the IVIS prior the bioluminescence measurement.
3. Use following Emission filters and exposure time settings until the age of two weeks:

   Emission filter 1: 590 nm, exposure time 300 sec

   Emission filter 2: 600 nm, exposure time 240 sec

   Emission filter 3: 620 nm, exposure time 180 sec

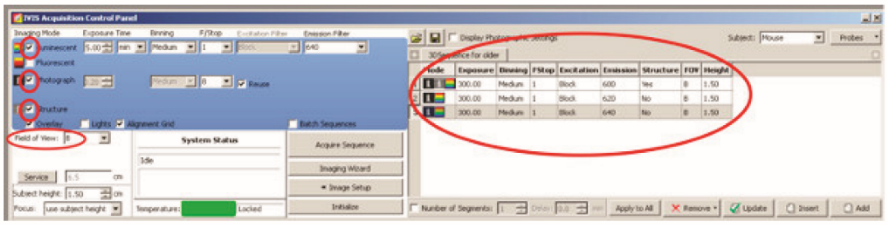
   Emission filter 4: 640 nm, exposure time 120 sec

   For rats older than P20

   Emission filter 1: 600 nm, exposure time 300 sec

   Emission filter 2: 620 nm, exposure time 300 sec

   Emission filter 3: 640 nm, exposure time 300 sec



Click here to view larger figure.

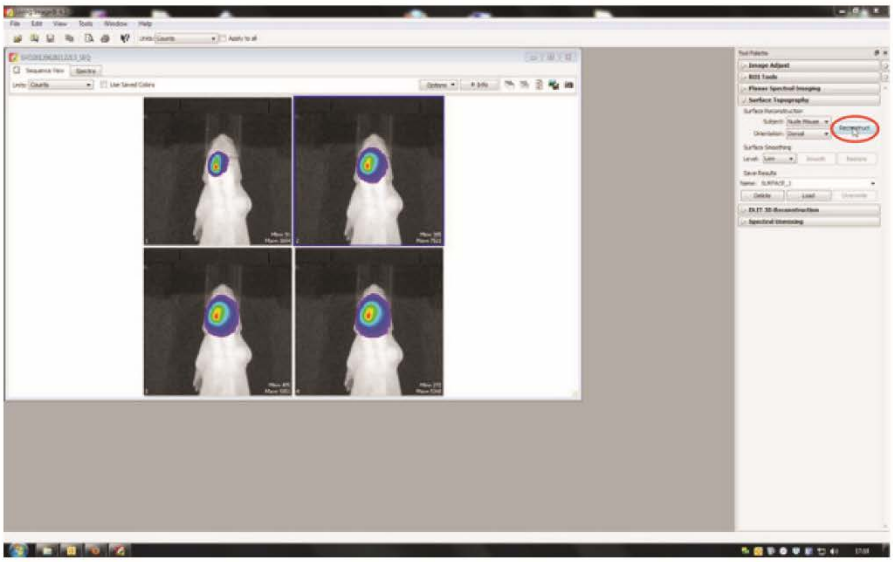
   Due to the decrease in signal strength in older animals, the exposure time is enlarged for the three best emission filters.
4. Set Stage level B
5. Set Binning to medium.
6. Set F/Stop at 1

9. After measurement, mark the rats by an earhole code to differentiate them from each other and to match them to the IVIS Live Imaging pictures
10. At the end of the measurement procedure, turn off isoflurane influx and keep the rat on the warmed plate for some minutes before returning it to its cage.

3. Analysis of Bioluminescence Images

The generation of 3D images, 3D movies and the quantification of the volume of the signal source is made by the Living Image software pre-installed on the IVIS Spectrum.

1. Generation of 3D images
   1. First, reconstruct a surface topography, therefore set threshold between 20-30%.



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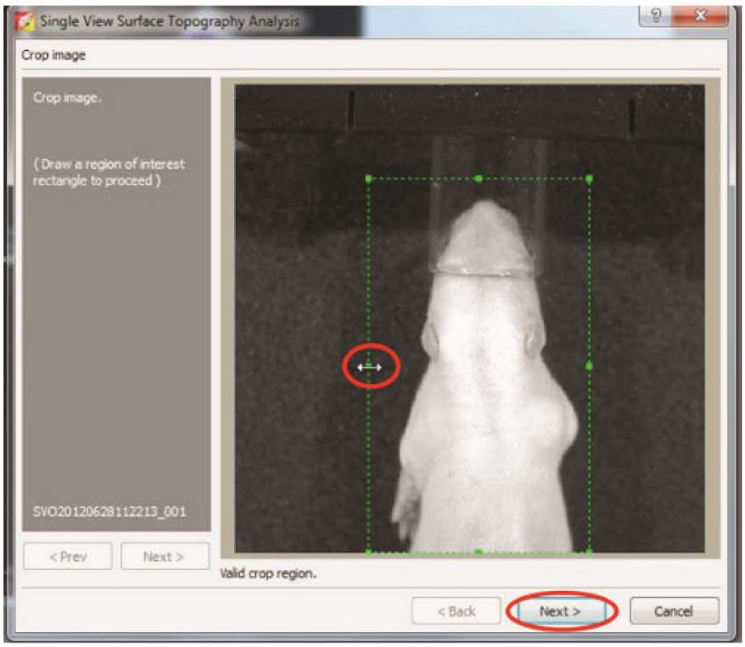
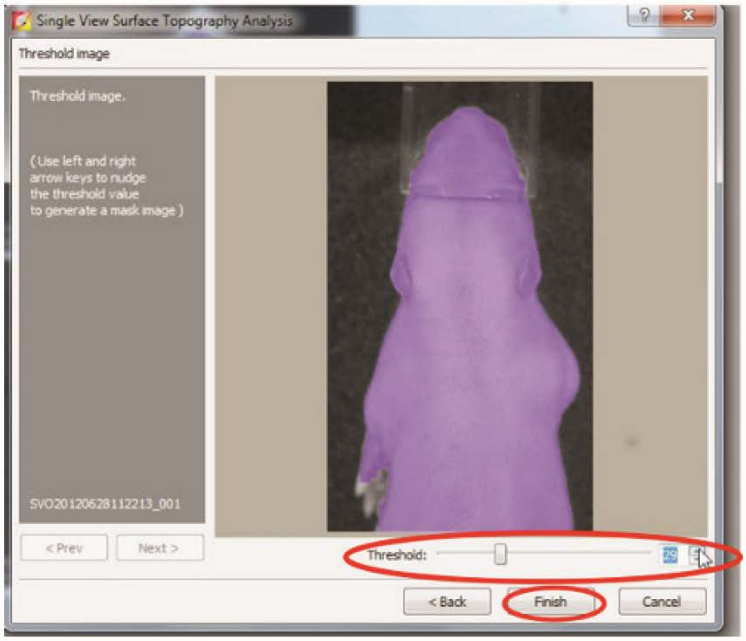
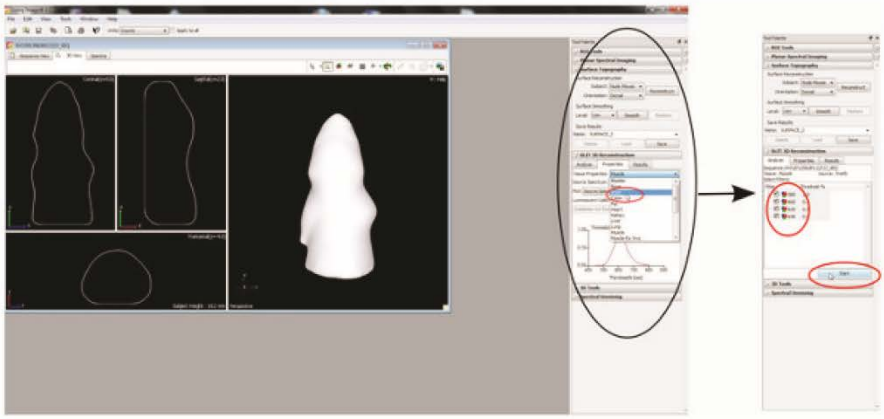




2. Start DLIT 3D reconstruction with an image threshold of 10% for each wavelength.



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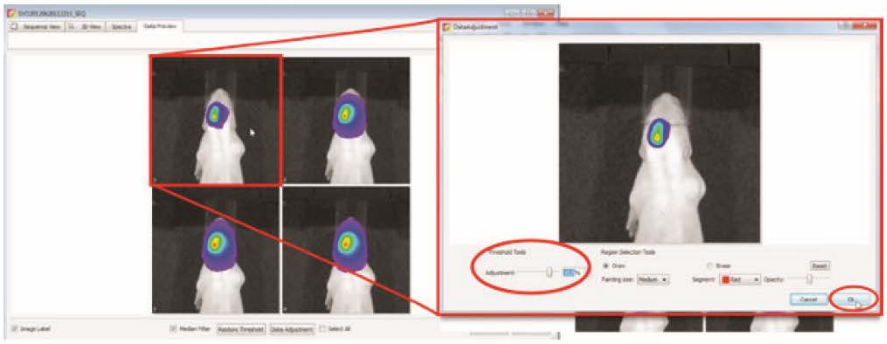
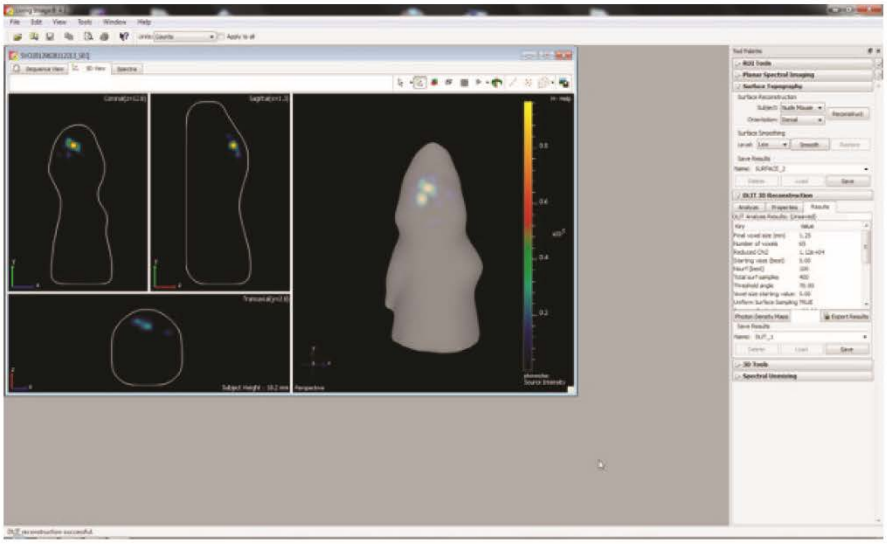


Click here to view larger figure.



Click here to view larger figure.

3. Create 3D movies by using the animate button within the 3D toolbar.
4. Choose different orientations of the 3D image, as well as zooming views as key frames and press the 'Record' button, recording the changeover from one position/orientation to another.

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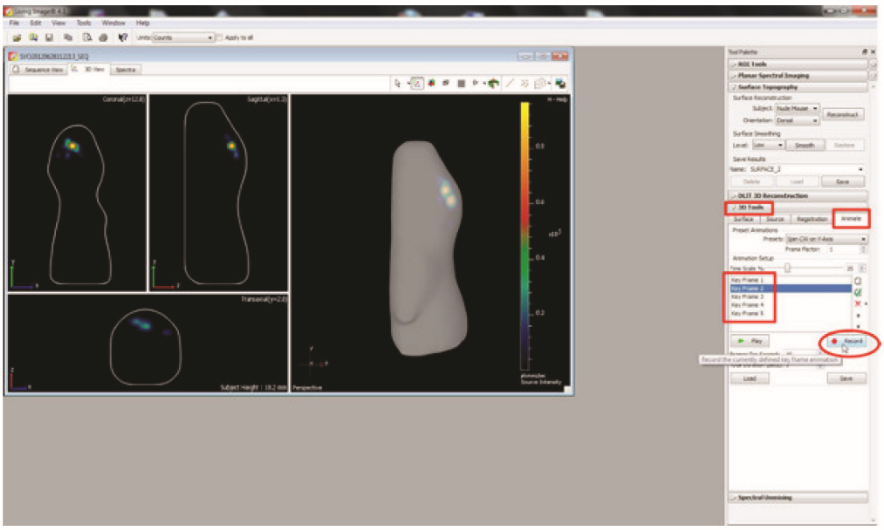

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4. Behavioral Testing

Behavioral testing was performed in order to determine whether IUE-mediated gene manipulations in the rat might initiate long-term effects that persist into adulthood. In the present particular case, the effect of transient, unilateral full length human DISC1 cortical overexpression after IUE was investigated by testing locomotion in an open field (OF), with and without a low dose of amphetamine, as a specific test for dopamine-related behavior [24]. In a similar procedure performed by Niwa *et al.* in IUE mice using DISC1 knockdown, IUE mice but not controls showed hypersensitivity to amphetamine[18].

Rats that were *in utero* electroporated with a DISC1 overexpressing vector were held under laboratory conditions with 12 hr light from 7 am to 7 pm and were fed *ad libitum*. At 3 months of age, rats underwent behavioral testing.

For quantifying locomotion as a readout of amphetamine effects, an open-field of a Tru Scan activity system situated in a sound- and light-isolated chamber was used. This system measures the duration time and distance the animal moves, time and distance spent in the margin or center of the open field, as well as rearing behavior[25].

1. On the first day, test after saline injection
    1. Weigh the animals.
    2. Inject intraperitoneally 1 µl/g body weight of a saline solution (1x PBS).
    3. Right after the injection, put the animal into the open-field and start the measurement of the TruScan system. Record for 15 min and subdivide data into 3 x 5 min parts.
    4. Put the animal back into its home cage.
2. Second day, test on amphetamine injection
    1. Weigh the animals.
    2. Inject intraperitoneally 1 µl/g body weight of a 0.5 mg/ml amphetamine solution.
    3. Right after the injection put the animal into the open-field and start the measurement of the TruScan system. Record for 15 min and subdivide data into 3 x 5 min parts.
    4. Return the animal to its home cage.
3. Analyze locomotion and rearing behavior generated by specific Tru Scan software. Create Graphs with GraphPad (Prism) and calculate statistics by SPSS Statistics software.

Representative Results

Figure 3 shows live imaging measurements of three rat pups at P7 after the injection of 150 mg luciferin/kg body weight. Differences of signal strength indicating the variability in the efficiency of the IUE are visible. Strong bioluminescence signals were recorded until P36 (**Figure 4**). In **Figure 5**, the ability to define cortical (**Figures 5A** and **5C**) and hippocampal (**Figures 5B** and **5D**) electroporation by bioluminescence imaging are depicted. Correlation of the bioluminescence signal (**Figure 7A**) with its fluorescence signal after skull removal (**Figure 7B**) and the

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corresponding GFP-electroporated cells in cryosectioned brain (**Figure 8**) at P14 are depicted in **Figures 6-8.** Of note, there was no detection of a fluorescence signal in live rats at any time point.

In vivo bioluminescence imaging enables approximate discrimination of different electroporated brain areas by 2D (**Figures 5A** and **5B**) which is greatly improved with the DLIT program of the IVIS Spectrum software generating 3D images (**Figures 5C** and **5D**). In the shown examples, electroporation of the prefrontal cortex and hippocampal could be distinguished. It should be noted that while aiming to electroporate the hippocampus, some progenitor cells for the cortex lying dorsal of the hippocampus can also be hit (**Figure 7**).

Rats unilaterally *in utero* electroporated with pCAX vector into the cortex and subsequently overexpressing full length human DISC1 were investigated for both spontaneous and amphetamine-induced hyperactivity as adults. Rats electroporated with pCAX-DISC1 were hypersensitive to a low dose of amphetamine. These rats moved significantly more after amphetamine treatment than after to saline injection, whereas control animals did not (**Figure 10**).

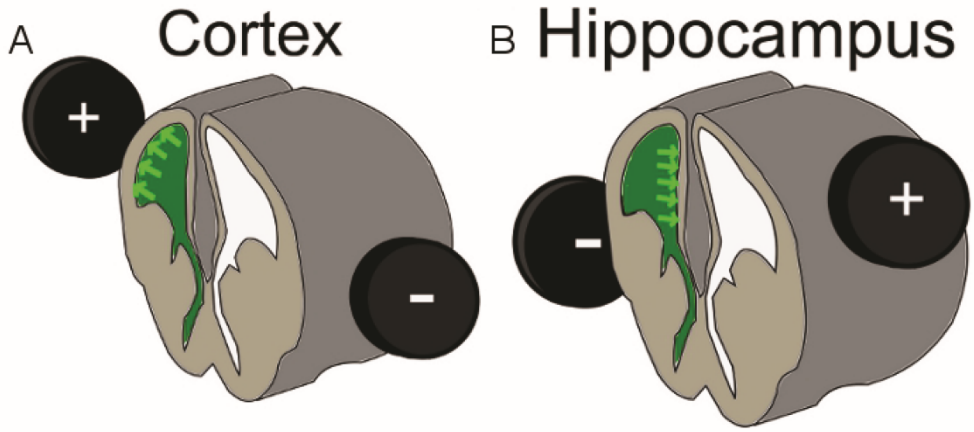

Figure 1. Scheme of electrode position for A) cortex electroporation and B) hippocampal electroporation; green = injected DNA-Mix within the ventricle.

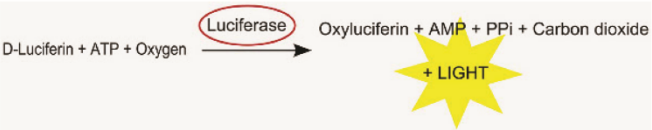

Figure 2. Luciferase reaction.

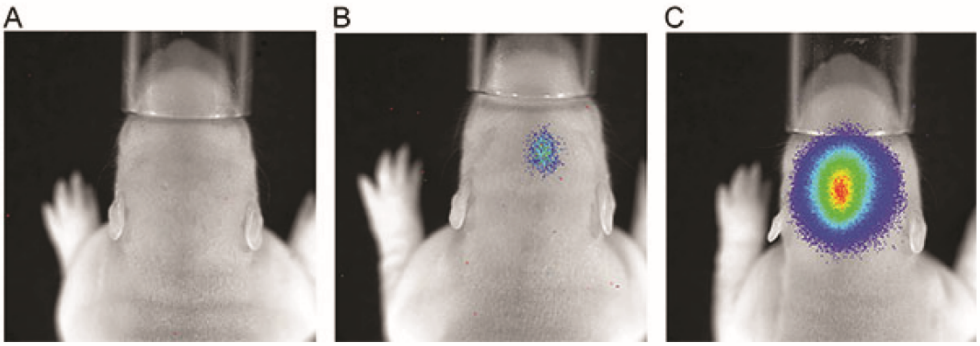

Figure 3. Luminescence measurement of P7 rats after injection of 150 mg/kg body weight luciferine; exposure time 180 sec; A) rat with no luminescence signal; B) rat with a weak bioluminescence signal; C) rat with a strong luminescence signal.

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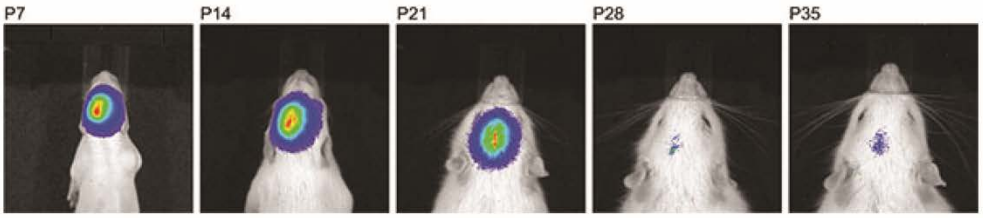

Figure 4. Time line of consecutive bioluminescence measurements of the same rat after injection of 150 mg/kg luciferine demonstrating a large time window where IUE success can be detected.

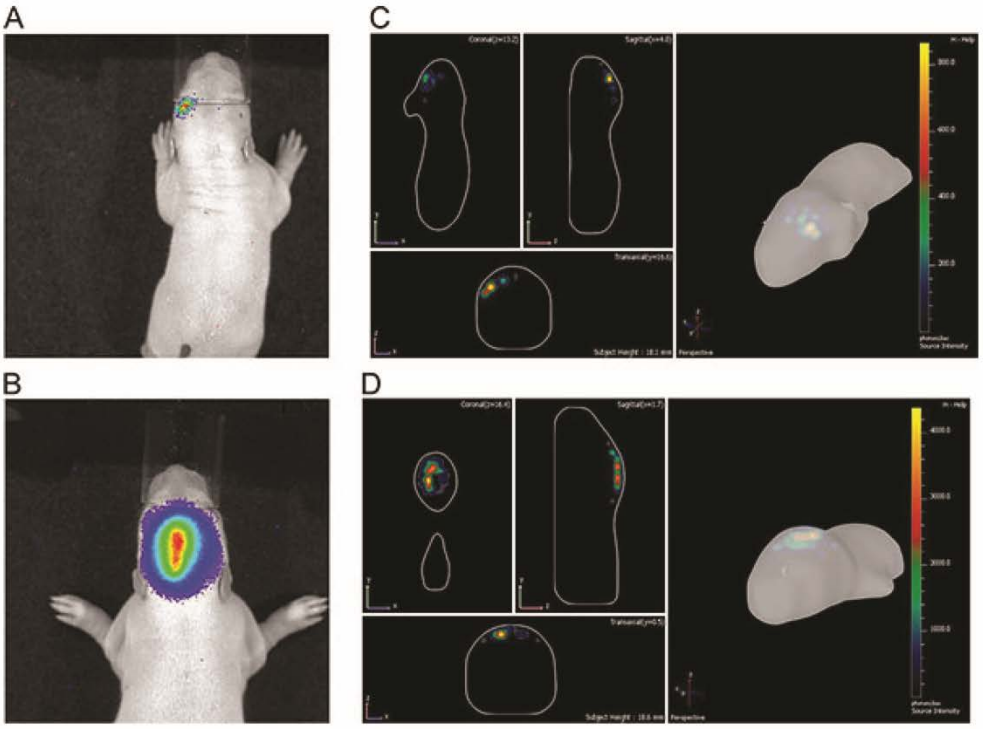

Figure 5. Illustration of differences between cortical and hippocampal electroporation at P7. **A)** & **B)** 2D pictures; **C)** & **D)** 3D pictures; **A)** & **C)** from cortex; **B)** & **D)** from hippocampus. Click here to view larger figure.

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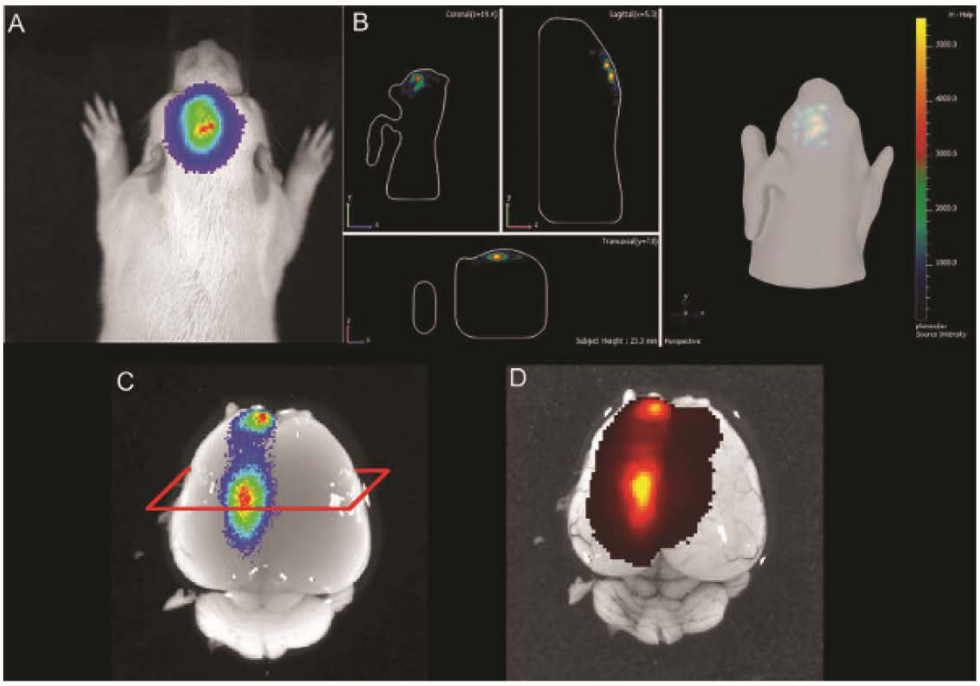

Figure 6. Illustration of E16 hippocampal electroporation of a rat pup at P14 A) 2D-picture of bioluminescence B) 3D illustration of the bioluminescence signal C) dissected brain with bioluminescence signal D) brain with GFP epi-fluorescence signal. Click here to view larger figure.

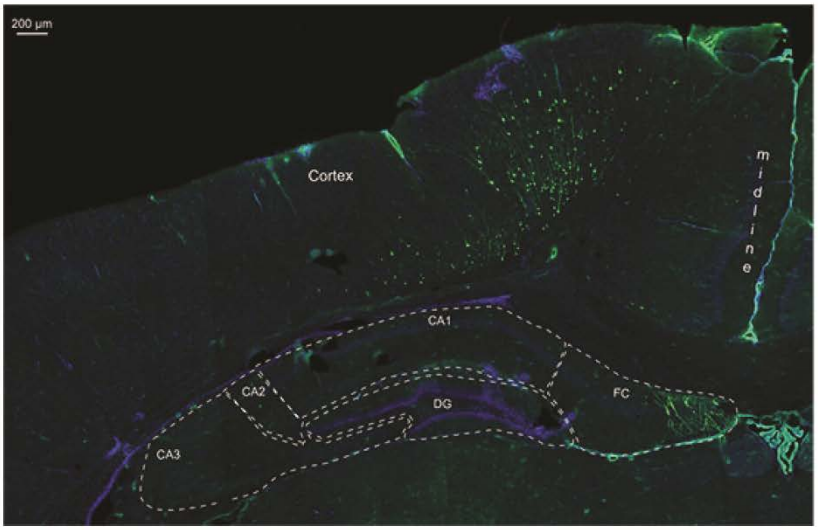

Figure 7. Detail of a fluorescence picture from a 20 µm cryo section of the same P14 rat brain electroporated with GFP containing plasmid and luciferase vector, nuclei staining with DAPI; CA1-3 = Cornu Ammonis 1-3; DG = Dentate Gyrus; FC = Fasciolarum cinereum.

Video 1. 3D animation of a hippocampus electroporated rat at P14.

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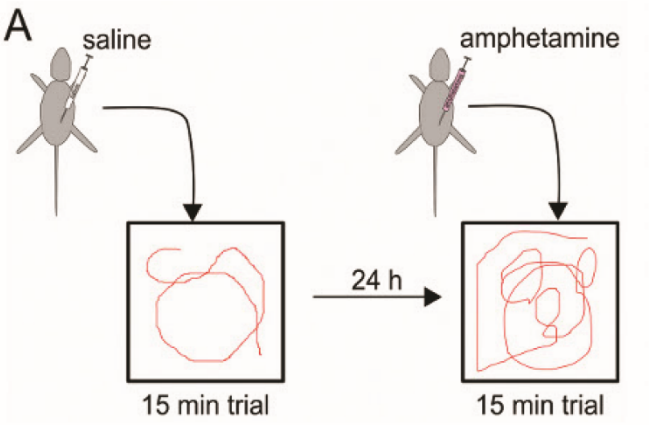

Figure 8. Amphetamine test scheme: first day saline injection before the 15 min trial, 24 hr later amphetamine injection before testing in the open field chamber.

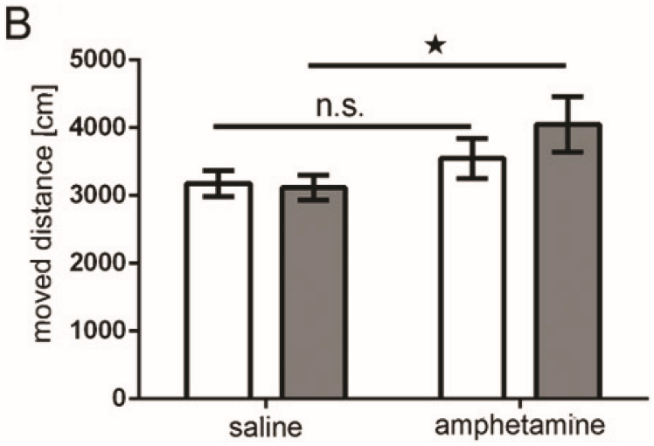

Figure 9. Amphetamine test. Bar graph showing moved distance (in cm) of the animal recorded by a TrueScan system over 15 min. White bars = control group; grey bars = DISC1 overexpressing group; control group n = 10; DISC1 overexpression group n = 11; ANOVA: geno*treatment p = 0.043; T-Test for total time saline vs amphetamine n.s.= not significant pcontrol = 0.172, pDISC1 = 0.001.

Discussion

Our study demonstrates that IUE is suited to generate adult rats with neurons expressing a transgene in a selective area of the brain and that, as a result of this intervention, these animals exhibit changes in behavior indicating functionality of the performed manipulation. In this study, as an example, rats overexpressing DISC1 unilaterally in a small part of the prefrontal cortex showed hypersensitivity towards amphetamine (**Figure 9**).

Selecting rats for electroporation success by *in vivo* bioluminescence imaging was effective in controlling for the inherent variability of IUE cell transfection and was applied to generate groups with a homogenous IUE area of low inter-subject variability for later investigations.

In this study, we were unable to select electroporated pups from the litter by detection of the co-electroporated GFP-induced fluorescence in the newborn animal, even though at the same time and in the same animal a bioluminescence signal of the equally co-electroporated luciferase could be detected after luciferin injection (**Figure 6**), and the GFP expressing neurons were still present in the brain at an age of six months. We conclude that, in the rat, the luciferase/luciferin reaction is well-suited to differentiate animals with successful electroporated brains (**Figure 3**).

The quantitative monitoring of IUE success relates to the strength of the bioluminescence signal which is measured by the counts of photons within the same exposure time (**Figure 3**) and corresponds to the enzymatic activity of co-expressed luciferase. Small bioluminescence signals are detectable by 100-200 counts of photons, and, at a radiance of ~ $1x10^4$ photons/sec/cm^2/steradian show 1,000-2,000 GFP-stained cells

in the histology in the 6 months old rat brain. The highest signal displayed a radiance of up to ~5x10^6 photons/sec/cm^2/steradian and ~64,000 counts.

In the Sprague Dawley rat strain used, we observed a weakening of the bioluminescence signal longitudinally with increasing age and the signal disappeared beyond the age of P35 (**Figure 4**). At this point, we do not know whether either the transient, plasmid vector-based expression of luciferase decreases, or if the bioluminescence signal weakens due to increasing brain mass, or both are the causes for the disappearing signal. For the present functional assay in the adult rats, the selection for behavioral studies was merely made based on the location of the signal, but not by bioluminescence signal strength.

Even though 3D quantitative bioluminescence monitoring allowed differentiation between different electroporated areas (**Figure 5**), its accurateness was limited for cells located in the depth dimension of the brain. **Figure 6** shows an example of a hippocampal electroporation where the bioluminescence measurement in the 2D and the 3D picture indicated a good positioning of the electroporation. In the dissected post mortem brain, a GFP-fluorescence signal was detected at about the same position as the bioluminescence signal, indicating correct targeting of the hippocampus. But histology shows that also cells in the cortex dorsal of the hippocampus had been targeted (**Figure 7**). This indicates that the bioluminescence assay is a useful tool to detect positive, IUE pups and also to have an idea of the electroporated area, but, ultimately, imaging cannot replace *post mortem* histology to exactly localize positively targeted cells.

Our demonstration indicates promise for the application of the IUE technology to generate subtle targeted manipulations of cortical or hippocampal brain regions to simulate aberrances in cortical migration or other neurodevelopmental defects that may influence the adult animal. While bilateral electroporation[26] has the advantage of a likely bigger effect on behavior, there is also more mortality of embryos. Unilateral electroporation was chosen in order to compare the two hemispheres with one as an internal control, as well as for showing that even IUE manipulating in an unilateral, small region is sufficient to change behavior. IUE-induced changes in connectivity or architecture between neurons may thus be induced without evoking a lesion and the required match of the to-be-IUE-manipulated region with the appropriate behavioral test is dependent on the scientific question.

Trouble shooting

Reduced litter size There are several suggestions regarding increasing the survival of the IUE pups. First, the use of very thin glass capillaries during electroporation in order to minimize tissue lesion is recommended. Second, do not electroporate the first embryo at the vaginal end of each uterus horn: death of the first-born embryo increases the chances of an abort of all other embryos. Third, after birth, mother rats often kill part of their progeny due to perinatal stress. In order to reduce additional stress, do not start with the live imaging right after birth, but wait for seven days.

GFP-fluorescence detection of the pups

At one week after birth, no signal of fluorescence by either using live binocular fluorescence microscopic imaging or fluorescence imaging with the IVIS Spectrum (epifluorescence and transfluorescence modes; for GFP excitation/emission: 465/520 nm and 500/540 nm). It is possible that both, the limited transmission of short wavelength excitation and emission light through tissue like the skull and the high autofluorescence background of the skin prevent using fluorescence under the described conditions in the rat. As shown in **Figure 6**, the luciferase signal in the living animal can also be detected in the dissected brain (without skull) and there, also a fluorescence signal is detectable (**Figure 6D**).

Differentiation of bioluminescence in closely spaced brain areas

Even in the 3D illustration the location of the bioluminescence area cannot be predicted to 100%. Especially cells on top of or below of the predicted area can also be accidentally targeted and transfected. The exact position has to be controlled by post mortem (fluorescence) histology (see **Figure 7**).

Disclosures

The authors of this study do not have financial interest in this study and have not been sponsored by industry for this study.

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4.2 Study II

Title	**Behavioral resilience and sensitivity to locally restricted cortical migration deficits induced by *in utero* knockdown of disabled-1 in the adult rat**
Authors	Sandra Vomund, M. Angelica de Souza Silva, Joseph P. Huston, and Carsten Korth
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Impact factor	8.665
Author's contribution (~90 %)	design of experimental setup *in utero* electroporation live imaging behavioral experiments intracardiac perfusion & brain dissection brain sectioning immunohistochemistry microscopy data analysis writing the manuscript

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Original Article

ORIGINAL ARTICLE

Behavioral Resilience and Sensitivity to Locally Restricted Cortical Migration Deficits Induced by In Utero Knockdown of Disabled-1 in the Adult Rat

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Abstract

Irregular neuronal migration plays a causal role in mental illnesses such as schizophrenia and autism, but the very nature of the migration deficits necessary to evoke adult behavioral changes is unknown. Here, we used in utero electroporation (IUE) in rats to induce a locally restricted, cortical migration deficit by knockdown of disabled-1 (Dab1), an intracellular converging point of the reelin pathway. After birth, selection of successfully electroporated rats by detection of in vivo bioluminescence of a simultaneously electroporated luciferase gene correlated to and was thus predictive to the number of electroporated neurons in postmortem histochemistry at 6 months of age. Rat neurons silenced for Dab1 did not migrate properly and their number surprisingly decreased after E22. Behavioral tests at adult ages (P180) revealed increased sensitivity to amphetamine as well as decreased habituation, but no deficits in memory tasks or motor functions. The data suggest that even subtle migration deficits involving only ten-thousands of cortical neurons during neurodevelopment can lead to lasting behavioral and neuronal changes into adulthood in some very specific behavioral domains. On the other hand, the lack of effects on various memory-related tasks may indicate resilience and plasticity of cognitive functions critical for survival under these specific conditions.

Key words: Dab1, bioluminescence live imaging, cortical migration deficits, in utero electroporation, neurodevelopment

Introduction

For major mental illnesses, such as schizophrenia and autism, neurodevelopmental abnormalities, including aberrant migration and mispositioning of neurons during corticogenesis, are discussed as possible causal variables (Murray and Lewis 1987; Weinberger 1995). For example, anatomical correlates of neurodevelopmental deficits in schizophrenia have been suggested to include abnormalities in number, positioning and function of interneurons, white matter abnormalities, and many others (reviewed in Lewis and Levitt 2002; Kochunov and Hong 2014).

The molecular and cellular mechanisms for potential neurodevelopmental abnormalities are only beginning to be elucidated. Several of those genes mutant in families of patients with schizophrenia, for example, Disrupted-in-schizophrenia 1 (DISC1) and neuregulin 1 (NRG1), have been shown to play a role in neurodevelopment when reverse-engineered in mice (Kamiya et al. 2005; Seshadri et al. 2010). An established, important pathway for neurodevelopment and corticogenesis is the reelin pathway (D'Arcangelo et al. 1995; Rice et al. 1998). Reduced reelin has been consistently seen in schizophrenia (Impagnatiello et al. 1998). Reelin signaling via the ApoER2 and VLDL receptors converges on the signaling protein, disabled-1 (Dab1; (Trommsdorff et al. 1999)), to provide signals for neuronal migration along glia fibers (Rakic 1971, 1972, 1981; Frotscher 1997; Cooper 2008). In humans, reelin mutations lead to microcephalus and premature death (Hong

et al. 2000), but mouse mutants deficient for reelin or Dab1, termed reeler or scrambler mice, respectively, are viable and both display an inverted cortical layering (Hamburgh 1963; Caviness and Sidman 1973; D'Arcangelo et al. 1995; Sweet et al. 1996; Tissir and Goffinet 2003). While profound ataxia is a dominant phenotype in mice deficient of *reelin/Dab1* (Falconer 1951; Hamburgh 1963; Sweet et al. 1996), the use of appropriate controls and heterozygous mutants has also revealed subtle behavioral phenotypes, including hyperactivity and perseverative behaviors, consistent with abnormalities in the dopamine system (Jacquelin et al. 2012; Michetti et al. 2014). A critical role of reelin signaling in the development of dopaminergic nuclei has been demonstrated (Kang et al. 2010; Bodea et al. 2014) and reelin is involved in dopamine release in a Snap25-dependent manner (Hellwig et al. 2011).

Over the last decade, in utero electroporation (IUE) has become a popular technique to study neurodevelopment (Tabata and Nakajima 2001; Kamiya et al. 2005; Tsai et al. 2005; Young-Pearse et al. 2007; Sapir et al. 2008). Using IUE, it is possible to genetically modify selected cells in specified regions with one or more genes and to avoid cellular compensatory mechanisms of gene dose changes, which is sometimes found in genetically engineered mice (Corbo et al. 2002). So far, IUE has not been extensively used for experiments with adult rats, for example, for investigating cellular pathology in relation to behavioral changes.

The aim of the present study was to test whether the introduction of a subtle migratory deficit by suppressing a key molecule in radial cortical migration, Dab1, in a relatively limited number of neurons by IUE would lead to long-term behavioral consequences in the adult. Subtle neurodevelopmental deficits of this kind have not been experimentally linked to behavioral processes. A full *reelin* or *Dab1* knockout mouse has been reported to lead to changes in the dopaminergic system (Michetti et al. 2014), as well as to deficits in attention, learning, and memory (Bliss and Errington 1977; Lalonde et al. 2004; Jacquelin et al. 2012). Accordingly, we chose to examine adult in utero electroporated rats for amphetamine sensitivity and subjected them to a wide range of tests of memory and learning, including tests of novel object preference, novel place preference, memory for temporal order, place habituation, T-maze alternation, and working memory.

Materials and Methods

Cell Culture and Western Blot

Human neuroblastoma NLF (NLF; Children's Hospital of Philadelphia, Philadelphia, PA, USA), cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin/streptomycin, L-glutamine, was transfected using Metafectene Reagent (Biontex, Martinsried, Germany) with either pCAGGS-Dab1-GFP (overexpression vector) + control shRNA plasmid, pCAGGS-Dab1-GFP + Dab1 shRNA plasmid (pENTR-U6), or pCAGGS-AFP (transfections control). After 24 h cells were lysed with VRL buffer (50 mM Hepes, 0.25 M sucrose, 5 mM EDTA, 0.1 M potassium acetate, pH 7.5), supplemented with 0.1% Triton X100, protease inhibitor, and DNase I.

Samples, equalized for protein concentration and mixed with SDS loading buffer, were run on a 10% SDS-Gel, before blotting on nitrocellulose membrane. Western blot using rabbit anti-Dab1 polyclonal antibody (EMD Millipore; AB5840, 1:2000) and mouse anti-α-Tubulin monoclonal antibody (Sigma-Aldrich, T9026, 1:10 000) as primary antibodies, as well as IRDye® 800 goat anti-rabbit and IRDye® 680 goat anti-mouse antibody (both LI-COR GmbH, 1:15 000) as secondary antibodies, were analyzed by ODYSSEY® CLx imager (LI-COR GmbH, Germany) and densitometry was analyzed by LI-COR software.

Animals

Thirty pregnant Sprague-Dawley rats purchased from Janvier Labs (France; http://www.janvier-labs.com/rodent-research-models-services/research-models/per-species/outbred-rats/product/sprague-dawley.html, last accessed 28 February 2016) were used for IUE at E16, with E1 considered to be the day after conception. The electroporated male outcome was used for behavioral testing (control group = 12; Dab1 shRNA group = 15). Animals were housed in groups of 2–4 animals, with a normal light–dark rhythm of 12 h and were fed ad libitum. All animal experiments were in accordance with National and European legislation and authorized by the responsible Landesministerium für Natur, Umwelt und Verbraucherschutz, North Rhine Westphalia, Germany.

In Utero Electroporation

The electroporations were performed over 3 consecutive weeks with 5 animals per day, 2 days per week. The pregnant rats were anesthetized at E16 with isoflurane and IUE was performed as previously described (Walantus et al. 2007; Rice et al. 2010). Shortly, the uterus horns were exposed and DNA was injected through the uterus wall into one of the lateral ventricles of the embryos with a thin glass capillary. The unilateral electroporation into progenitor cells of the medial–dorsal ventricular zone was performed with 5 pulses of 55 V using a Square wave pulse electroporator (CUY21SC; Napa Gene Co., Ltd, Japan). For Dab1 silencing, a Dab1 shRNA plasmid (pENTR-U6, 1.5 μg/μL; provided by Tracy L. Young-Pearse (Young-Pearse et al. 2007)), with the target sequence "gcatcaatgcgagctcatggag" (targeting exon 13 of transcript variant X1, XM_008763854.1), was injected into one of the lateral ventricles. As a control, a scrambled shRNA (1.5 μg/μL, kind gift from Tracy L. Young-Pearse) was used. For evaluating the success of electroporation, a luciferase vector (pCAX-Luciferase; 0.5 μg/μL) was co-injected for in vivo imaging as well as a GFP-containing plasmid (pCAGGS-AFP; 0.5 μg/μL) for histological analysis.

Live Imaging

Electroporation success was examined by in vivo imaging of P7 rat pups from the electroporation litters (Vomund et al. 2013). The bioluminescence that ensues after metabolism of intraperitoneally injected D-Luciferin through co-electroporated Luciferase was detected in an IVIS Live Imaging System (PerkinElmer). The emitted light from this reaction allows a calculation of an approximate positioning of the targeted area via 3D illustration.

Correlation analyses of live imaging signal with histological findings were done using Spearman's rank correlation coefficient by GraphPad Prism software.

Behavioral Tests

All male rats showing bioluminescence signal at P7 (Dab1 shRNA = 15, control shRNA = 12) were subjected to behavioral tests, starting at an age of around 3 months until the age of around 6 months, for simplicity in the following just referred to as P90 and P180, respectively.

The choice of behavioral tests was based partly on the findings with the *Dab1* knockout mouse indicating changes in the dopaminergic system (Michetti et al. 2014) and deficits in attention, learning, and memory (Jacquelin et al. 2012), as well as on

behavioral phenotypes reported in mice with migrational deficits induced by IUE (amphetamine supersensitivity; Niwa et al. 2010). The cognitive tests employed sample a wide range of subtypes of memory processes, including memory for objects, place, and temporal order, attention, and working memory in the T-maze and behavioral habituation to place.

Tests for recognition memory of objects, places, and temporal order were carried out based on the description by Barker et al. (2007). Each of these memory subtypes involve different neural forebrain mechanisms, which would be expected to be influenced by migration deficits in cortical regions (de Souza Silva et al. 2015). The studies were carried out in an acrylic open-field box with dimensions of 40 × 40 × 30 cm (w/d/h) within a sound and light isolated chamber. The behavior was monitored by a video camera and analyzed by EthoVision Software (Noldus Information Technology). Time at the novel or known objects, their visiting frequency and the distance moved by the animals was recorded.

Habituation

First, at P90, the animals were habituated to the open field by allowing them to freely explore it for 15 min on 2 consecutive days. In addition to locomotor behavior (distance traveled), the duration of rearing behavior was recorded.

Object-Recognition Task

The object-recognition test is based on the finding that rodents prefer to explore a novel object more than a familiar old one, indicating that they can recognize (remember) the old object (Ennaceur and Delacour 1988). Two identical objects (1.5-L plastic flasks with a height of 30 cm and filled with either clear or red colored liquid) were placed in defined corners of the box and the animal was allowed to explore them for 10 min (sample trial). After an intertrial interval (ITI) of 1 h, one object was exchanged by another different flask, and the animal's exploration was recorded for 10 min (test trial).

Object Place-Recognition Task

Rodents explore an object that has been displaced more than one left at the same location, indicating that they recognize the displacement and, thus, show memory for place (Ennaceur and Aggleton 1997). As in the object-recognition task, 2 identical objects were presented during the sample trial. During the test trial, one of these objects was relocated to another corner of the box.

Temporal-Order Memory Test

One week later, the animals were studied in a temporal-order memory test (Mitchell and Laiacona 1998). The rats underwent 3 trials (2 sample trials and 1 test trial). In the first sample trial, 2 identical objects were placed in 2 corners of the box; during the second trial, both flasks were exchanged by 2 different, but identical flasks. In the test phase, one object of sample 1 (old familiar object) and one object of sample 2 (recent familiar object) was presented. Again, the duration of the trials was 10 min with 1 h ITIs. Rats explore an object encountered previously in sample 1 more than a recently presented object from sample 2, demonstrating memory for recency (Mitchell and Laiacona 1998).

Re-trial Open-Field Exploration

At the age of approximately 5 months (P150), the animals were tested again for 15 min in the open field. For this purpose, the surface of 2 walls was changed by sticking a structured tissue onto the walls and placing an object (1.5 l plastic flasks with a height of 30 cm and filled red colored liquid) into the center of the Open Field. Using EthoVision Software (XT8), we assessed amount of locomotion, the length of time at the walls and number of direction changes during locomotion.

T-Maze

At P150, rats were also tested for spontaneous alternation behavior on 2 tasks using a T-Maze, with a 70-cm stem and 50-cm arms, all enclosed by walls with the height of 30 cm, and made of wood with dark brown coloring.

Continuous Spontaneous Alternation Behavior

The animals were placed into the starting arm of the maze and given 5 min to explore the maze. Triplets of visited arms (alternating arm entries, where all 3 arms were visited before entering one arm that was visited before) were recorded as a measure of exploratory behavior and working memory (Hughes 2004).

Two-Trial Alternation Behavior

One or 2 days after the continuous spontaneous alternation behavior (cSAB), the rats received a session of two-trial alternation behavior (2tSAB) with 6 trials and an ITI of 10 min, to study spatial working memory and responsiveness to novelty; for a review of this task and its interpretation see (Lalonde 2002; Hughes 2004). On each trial, the rats performed 2 runs in the T-maze with an inter-run interval (IRI) of 15 s, whereby the first run was forced by blocking one of the goal arms. The second run was a free-choice run where the animal could visit either the previously visited arm or the blocked one. The numbers of arm entries and time to enter a goal arm were measured.

Amphetamine Hypersensitivity Test

An enhanced behavioral response to an amphetamine challenge indicates supersensitivity of dopamine receptors (Seeman 2011). At P180, the animals were tested on 2 consecutive days for amphetamine hypersensitivity. On the first day, all animals received an intraperitoneal injection of phosphate-buffered saline (PBS; 1 mL/kg) directly before they were allowed to explore an empty open field (same as used for the memory tests) for 15 min. On the next day, a low dose of D-amphetamine (0.5 µg/g; Sigma-Aldrich, dissolved in PBS) was injected (1 mL/kg) before the 15 min trial. Behavior was recorded by a video camera situated centered above the box. Locomotion (total distance moved) and rearing (duration of rearing up on hind legs) were detected and analyzed using the EthoVision Software.

Staining and Microscopy

Animals were sacrificed with an overdose of isoflurane, except embryonic rats which were put on ice, and intracardially perfused with PBS. Brains were fixed in 4% paraformaldehyde. After cryo protection with 30% sucrose solution, brains were shock frozen in isopentane and stored at −80°C. A Cryostat (Leica 1900) was used to cut 25-µm sections.

For fluorescence microscopy, the brain sections were defrosted at 37°C and washed with PBS before dehydration in 70% Ethanol and 10 min incubation with 0.5% Sudan Black B solution (in 70% Ethanol). After washing in 70% Ethanol, the slices were rehydrated in PBS and mounted with Prolong Gold + Dapi. For antibody staining, after defrosting and washing, the sections were blocked with 10% normal serum in antibody diluent (Dako) for one hour before incubation with the first antibody overnight. Antibodies used were

rat anti-c-tip 2 (1:200, abcam, ab18465), rabbit anti-CDP (M-222) (Cux1; 1:200, Santa Cruz, sc-13024), and mouse anti-GFP (1:1000, Roche). For layer marker stainings, the sections had to be pretreated by cocking in "Target Retrieval Solution" (Dako, S1699) for 20 min. As secondary antibodies anti-rabbit Alexa Fluor® 594 and anti-mouse Alexa Fluor® 488 and anti-rat Alexa Fluor® 594 were incubated for 2–3 h. After washing, the sections were stained with Sudan black and mounted with Prolong Gold + Dapi.

To create fluorescence microscopy pictures a Zeiss Apotome II was used. Cell countings of GFP expressing/IUE targeted cells were done manually using ZEN 2012 software.

Results

Efficiency of Dab1 shRNA Plasmid

To validate the effects of Dab1 shRNA, NLF neuroblastoma cells were transiently transfected with a Dab1 overexpression vector and co-transfected with either control shRNA or the Dab1 shRNA plasmid. Cell lysates analyzed by western blot revealed a clear downregulation of *Dab1* expression in cells co-electroporated with the Dab1 shRNA (Fig. 1*B*). Densitometric evaluation with LI-COR software resulted in 61% reduction of Dab1 signal intensity in the cells co-transfected with Dab1 shRNA.

Furthermore, analysis of brain sections of in utero Dab1 shRNA-transfected rat brain revealed the expected migrational deficits (Figs 1*A* and 2). While, in P180-day-old control-treated rats, GFP-positive cells were spread throughout the cortex layers IV, V, and VI (Fig. 2*A*,*C*), neurons in the Dab1 shRNA-treated brains were basically found just above the white matter, with only some cells reaching layer V and VI (Fig. 2*B*,*D*). In Figure 1*A*, the difference in migration between Dab1 shRNA- and control shRNA-targeted cells is displayed in brain sections of rats at E22.

Identification of Successful In Utero Electroporation by Bioluminescence Live Imaging and Histological Findings

To select only those rats that were successfully in utero electroporated, in individual pups bioluminescence after luciferin injection was screened at 1 week after birth (P7) to detect co-transfected luciferase. The litters of 30 mother rats, either electroporated with Dab1 shRNA or control (scrambled) shRNA, were analyzed. From 77 rat pups electroporated with Dab1 shRNA, 22 (29%) individuals showed a signal elicited by luciferin, while the rest did not show any bioluminescence; for the control group, 26 out of 78 (33%) pups were positive. Only bioluminescence-positive animals were further analyzed. Later behavioral testing was performed exclusively with males. Accordingly, a group of 15 Dab1 shRNA rats and one group with 12 control shRNA were formed. Pathological findings after dissection of the brain at the end of all experiments (P180) revealed that 7 animals from the Dab1 shRNA group and 1 control rat showed abnormal formation of the cerebellum. The datasets of these animals were excluded from all prior experiments, for avoiding possible confounding effects of a dysplastic cerebellum on behavioral readouts.

As one major result of our imaging study using co-transfected luciferase and green fluorescent protein (GFP) to determine IUE efficiency, P7 luciferase signal strength was positively correlated (control: $r = 0.6818$, $P = 0.0251$; Dab1 shRNA: $r = 0.9759$, $P = 0.0003$; Spearman's rho) with the number of GFP-positive cells found in postmortem histological analysis in 6-month-old rats (Fig. 3*A*). This means that a high fluorescence signal in the young living pup predicts a large number of targeted cells also in the adult animal. Of note, none of the Dab1 shRNA animals exhibited a number of GFP-positive cells higher than 20 000, and, accordingly, at P7 no luciferase signal above $3.11 \times 10^{+06}$ photon counts was detected.

Migration Deficits and Neurodegeneration of Dab1

To investigate that the difference in targeted cells between controls and Dab1 shRNA animals at the age of 6 months (Fig. 3*B*; Mann–Whitney *U*-test: $P = 0.0202$) was not an effect of electroporation variability, histological analysis of electroporated brains before P7 (at E22 and P1) was performed. As shown in Figure 3*B*, even though an approximately equal number of cells were GFP-positive at E22, 2 days later, at P1, there were significantly fewer Dab1 shRNA cells GFP-positive compared with controls ($P = 0.013$; *t*-test), indicating that between E22 and P1 degeneration of Dab1 expressing cells had occurred.

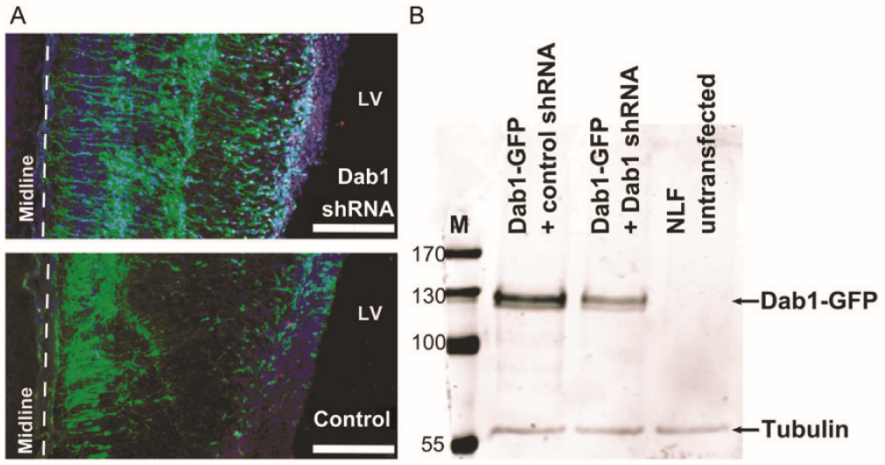

Figure 1. Evidence of Dab1 shRNA efficacy. (*A*) Sample E22 coronal sections of Dab1 shRNA (upper panel) and control shRNA (lower panel) electroporated brains showing prenatal migrational differences between the groups. LV, lateral ventricle; scale $bar_E = 20$ µm. (*B*) Western blot of NLF lysates from cells either transfected with Dab1-GFP (overexpression) and control (scrambled) shRNA, Dab1-GFP and Dab1 shRNA, or untransfected, showing an ~61% reduction of Dab1-GFP in cell lysates with co-transfected Dab1 shRNA.

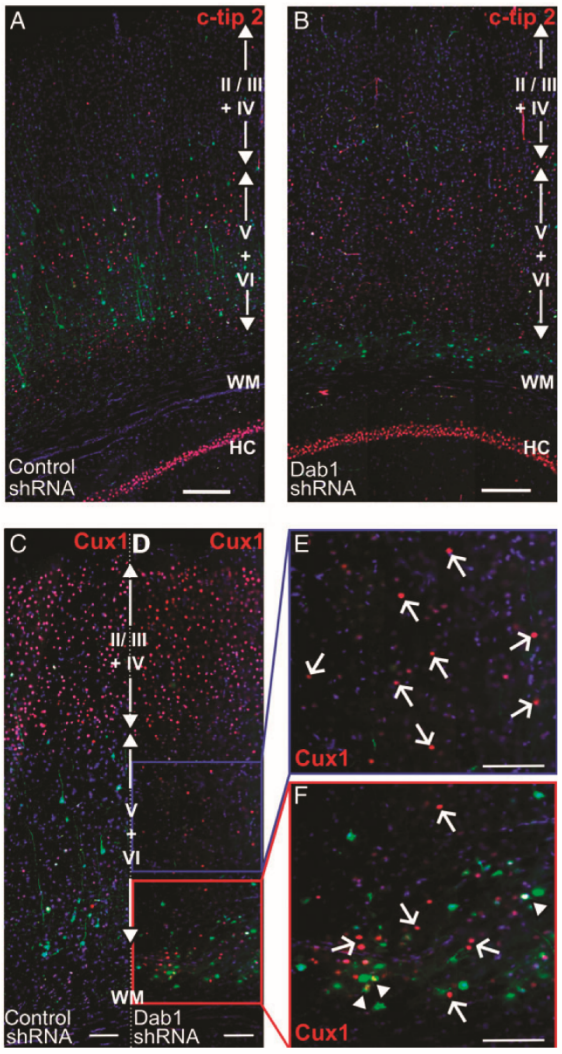

Figure 2. Migratory defects in the Dab1 shRNA-electroporated rat cortices. Sample coronal brain sections of 6-month-old rats electroporated with either Dab1 shRNA (*A*, *C*) or control shRNA (*B*,*D*) and stained with the layer markers c-tip 2 antibody (*A*,*B*) or Cux1 antibody (*C*,*D*). This illustrates the migration deficit of Dab1 shRNA-electroporated cells, which are ectopically situated underneath the c-tip2-positive layer and also recruit Cux1 cells in their subcortical heterotropia. (*E*,*F*) Magnified details of the Cux1-stained, Dab1 shRNA-electroporated section (*D*), to highlight the subcortical band heterotropia (*F*) and ectopically located Cux1-positive neurons (*E*,*F*). White triangles indicate GFP/Cux1-doubled stained cell; white arrows indicate examples for ectopically found Cux1-positive cells, HC, hippocampus; WM, white matter; scale $bar_{A+B} = 200$ μm; scale $bar_{C-F} = 100$ μm.

Fluorescence microscopy showed that most cortical areas targeted by IUE were located in the cortex between Bregma −0.5 mm and Bregma −4.5 mm (± 2 mm). Layer marker staining's with Cux1 and c-tip2 antibodies (Fig. 2) revealed that, in the control brains, the targeted cells had migrated mainly to layers V–VI (c-tip2 positive), with some cells reaching layer IV (Cux1 positive). In contrast, most Dab1 shRNA-targeted neurons stayed underneath the c-tip2-stained layer VI, and here, Cux1-positive cells, which refer to layer II/III and IV, were found ectopically in layers V and VI, as well as in the area with the GFP-positive cells just above the white matter (white arrows and triangles in Fig. 2F). This abnormal migration could be documented for 4 of the Dab1 shRNA in utero electroporated rats where a distinct area of GFP-positive cells could be identified in postmortem brain histology (P180).

Whereas in all control shRNA brains, neuronal GFP-fluorescing projections could be detected, reaching from the targeted area to the hippocampus and the thalamic formation, no such projections from GFP-positive cells were seen in the Dab1 shRNA animals (Supplementary Fig. 2), indicating that the effects of Dab1 shRNA electroporation had also consequences for remote connectivity, extending beyond the locally electroporated area.

Amphetamine Supersensitivity

Two-way ANOVA was used to analyze the distance moved (main effect for treatment $F_{1,16} = 34.468$; $P = 0.0001$) and duration of rearing (main effect for treatment $F_{1,16} = 10.727$; $P = 0.005$). These results indicate a higher behavioral response of the Dab1 shRNA rats to a low dose of amphetamine that was not sufficient to elicit a motor response in the control group. The Dab1 shRNA group ($n = 8$), but not the control group ($n = 11$), showed more locomotion (Fig. 4H, $P = 0.004$; post hoc paired t-test) and an increase in duration of rearing (Fig. 4I, $P = 0.04$; paired t-test) after the amphetamine injection compared with the saline injection given on the previous day.

Correlation of behavior with the localization (Bregma position) of the Dab1 shRNA-transfected area (Fig. 5D,E) for the amphetamine response revealed a higher locomotion response to more posterior transfected regions (Spearman $r = -0.8857$, $P = 0.0333$). Bregma position was defined by highest amount of counted GFP-positive cells in the postmortem brain. Due to the occurring cell death between E22 and P1 (Fig. 3B) in Dab1 shRNA-transfected cells, a correlation of the number of transfected cells (GFP-positive at P180 or bioluminescence at P7) with behavioral variables did not yield significant results.

Object Recognition, Object Place Recognition, and Temporal Order Memory

The 3-month-old IUE rats were tested in memory tasks, including object recognition, object place recognition, and temporal-order memory. During the performance of these tests, time at the novel or known objects, their visiting frequency, and the distance moved by the animals were recorded. The results indicated that the Dab1 shRNA animals and controls performed successfully on all 3 tasks and that there were no group differences on any task (data not shown). Rats from the Dab1 shRNA group, as well as control group animals also displayed comparable within-trial habituation to the open-field testing box during an initial habituation test, as shown by comparison of total distance traveled during the 10-min trials on 2 consecutive days (Fig. 4A).

Activity Level and Behavioral Habituation

Although there was no significant difference between the groups in object recognition itself, there was a difference in total distance moved (ANOVA: main effect on trial $F_{1,16} = 8.122$, $P = 0.012$; paired *t*-test for controls comparing the performance between the 2 trials: $P = 0.006$). While the control group displayed significantly less overall locomotion in the second (test) trial, the Dab1 shRNA animals traversed a similar distance in the test trial as in the sample trial. Since both groups exhibited comparable amounts of locomotion during the sample trial, the difference in overall locomotion during the test trial can be

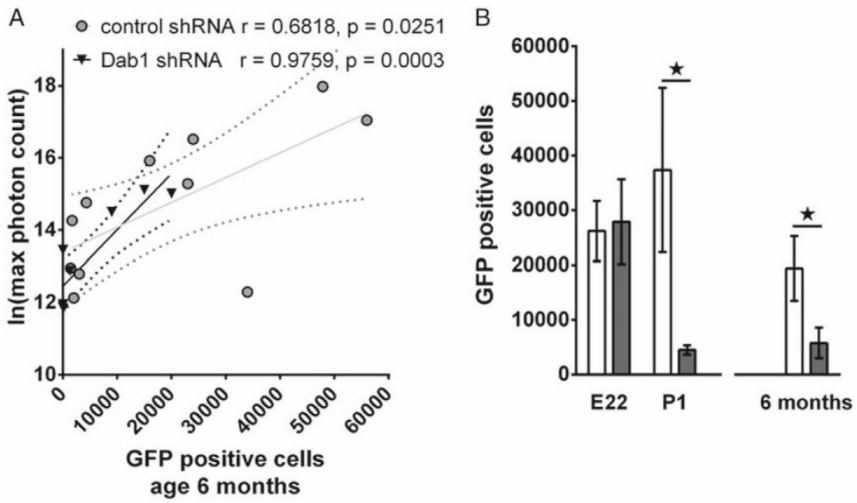

Figure 3. Correlations of Luciferase Luciferin Assay at P7 and histological findings in the brains of 6-month-old rats. (*A*) Correlation of number of GFP-positive cells from histological analysis in 6-month-old rat brains with bioluminescence signal from Luciferase assay derived from 1-week-old living rat pups (control: Spearman $r = 0.6818$, $P = 0.0251$; Dab1 shRNA: $r = 0.9759$, $P = 0.0003$; Spearman's rho). Thus, the number of transfected cells in the adult rat brains is predicted by the magnitude of the luciferase signal in the young pups in the control as well as in the Dab1 shRNA group. (*B*) Graph comparing histological findings of control group animals and Dab1 shRNA rats by displaying the number of GPF-positive neurons at E22 and P1 and old rat brains. At 6 months and at P1, there were significantly fewer GFP-positive cells in Dab1 shRNA rats than in control animals (P1:*$P = 0.048$, *t*-test; control shRNA $n = 3$, Dab1 shRNA $n = 4$; 6 months: *$P = 0.0202$, Mann–Whitney *U*-test; control shRNA $n = 8$, Dab1 shRNA $n = 11$). On the day before birth, at E22 (control shRNA $n = 6$, Dab1 shRNA $n = 3$), the number of targeted cells between Dab1 shRNA animals and controls did not differ significantly. Error bars displaying SEM values.

interpreted in terms of overall less behavioral habituation by the Dab1 shRNA group (Fig. 4*B*).

Two months later, they were again exposed to an open field in which the surface of 2 walls and wall cues were changed, and a single object was placed in the middle of the box. The total distance moved during the 2 trials on consecutive days revealed no evidence of habituation in either group (Fig. 4*C*). However, only the control animals spent significantly less time at the walls (ANOVA: for trial $F_{1,16} = 5.648$, $P = 0.030$; *t*-test, trial 1 vs. trial 2: $P = 0.008$), again, indicative of less habituation to the environment (Fig. 4*D*). Also, the control shRNA animals, unlike the Dab1 shRNA rats, exhibited significantly fewer changes in direction on trial 2 compared with trial 1 (ANOVA main effect on trial × genotype $F_{1,15} = 4.976$, $P = 0.041$; *t*-test, controls, trial 1 vs. trial 2: $P = 0.006$) (Fig. 4*E*). This result again may suggest less behavioral habituation in the Dab1 shRNA animals.

There were no indications for behavioral asymmetries in the Dab1 shRNA animals. Analysis of the side of the vibrissae employed in exploring the walls of the open field showed that there was no significant difference in vibrissal thigmotaxis (scanning of the walls) between the side of the face situated ipsilateral and contralateral to the electroporated hemisphere.

Bregma position (antero-posterior) of the main Dab1 shRNA-transfected area seemed to play a role in the re-trial open-field exploration. The distance moved during this trial correlated with the Bregma position of transfected are in the Dab1 shRNA animals on the second exploration day (Fig. 5*C*; Spearman $r = -0.8857$, $P = 0.0333$), meaning that the more posterior the main transfected area in the brain was, the higher the locomotion of the animal on the second exploration day.

Working Memory in T-maze

Working memory was assessed via 2 T-maze alternation tests. The cSAB test revealed no significant differences between the groups in triplets of visited arms (alternating arm entries before entering one arm that was visited before). In the 2tSAB, both groups performed above chance level (Fig. 4*F*, control $P = 0.002$; Dab1 shRNA $P = 0.003$; *t*-test) and no significant difference in alternation was observed. The Dab1 shRNA animals took longer than the controls to enter the familiar arm during the free-choice run (Fig. 4*G*, ANOVA: effect for genotype $F_{1,15} = 4.928$ $P = 0.042$; *t*-test $P = 0.011$), while time to enter the alternating/nonfamiliar arm was similar for both groups. This hesitation could be indicative of conflict in decision-making behavior, but no other comparable behaviors that would support such an interpretation were observed. On the other hand, this hesitation also argues against a hypothesis that the Dab1 shRNA animals would exhibit more "impulsive"-like behaviors.

Discussion

The main findings of this work are that 1) IUE of Dab1 shRNA in rats at E16 led to a migratory deficit and neuronal degeneration; 2) The success of IUE was reliably detected by in vivo bioluminescence screening; 3) The subtle migratory deficit in adults increased sensitivity to amphetamine challenge, suggesting a likely influence on dopamine-related neurotransmission; and 4) Behavioral habituation was impaired, whereas performance on memory tasks assessing working memory, memory for objects, place, and temporal order was intact and apparently resilient to the lesion.

First, neurodevelopmental origins of behavioral disorders such as schizophrenia have long been hypothesized and considerable experimental evidence supports this view, like reduced cortical thickness, enlarged ventricles, and increased neuronal density (Murray and Lewis 1987; Weinberger 1987; Selemon et al. 1995; Selemon and Goldman-Rakic 1999). As recently reviewed in Schmidt and Mirnics (2015), several genes/proteins

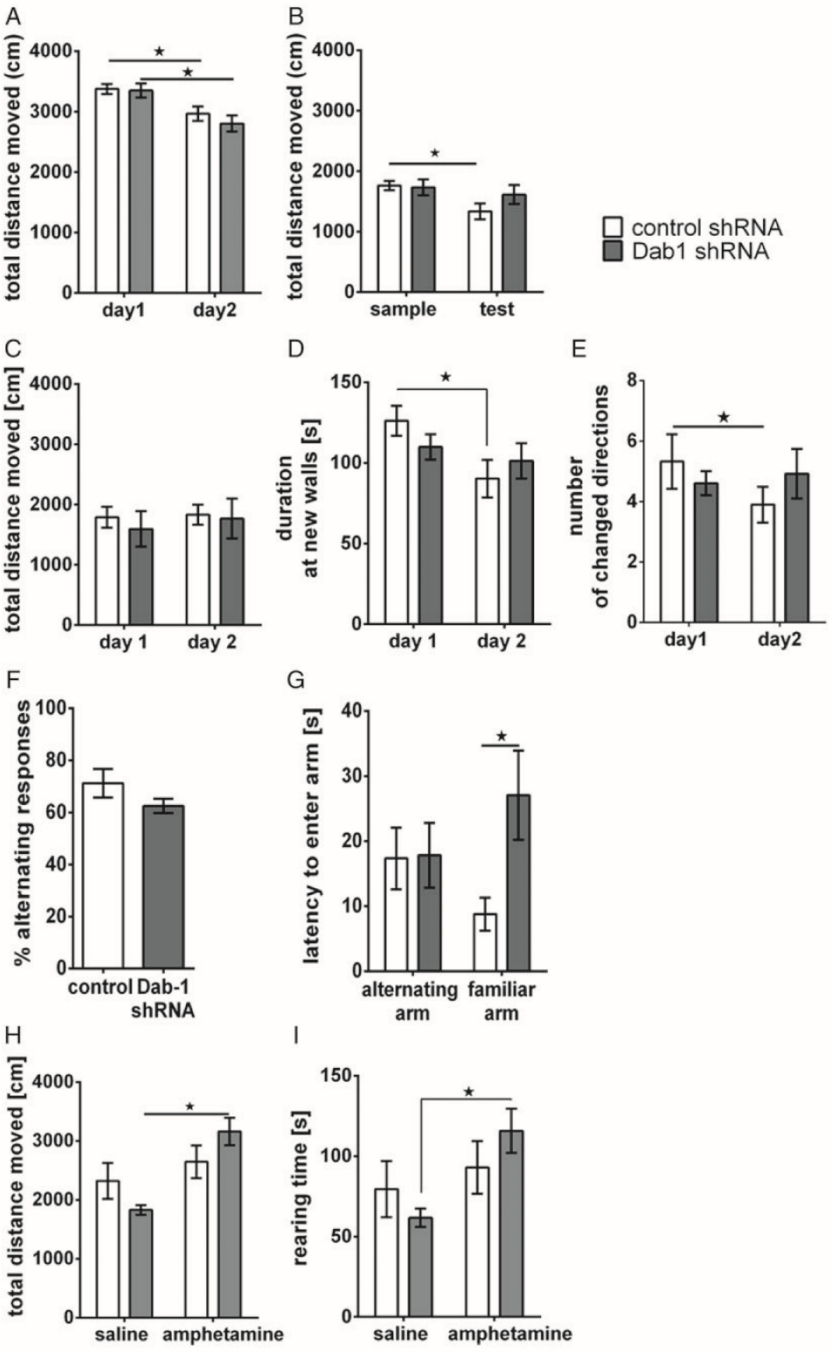

Figure 4. Open-field tests. (*A*) Both groups showed comparable behavioral habituation to the open field by virtue of exhibiting less locomotion on trial 2 versus trial 1 (control $P = 0.01$; Dab1 shRNA $P = 0.048$; paired *t*-test). (*B*) During the object-recognition test, only the control animals covered significantly less distance (behavioral habituation) during the test trial (ANOVA: main effect of "trial" $F_{1,16} = 8.122$, $P = 0.012$; $^{\star}P = 0.006$, paired *t*-test). (*C–E*) Retesting for habituation in a new open-field: (*C*) There was no significant reduction in total distance moved from trial 1 to trial 2 in either group. (*D*) Only the control animals spent significantly less time at the walls on trial 2 than on trial 1, indicating behavioral habituation (ANOVA: effect for "trial" $F_{1,16} = 5.648$, $P = 0.030$; controls: $P = 0.008$; paired *t*-test). (*E*) Only the control group exhibited significantly fewer changes in walking direction. (ANOVA main effect on trial × genotype $F_{1,15} = 4.976$, $P = 0.041$; *t*-test, controls, trial 1 vs. trial 2: $P = 0.006$). "T-maze: 2-Run alternation test": (*F*) Percentage of alternating responses in the free-choice run of 7 trials, showing no differences between the groups in alternation behavior. (*G*) Latency to enter the alternating and familiar arms of the T-maze during the free-choice run. While both groups needed the same amount of time to enter the alternating arm, the control group was significantly faster in entering the familiar arm (ANOVA: effect for genotype $F_{1,15} = 4.928$ $P = 0.042$; *t*-test: $^{\star}P = 0.011$). "Amphetamine challenge": Distance moved of control and Dab1 shRNA animals after saline injection on day 1 and amphetamine injection (0.5 µg/mg) on day 2. (*H*) Distance moved after amphetamine challenge was significantly longer than after the previous saline challenge only in the Dab1 shRNA group (main effect for treatment $F_{1,16} = 34.468$; $P = 0.0001$; paired *t*-test: $^{\star}P = 0.001$). (*I*) Total duration of rearing after low-dose amphetamine was significantly longer than after previous saline challenge only in the Dab1 shRNA group (main effect for treatment $F_{1,16} = 10.727$; $P = 0.005$; paired *t*-test: $^{\star}P = 0.01$). Error bars displaying SEM values.

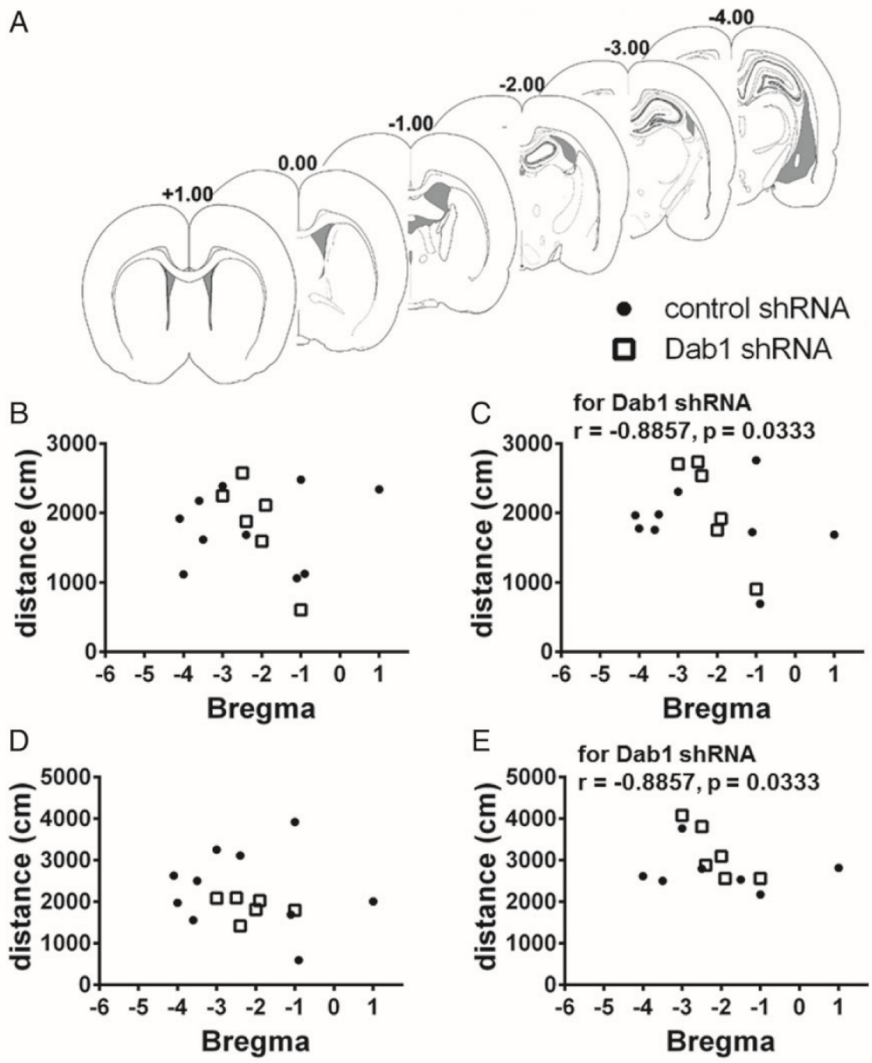

Figure 5. Correlation of localization of migration deficit with behavior. Main targeted area defined by the Bregma position of majority of GFP-positive cells in P180 brains. (*A*) Scheme of brain architecture with approximate Bregma position of the main targeted areas (according to atlas (Paxinos and Watson 2007)); (*B*,*C*) Distance moved in the re-testing for habituation in a novel open field. Whereas no significant correlation was found for either group on the first day in the open field (*B*), at re-testing for habituation (P150) only the in utero Dab1 shRNA-treated rats exhibited a significant negative correlation with localization of the induced area (*C*; Spearman $r = -0.8857$, $P = 0.0333$). (*D*,*E*) Locomotion after amphetamine challenge: (*D*) Upon saline injection, neither groups exhibited significant correlation with targeted area; (*E*) Dab1 shRNA animals exhibited behavioral differences dependent on the Bregma position (Spearman $r = -0.8857$, $P = 0.0333$). The negative correlation indicates that the more posterior the main transfection area in the cortex, the higher the level of locomotion in both tests.

connected to neurodevelopment, such as NRG1 and DISC1, are aberrantly expressed in schizophrenic patients. Also, reelin expression has been reported to be reduced in neuropsychiatric disorders, such as schizophrenia and autism (Impagnatiello et al. 1998; Fatemi 2005). Therefore, we decided to disrupt the reelin signaling pathway by knock down of Dab1 to induce a subtle migratory deficit.

In the present study, we paradigmatically show a clearly characterized migratory deficit in a limited number of 10–50 000 neurons targeted with Dab1 shRNA by IUE which, perinatally, leads also to the disappearance of a significant number of these neurons. Likely, the local disturbance of connectivity, as evidenced by the lack of projections from Dab1 shRNA-targeted cells (Supplementary Fig. 2), contributes to this effect. It is tempting to speculate that the in utero induced, local genetic alteration of the reelin/ Dab1 pathway has led to an axonal pathfinding problem very different from that of a complete Dab1 knockout animal with inversion of cortical layering where, on the level of immediate neighboring connections, connectivity is largely conserved.

The migratory deficit of cells targeted with Dab1 shRNA, was very distinct (Fig. 1A; Fig. 2). Whereas in the control group the targeted, GFP-positive neurons were distributed over cortical layers IV, V, and VI and their projections extended to parts of the limbic system (Supplementary Fig. 2), in the Dab1 shRNA animals, GFP-positive neurons mainly stuck just on top of the white matter and no projections from these cells to the hippocampus or thalamic formation were detected. Missing connections to the thalamus

can be a result of failure of the targeted neurons to pass the subplate to reach their destination layers V and VI from where trajectories to the thalamus extend (Crandall and Caviness 1984; De Carlos and O'Leary 1992).

The bioluminescence strength at P7 as well as the number of GFP-positive cells was distinctly lower in rats electroporated with Dab1 shRNA than in controls (Fig. 3). Since this is unlikely to be due to the variability of IUE success (no such high variability was seen within the control group), the large difference between the groups suggests that Dab1 shRNA-targeted neurons died during brain development. We narrowed down the time point of their death as being between E22 and P1 (Fig. 3B). This time point corresponds to a developmental phase where many cells are subject to programmed cell death (PCD). Between the end of prenatal and early postnatal development cells undergo apoptosis especially within zones of proliferation (Blaschke et al. 1996). Programmed cell death fulfills multiple functions in brain development, occurs during regression of vestigial organs, during morphogenesis of organ anlage as well as during remodeling of tissues (Kuan et al. 2000). We propose that cells that did not migrate properly due to Dab1 knockdown and were forced to stay in/near the zones of proliferation, respectively, underneath the transient subplate, and then underwent PCD, like the majority of cells in this area (Kostovic and Rakic 1990). In contrast, control cells correctly reached the cortical plate, which is not as prone to PCD as the proliferative zones (Fig. 2). Alternatively, Dab1-silenced neurons could undergo neurodegeneration due to malfunction, caused by disturbed neuronal maturation like dentrite outgrowth, synaptic plasticity, and hence building-up cell connectivity, wherein Dab1 protein is also involved (Niu et al. 2004; Beffert et al. 2006). It is tempting to speculate that birth-associated changes, like stress or the on-switch of certain brain functional circuitry could play a role in the PCD of these neurons.

In studies of similar design, using Dab1 knockdown by IUE but with mice (Olson et al. 2006; Sekine et al. 2011), no massive perinatal cell death was observed. To explain this discrepancy, we propose differences in target specificity of the shRNA construct used and/or species differences in the functions of mouse and rat Dab1. While mouse Dab1 is spliced into 8 variants, rat Dab1 is only spliced into 2 variants. As reviewed by Gao and Godbout (2013), different splice variants have different functions and expression patterns during brain development. In most reports using mice, the phosphorylation region of Dab1 is the shRNA target site, and sometimes a combination of several Dab1 shRNAs to different transcripts was used (Olson et al. 2006). We used a shRNA targeting nucleotides 2388–2409 of transcript variant X1 of rat Dab1 that has been successfully used in rats by Young-Pearse et al. (Young-Pearse et al. 2007). Unfortunately, Young-Pearse et al. did not look at the fate of Dab1 shRNA-electroporated migrating neurons beyond E19. Furthermore, there is evidence for species differences between rats and mice in cortical migration. For example, in mice, laminar dysplasia after knockdown of doublecortin was observed, whereas in rats subcortical band heterotropia was observed (Ramos et al. 2006), quite similar to what we discovered in Dab1 shRNA-electroporated rats.

Second, we demonstrated that the number of in utero electroporated neurons in postmortem brain correlated to the in vivo bioluminescence signal at the age of 1 week allowing monitoring of IUE success with the IVIS Spectrum imager shortly after birth (Fig. 3A). We earlier reported the possibility of detecting a bioluminescence signal up to P30 (Vomund et al. 2013), but did not validate these data with histological results. The present study, therefore, demonstrates for the first time a correlation of IVIS live imaging bioluminescence signal in 1-week-old living animals with the number of GFP-positive cells in brain sections of 6-month-old rats (Fig. 3A), thus validating that in vivo bioluminescence detection for controlling IUE success is predictive of the number of electroporated neurons at 6 months of age. These finding indicate that it is possible to predict the adult amount of IUE cells correctly from perinatal bioluminescence imaging.

Third, the Dab1-silenced rats displayed several subtle behavioral abnormalities: they exhibited an increased sensitivity to an amphetamine challenge, akin to similar changes in the dopamine system that has been linked to schizophrenia (Seeman 2011). They also exhibited more exploration/locomotion upon repeated testing in a familiar environment, indicative of deficient behavioral habituation or attention. The increase in locomotion was significantly correlated with more posterior localizations of the IUE-transfected areas (Fig. 5).

Here, similar to other prenatal lesions, such as an unspecific immune activation (Vuillermot et al. 2010), or genetic lesions (Niwa et al. 2010; Vomund et al. 2013), the disruption of homeostasis of the dopamine system may be a converging point. The rats in utero electroporated with Dab1 shRNA exhibited an enhanced behavioral response to a low dose of amphetamine. Such an enhanced sensitivity to amphetamine challenge is indicative of a supersensitivity of the brain's dopamine receptors and is one of the most important models for schizophrenia (Seeman 2011). Niwa et al. found that knockdown of DISC1 by IUE in cells of the prefrontal cortex of mice resulted in hypersensitivity to administration of methamphetamine in adult (P56) mice, consistent with a dysfunction of the dopaminergic system (Niwa et al. 2010). Unlike Niwa et al. (2010), however, we found no evidence for impaired novel object recognition, nor deficits in any of the various learning/memory paradigms we examined, which could indicate different functions of the DISC1 versus reelin systems, or be a result of a lower extent of neuronal disruption evoked by unilateral electroporation in our preparation.

Finally, we expected to find behavioral changes in the electroporated rats akin to those reported with the full Dab1 knockout mouse, in which behavioral changes indicative of deficits in attention, learning and memory were present (Jacquelin et al. 2012). However, we found no evidence for deficient memory for objects, for place memory or memory for temporal order. Neither did we find changes in spontaneous alternation in the T-maze, nor in working memory. The intact performance on these cognitive tasks may indicate a resiliency to the neuronal changes incurred by the in utero insult, but may also indicate that number of affected neurons were too small.

The lack of effect on various cognitive parameters may indicate a resilience of the brain to a short-lasting disruption of neuronal migration related to reelin. Resilience to early insult can also reflect redundancy in the neural circuits that underlie learning and memory processes, or it may suggest compensation for early disruption via functional neuronal reorganization/plasticity of neural/behavioral systems that are critical for survival. It should also be considered that our IUE was administered unilaterally, which may have prevented lasting behavioral disruption via compensatory processes as well as redundancy provided by the intact hemisphere. Of note, although the IUE was carried out unilaterally, the adult animal exhibited no signs of behavioral asymmetries which might be expected by a unilateral insult to the brain (data not shown), again suggesting robustness in the development of sensory-motor systems in face of early damage.

Although the unilateral electroporation did not lead to memory deficits in object recognition, object place recognition, and temporal-order memory and working memory, the results are

suggestive of a deficit in behavioral habituation. A persistency in responding to a familiar environment can indicate an attenuated habituation to environmental stimulation (Cerbone and Sadile 1994). This can, in turn, be a result of either 1) deficient attention to the environment, 2) deficient memory for a familiar set of stimuli, or 3) an overall higher responsiveness to the environment. A higher responsiveness, in turn, could be indicative of higher level of arousal, lack of inhibition and impulsiveness, which might all be expected in an animal exhibiting higher sensitivity to a dopaminergic stimulant, as the Dab1 shRNA animals displayed to the amphetamine challenge. Interestingly, an increase in behavioral responsiveness to amphetamine challenge along with deficient attention could indicate a schizophrenia-related phenotype, as would be predicted from an association study which links reelin signaling with schizophrenia (Verbrugghe et al. 2012).

In summary, our data suggest that disruption of reelin signaling of a limited number of neurons during migration can have lasting neurological and subtle behavioral consequences in the adult animal. On the other hand, the lack of effect on various cognitive parameters (object recognition, spatial memory, temporal-order memory, working memory) suggests a resiliency of the brain to a short-lasting disruption of neuronal migration involving reelin pathways, which may either indicate redundancy in the neural systems that subserve cognitive functions, or robust ability of behavioral systems critical for survival to compensate for such disruption via functional neuronal reorganization.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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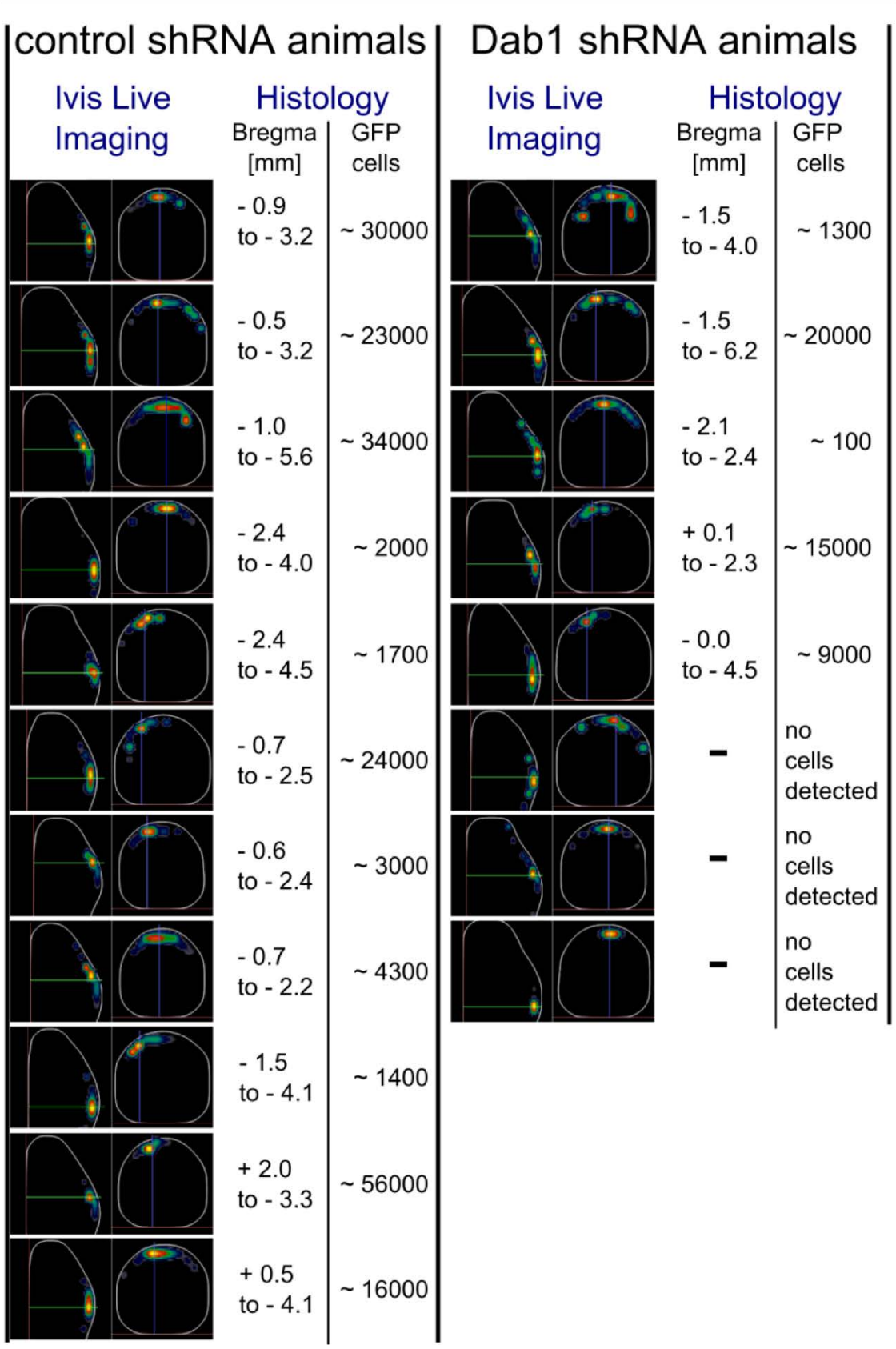
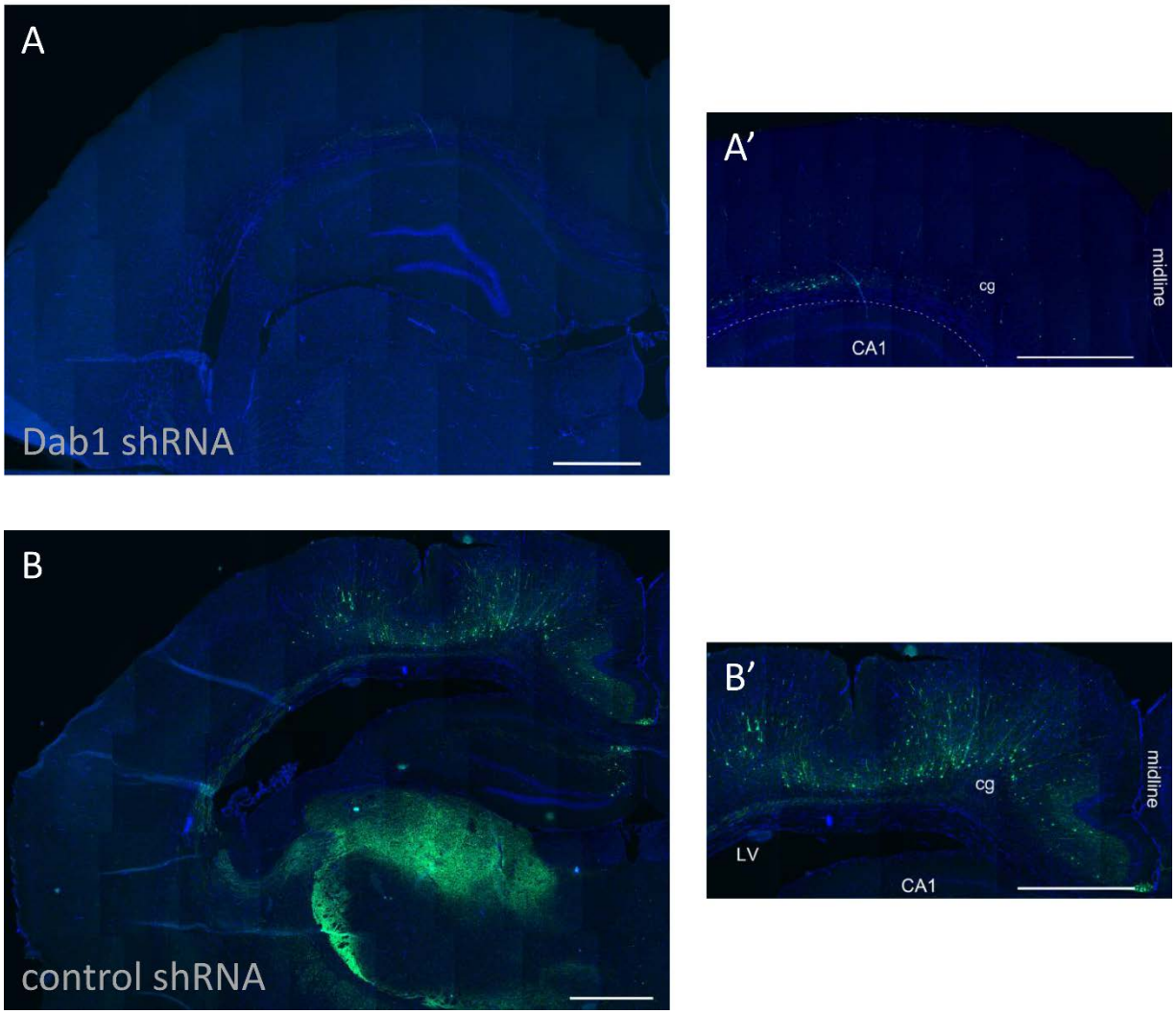
Supplement:

S1



S2


A
Dab1 shRNA
A'
cg
midline
CA1
B
control shRNA
B'
cg
midline
LV
CA1

Supplemental Figure 1

Bioluminescence and histological examination of the rats used for behavioral studies

Comparison of results from bioluminescence analysis after D-luciferin injection at P7 with coronal and sagittal view of IVIS Spectrum 3D animation and the histological results with number and approximate position of GPF positive (=targeted) cells in the brains of every P180 rat used for behavioral experiments.

Supplemental Figure 2

Migratory and connectivity differences between Dab1 shRNA and control shRNA electroporated brains

A) Coronal brain section of a six-months old rat electroporated with Dab1 shRNA (with higher magnification shown in A'), showing targeted GFP positive cells located in the cortex just above the with matter without any green trajectories to the thalamus or the hippocampus.

B) Coronal brain section of a six-months old rat electroporated with control shRNA (with higher magnification shown in B'), showing spreading of targeted cells in the cortical plate. GFP expressing trajectories from the cortex to the thalamus and also connections to the hippocampus are visible.

4.3 Study III

Title **Efficient clearance of amyloid-β in a novel mouse model displaying somatic mutations for Alzheimer´s disease in single neurons**

Authors Sandra Vomund, Hannah Hamburg, Andreas Müller-Schiffmann, Carsten Korth

- Manuscript in revision -

Author's contribution

(~80 %)

design of experimental setup

in utero electroporation

live imaging

intracardiac perfusion & brain dissection

brain sectioning

immunohistochemistry & histological stainings

microscopy

data analysis

writing the manuscript

Efficient clearance of amyloid-β in a novel mouse model displaying somatic mutations for Alzheimer´s disease in single neurons

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keywords:

Alzheimer's disease, amyloid precursor protein, *in utero* electroporation, somatic mutations, mouse model

Main text

Alzheimer's disease (AD) is an invariably fatal neurodegenerative disorder and the most common form of dementia. Even though the identification of familial cases has helped elucidating essentials of the disease mechanism focusing on aberrant amyloid precursor protein (APP) processing as a main inducer of downstream changes like tau hyperphosphorylation and neuronal cell death, the molecular cause of the majority of sporadic forms of Alzheimer's disease has remained unclear.

As one possible cause of sporadic Alzheimer's disease, the occurrence of somatic mutations within the three major AD genes, APP, presenilin (PSEN) 1 and 2 has been hypothesized. The idea is that somatic mutations could lead to aberrant production of aggregation-prone amyloid-β (Aβ) species that would provide nuclei for resident extracellular Abeta monomers or oligomers and thus trigger Aβ aggregation with its known downstream effects.

Indeed, recently, using single cell quantitative PCR and peptide nucleic acid fluorescent *in situ* hybridization, it was reported that in brains of AD patients, increased APP copy numbers or variations thereof are present in small cohorts of neurons especially in the cortex (Bushman et al., 2015). Furthermore, using a deep sequencing approach, the presence of mosaic single nucleotide variants having a mutant allele frequency of 1% could be determined (Sala Frigerio et al., 2015). However, to this end, the actual mechanistic impact of somatic mutations to trigger AD or AD-like neuropathology, for example, via providing Aβ plaque seeds, has not been studied in suitable animal models.

Here, we report a technique to model somatic mutations in a limited number of cortical cells in the mouse. *In utero* electroporation (IUE) is a technique to manipulate gene expression in specific brain regions by inducing genes or silencing constructs during embryogenesis via electroporation (Tabata and Nakajima, 2001). So far, IUE has mainly been used to study

neurodevelopment, mostly related to major mental illnesses (for review see Taniguchi et al., 2012). We previously discovered that IUE could also be used to model the influence of neuronal migration deficits on adult behavior and demonstrated protein expression of IUE transfected genes up to 6 months in rats (Vomund et al 2016).

We investigated the use of IUE to model somatic mutations of the pathogenesis of AD-like neuropathology, specifically Aβ formation. Since AD-like pathology in humans as well as in many rodent models develops only in advanced age, the first question was whether gene expression of IUE administered genes would still be present after 12 or more months. The second specific question was whether the prolonged expression of mutant APP in conjunction with mutant PSEN1 would lead to an accumulation of Aβ in the vicinity of the transfected cells.

Human APP (harboring the Swedish (KM670/671/NL) and Indiana (V717F) mutations), as well as mutated presenilin 1 (L166P) was electroporated into neuronal progenitor cells of the cortex of NMRI mice at embryonic day 14. Both mutant proteins have been shown to increase the level of aggregation prone $A\beta_{42}$ (Murrell et al., 1991; Mullan et al., 1992; Moehlmann et al., 2002). In order to identify and track successfully IUE mice (NMRI strain), we used *in vivo* bioluminescence imaging through a co-transfected luciferase gene (Vomund et al., 2013; Vomund et al., 2016). We successfully demonstrate a solid bioluminescence signal up to the age of 1.5 years, although the signal decreased in adult ages (Figure 1). Furthermore, signals in 8 months old mice correlate with the initial signals at the age of 1 week (Figure 1E). Signal reduction can be caused by enlarged tissue thickness of the grownup brain, due to decreasing tissue penetration of light. Alternatively, reduced permeability of the blood-brain-barrier for substrate luciferin in the adult brain may impact bioluminescence measurement since brain access for luciferin is limited (Lee et al., 2003; Berger et al., 2008) (Ayzenberg et al., 2015). BBB disruption might explain two outliers in Figure 1 C which actually showed increased bioluminescence upon live imaging before dying unforeseenly.

Immunohistology was performed on *post mortem* brain sections at different ages. To easily identify the targeted area of the electroporation, the signal of co-electroporated green fluorescence protein (GFP) was quantified (Figure 2 A). An extrapolation of counted GFP positive cells in 1.5 year old mice (n=3) revealed that the electroporated area included an approximate number of 6000 cells. We can thus conclude that, surprisingly, IUE of a supposedly episomal plasmid still leads to gene expression 1.5 years later, and, thus, that this procedure is well suited to model somatic mutations.

Immunohistological staining with human-specific Aβ antibody 6E10 in 18 months old brains showed fluorescence in cells in the targeted cortical area (Figure 2 B), indicating that recombinant human mutant APP was indeed expressed. However, Aβ plaques were not detected, neither in the vicinity of 6E10-positive cells nor remote from the electroporated area, unlike in the CRND8 transgenic mouse model where Aβ plaques were abundant at that age (data not shown). This indicates that even a locally restricted induction of AD-like pathology did not occur, in contrast, for example, to germline overexpression of APP_{SWE} and *PSEN1 (L166P)* genes (Radde et al., 2006).

We conclude that around 6.000 cells harboring pathogenic APP/PSEN1 mutations and thus likely to lead to increased levels of insoluble Aβ are not sufficient to lead to Aβ plaque pathology. We conceive several possible explanations: 1. 6.000 Aβ aggregation-producing cells are not enough to set a nucleus of an Aβ plaque against a steady physiological clearance of insoluble Aβ, 2. The somatic mutations need a specific extracellular proteome background, for example expressing sufficient amounts of human APP (Aβ) in order to trigger nucleation of Aβ plaques.

In summary, we demonstrate that IUE combined with *in vivo* bioluminescence is a convenient technique to investigate somatic mutations and their impact on chronic brain diseases.

Abbreviations:

Aβ= Amyloid beta

AD = Alzheimer's disease

APP = amyloid precursor protein

GFP = Green fluorescence protein

IUE = *in utero* electroporation

PSEN = presenilin

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Authors' contributions

CK conceived, planned and supervised the experiments, interpreted results, and drafted the manuscript; AMS planned and supervised the experiments, interpreted results, and drafted the manuscript; HH was involved in histological analysis; SVO created the mouse model, performed experiments, interpreted results, and drafted the manuscript

None of the authors has any competing interests of any kind in the present manuscript.

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Figure Legends

Figure 1: Identification and tracking of electroporated areas and bioluminescence intensities at different time points

A+B) *In vivo* imaging of the same mouse at the age of 1 week and 1.5 years shown in a 3D reconstruction (right panel) and the three sectional planes. Color scale bar indicates bioluminescence intensity in photons counts per seconds.

C) Comparison of the mean radiance (pixel/second/cm²/steradian) of the bioluminescence signal from 1 week old and 3 months old mice (n = 3). The graph shows that only 0,2 % of the initial luminescence signal strength is recorded in the adult mice.

D) Bioluminescence detection over time for all mice with a minimum age of 8 months displays a strong reduction in the relative luminescence unit (RLU) after the 1 week measurement, while after 8 months the detected signal stays on the same level.

E) Comparison of bioluminescence signal strengths of 1 week and 8 months old mice shows significant correlation for the two time points (spearsons r = 0.7366, p = 0.0063, n = 12).

Figure 2: Immunohistological analysis of brain sections

A) 4 µm paraffin sagital brain section of an 1.5 year old electroporated NMRI mouse, that displays GFP expressing cells in the cortex. Indicating the electroporated area and amount of targeted cells. Scale bar 1000 µm

B) Detection of IUE targeted cells with GFP (green) and expression proof of electroporated target gene with anti-human APP (6E10) antibody (red) in a 4 µm paraffin section of a 1.5 year old mouse cortex. Scale bar 100 µm

Figure 1

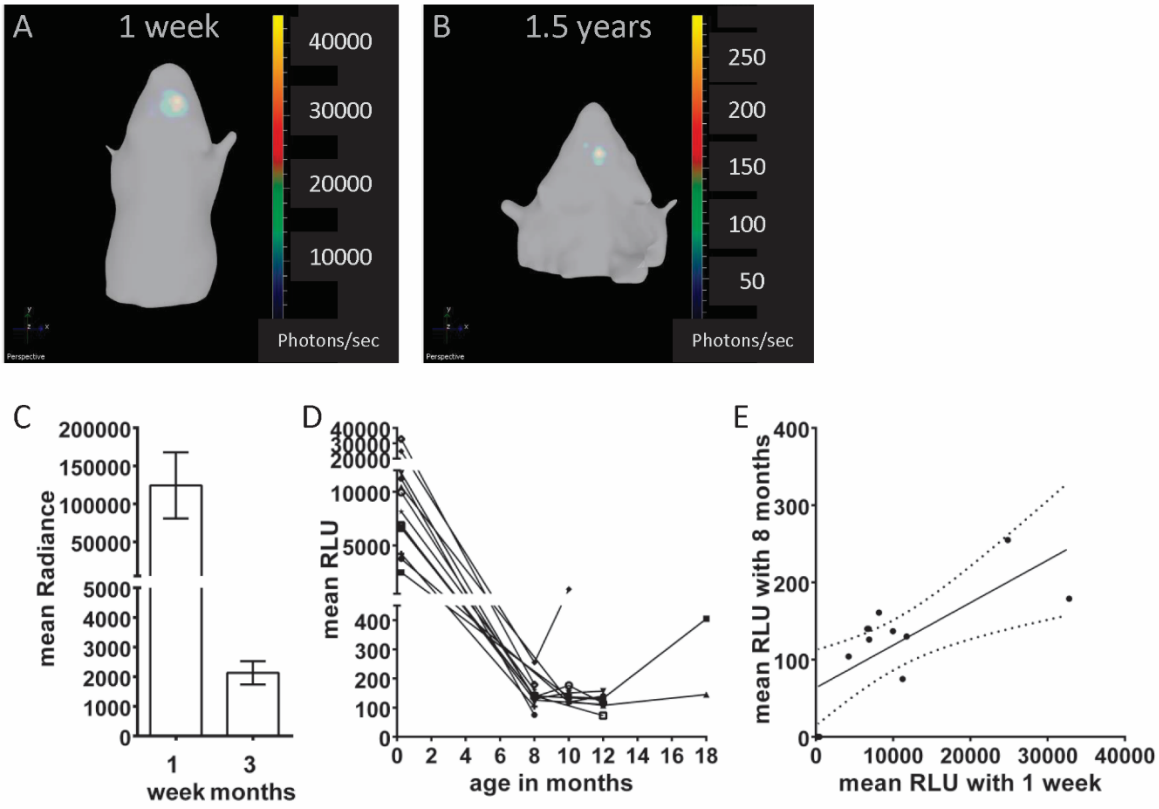
A
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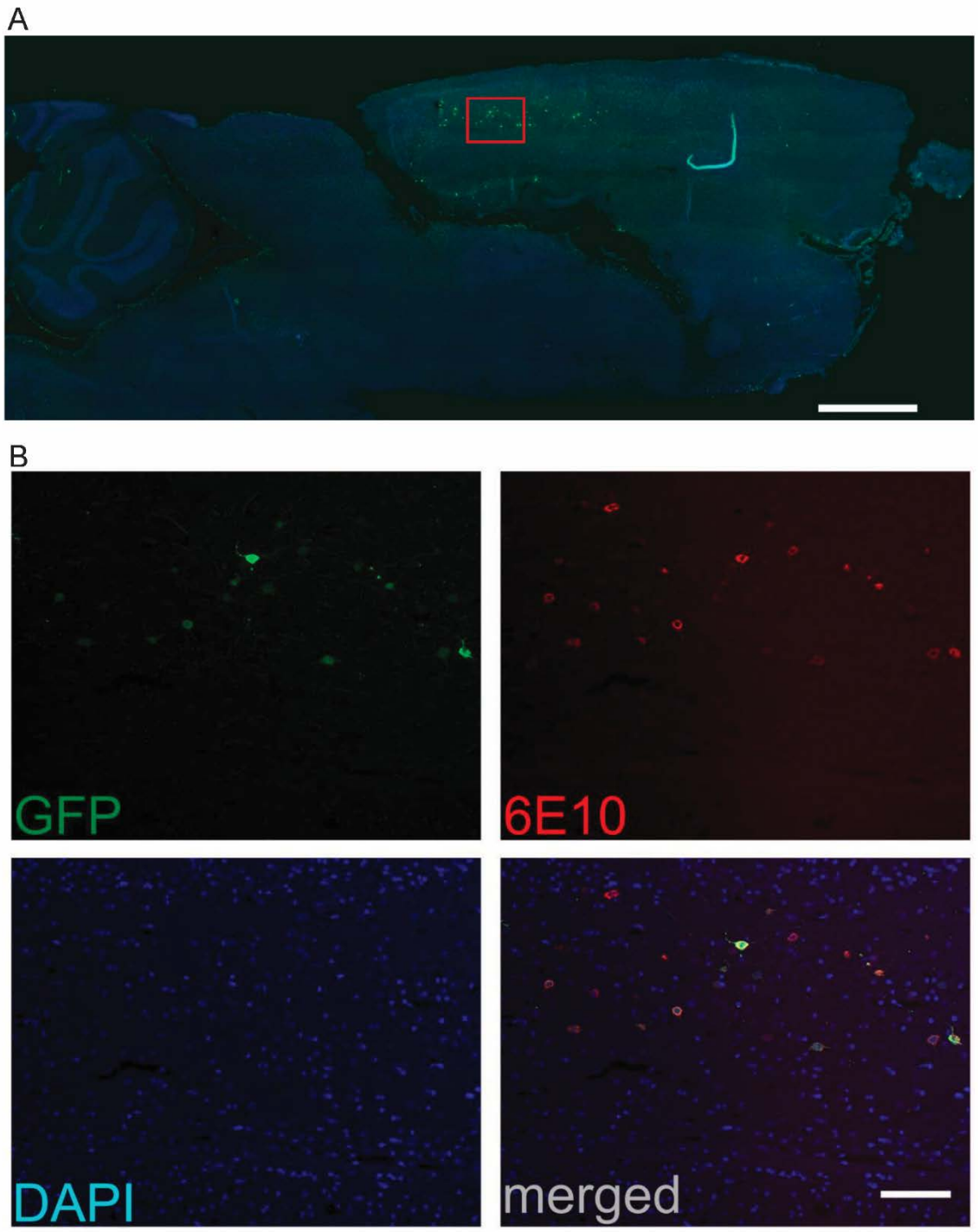
Figure 2



5 Discussion

This thesis aimed at the modeling of human brain diseases in rodents, to study the underlying mechanisms of the diseases. The following objectives have been pursued 1) establishment of *in utero* electroporation to study behavioral or neuropathological consequences, 2) creation of a method combinable with IUE to select born animals for electroporation success, and 3) investigation of IUE suitability for modeling somatic mutations. As discussed in detail in the next paragraphs these aims have successfully been achieved.

In utero electroporation

The *in utero* electroporation technique with rats and mice could be established to model behavioral and neuropathological phenotypes. The IUE protocol was adjusted to the animal facility conditions and the choice of animal strain was evaluated. For example, instead of Wistar rats that were available but not breeding very well in the animal facility, Sprague Dawley rats purchased from Janvier Labs (http://www.janvier-labs.com/rodent-research-models-services/research-models.html) were used. For IUE with mice NMRI mice were employed. These mice are known to be good breeders compared to the C57BL/6, FVB and SJL mice, which were first considered as mouse models. The anesthesia method was also changed from injected substances, namely a ketamine/xylazin combination, to the inhalational anesthetic isoflurane. With isoflurane the anesthesia could be induced more rapidly, animals used to wake up shortly after surgery and therefore the overall investigation time was reduced.

Although IUE worked in both rats and mice, a comparison between the rats used in study II and the mice used in study III revealed that the performance in mice was superior (Figure *5.1*). While the mean number of embryos per mother at the IUE time point, of electroporated embryos and even of born pups are comparable between the two species, the number of positive pups shown by live imaging at the age of one week was drastically reduced in rats. In mice, approximately 75 % of the born pups showed a positive signal in the bioluminescence screen at the age of one week. In rats only about30 % of the born pups showed a positive bioluminescence signal. The percentage of one week old positive pups compared to the originally electroporated embryos was 52°% in mice and only 16 % in rats.

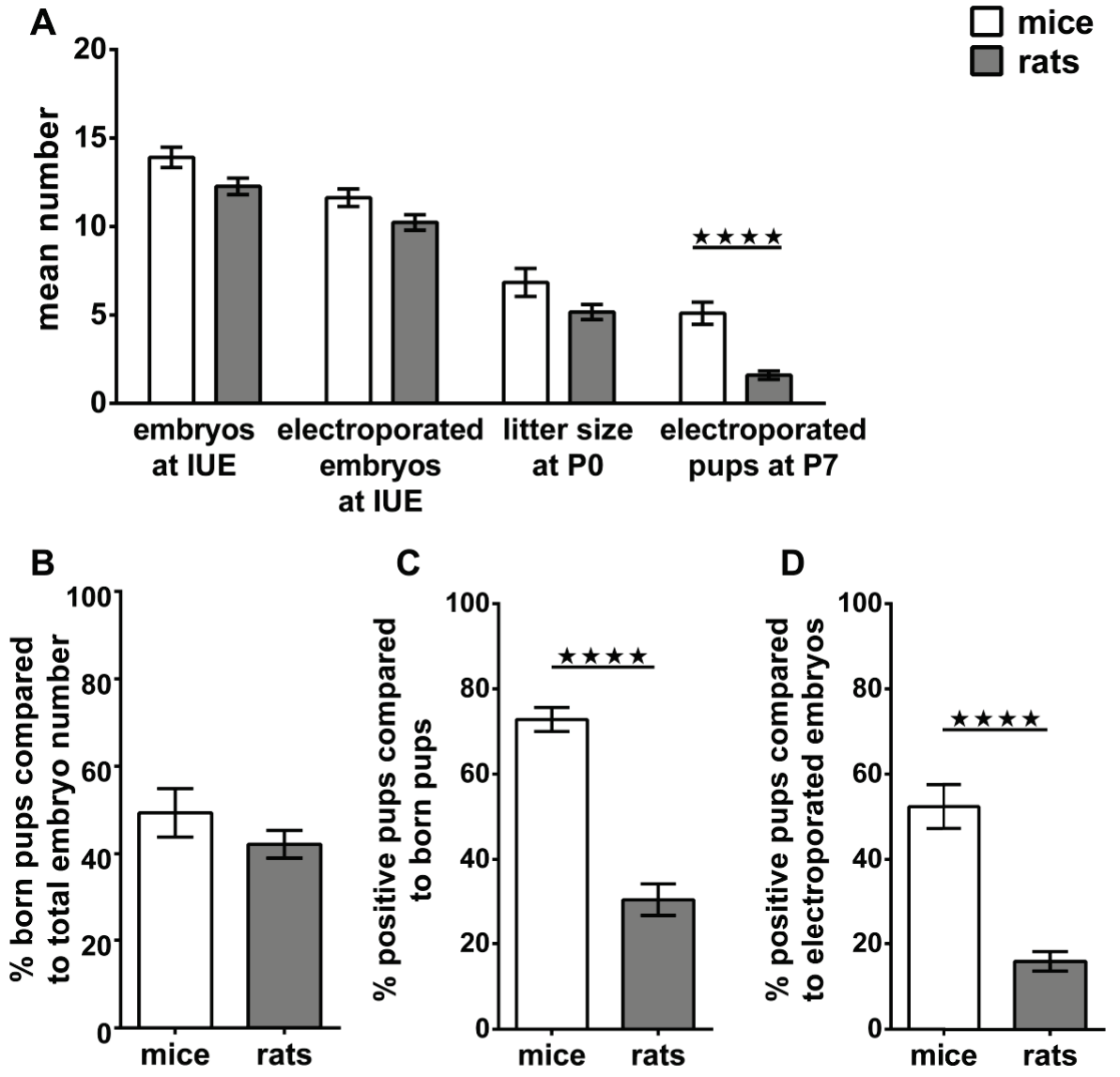


Figure 5.1 IUE comparison of mice and rats

In utero electroporation success for rats and mice from studies II and III, displayed by the mean numbers or the calculated percentages of embryos or born pups from 32 mother mice and 30 mother rats. A) Comparison of the mean number of embryos at the IUE time point, the number of embryos used for electroporation, the number of born animals (litter size), and the amount of 1-week old positive electroporated pups (defined by bioluminescence signal) showed a significant lower number of successfully electroporated rats compared to mice. B) Percentage of living pups at birth in relation to embryo number at the electroporation time point revealing no significant differences between rats and mice. C) Comparison of percentages of successfully electroporated pups in relation to born pups showing a significant lower percentage for rats than for mice ($p = <0.00001$). D) Comparison of percentages of successfully electroporated pups in relation to electroporated embryos at IUE time point showing a significant lower percentage for rats than for mice ($p = <0.00001$)

This is partially a result of the survival rate of only about 49 % in mice and 42 % in rats. Baumgart and Grebe ((Baumgart and Grebe, 2015) showed for C57BL/6 mice, that especially voltage strength and age at electroporation time point are important factors for the survival rate. Voltages have to be chosen carefully to have a balance between electroporation efficiency and survival rate. Other studies showed higher survival rates. For example, Saito and Nakatsuji showed a survival rate of above 90 % for voltages of 30 – 40 V in IRC mice with 54 - 69 % positive embryos (Saito and Nakatsuji, 2001). For Sprague Dawley rats dal Maschio et al showed a survival rate of about 70 % and an electroporation success in cortical areas with a tweezer electrode of 50 – 80 % (dal Maschio et al., 2012). In contrast to the present study, survival rates in the mentioned studies were obtained two or three days after electroporation still at embryonic stages. These embryos therefore did not endure birth stress, which might also have an impact on survival. Another point important for the survival rate of embryos seems to be survival of the neighboring embryos. Therefore, both embryos next to the uterine fundus and embryos difficult to access without damaging were omitted. So just about 80 % of the embryos were electroporated during IUE.

Nevertheless, using the IUE technique to generate transgenic mice and rats reduced the amount of animals used to study behavior and neuropathology by manipulating the brain region of interest and the gene of interest directly in the investigated animal. This saved much time compared to conventional generation of transgenic animals with all the breeding steps.

In vivo bioluminescence imaging

IUE is mostly used to study brain development and changes of development after manipulation in the targeted cells. Proliferation, migration, neurite outgrowth and cortex patterning can be determined and involved genes or proteins can be identified (Fukuchi-Shimogori and Grove, 2001; Kamiya, et al., 2005; Tsai, et al., 2005; Ramos, et al., 2006; Sapir, et al., 2008; Pacary, et al., 2012). Therefore, in most IUE studies pathology or tissue and cell harvesting is done some days after surgery, in pre- or early postnatal days. At this stages targeted areas and electroporation success can be easily determined in the dissected brain or even within the embryonic head by fluorescence detection of co-electroporated fluorescence genes like GFP (Rice, et al., 2010). GFP measurement in the brains of born mice and rats is not possible. The main reason therefore is the limited tissue penetration of 1-2 mm for visible light of the GFP wavelength (about 500 nm), due to tissue absorption and scattering of the emission as well as the absorption light (Cassidy and Radda, 2005). To reach the target within the brain the light has to cross fur, skin, skull (bone tissue), meninges with the blood vessels, as well as grey and white matter. All these tissues have different absorption and light scattering properties, even different brain areas show different scattering

(Al-Juboori et al., 2013). Tissue penetration is also dependent on light wavelengths, the higher the wavelength the deeper the penetration. Near infrared light (~650-900 nm) is the most promising light for noninvasive *in vivo* fluorescence imaging, because at these wavelengths the absorption coefficient of the major absorber hemoglobin is lowest (Weissleder, 2001). Another problem for fluorescence imaging is autofluorescence, especially of blood and skin for example with emission peaks at 590 nm and 630°nm for blood (Masilamani et al., 2004). Therefore, for *post mortem* brain analysis the animals were perfused with PBS before dissection to remove blood from the brain. To reduce background through autofluorescence in the *in vivo* imaging, bioluminescence instead of fluorescence was used. A comparative study of fluorescence and bioluminescence showed, that luciferase-luciferin reaction based luminescence imaging has a higher sensitivity, a wider dynamic signal bandwidth, and a deeper tissue penetration than GFP fluorescence imaging (Choy et al., 2003). Study I showed, that bioluminescence imaging was a very useful tool to detect the area of *in utero* targeted cells and to determine the approximate position by 3D reconstruction. Furthermore, in study II a correlation of the bioluminescence signal in one week old rat pups could be correlated to the number of GFP positive (=IUE targeted) cells in *post mortem* brain analysis of six months old rats. But although luciferase based imaging has a deeper tissue penetration, there are still limitations. In the used transgenic rat model signal detection was limited to adolescence ages (~5 weeks), most likely due to enlarged thickness of the rats head. In the mouse model, which is characterized by a much smaller size, a bioluminescence signal could be detected up to at least 1.5 years (study III).

Overall, the *in vivo* bioluminescence detection proofed to be a useful tool to evaluate IUE success in born rats and mice to investigate behavioral and neuropathological phenotypes in the adult animals.

Histological examination

The coelectroporated GFP vector, although not helpful for *in vivo* imaging, was useful for the *post mortem* neuropathological analysis. GFP expressing cells revealed the position of IUE targeted cells in the histology. Therefore, it could be easily shown, that in study II the electroporated Dab1 shRNA led to the intended migrational deficits in the targeted cells compared to control shRNA electroporation. Knockdown of the intracellular adapter protein Dab1 led to ectopically located neurons and in some cases to heterotopia formation. Cells within these heterotopias also expressed a marker associated with cortical layers II-IV, indicating that these cells were initially supposed to migrate further to higher cortical layers. A recent paper showed that using IUE to overexpress different proteins associated with the reelin-Dab1 pathway, namely reelin, Cdk5 and Fyn, led to heterotopia formation similar to the

ones reported in study II (Ishii et al., 2015). This indicates that a homeostasis of the proteins of this pathway is important for the neuronal migration process.

Furthermore, in study II a drastically reduced number of transfected cells in the *post mortem* brains of rats electroporated with Dab1 shRNA compared to controls was found. The differences in cell numbers were such striking, that an examination of cell fate was performed to see whether the lower cell number was due to cell death at some time point of development. The validation revealed a loss of Dab1 shRNA cells after the embryonic day (E) 22. At that time point the number of GFP expressing cells was similar to the control, whereas two days later and one day after birth of the rat pups at postnatal day (P) 1, GFP positive cells were drastically reduced in the Dab1 shRNA rats. As argued in the discussion of study II, the loss of neurons might be due to programmed cell death, which belongs to normal brain development.

GFP fluorescence of cryo sections from brains of intracardiac perfused animals could directly be visualized under the microscope. Imaging of paraffin sections and brain sections from animals without perfusion needed further treatment using anti-GFP and secondary fluorescence (AlexaFluor, Thermo Fischer Scientific) antibody to intensify fluorescence signals and to reduce background autofluorescence. Reduction of autofluorescence was also achieved by application of sudan black staining. Thereby, the lysochrome diazo dye sudan black stains triglycerides and lipids and reduces lipofuscin-like autofluorescence in a concentration-dependent manner (Schnell et al., 1999). This was especially important for older animals due to lipofuscin accumulation with age (Brizzee et al., 1974). GFP- and AlexaFluor antibody fluorescence was not affected by sudan black, therefore this treatment improved the immunohistological analysis.

In general, immunofluorescence staining protocols needed to be individually adjusted to the used antibody and the tissue preparation. For example, the layer marker stainings in study II were only successful after pretreatment with antigen retrieval, meaning 20 minutes cooking in citrate buffer. However, this reduced the GFP fluorescence and a costaining with GFP antibody was necessary.

Overall, after adjustment of staining protocols, fluorescence imaging yielded evaluable and quantifiable pictures, which resulted in clear pathological findings. These findings mainly involved information about number of targeted cells, their position in the brain and therefore cell fate during development. For study III histological analysis was used to search for neuropathological changes corresponding to Alzheimer's disease pathology. Investigations with various antibodies against APP and staining for amyloid fibrils using congo red did not yield any Alzheimer's disease relevant pathological finding at any age of the mice. The main

reason for that might be the too low number of targeted cells, as revealed by counting the GFP positive cells in *post mortem* brains. Nevertheless, in this study using immunohistological staining, it could be shown for the first time that gene expression from plasmids integrated via IUE is detectable in old ages of mice. Therefore, in principle investigations to study neurodegenerative illnesses the use of IUE should be possible.

Behavioral examination

One main goal of this thesis was to study disease relevant behavioral phenotypes in the adult animal due expression changes of neurodevelopmentally important proteins. The overall goal of these investigations was to determine the neurodevelopmental hypothesis of mental illnesses such as schizophrenia. As mentioned in 3.2.1, schizophrenic patients do not show obvious structural changes in brain anatomy, but short developmental disturbances might not displayed by huge changes, but rather could be expressed by slightly enlarged ventricles as seen in SCZ patients (Murray and Lewis, 1987). In 2011, a first study by Niwa et al. investigated IUE generated transgenic mice for behavioral phenotyping in regard of SCZ (Niwa, et al., 2010). By knockdown of the SCZ susceptibility factor DISC1 in the frontal cortex of mice, they showed behavioral abnormalities of information processing and cognition in adulthood, such as amphetamine hypersensitivity. In study I, I could show that also overexpression of DISC1 in the rat cortex lead to amphetamine hypersensitivity. This indicates that the homeostasis of DISC1 during development is important for brain maturation. DISC1 has been shown to interact with a huge amount of proteins and therefore is involved in different functions within brain development, such as proliferation and migration.

To investigate further if behavior is also affected by disruption of the migratory signaling pathway, knockdown of Dab1 and adult animals behavior testing was performed in study II. The histologically validated migrational deficit together with a perinatal loss of neurons in the cortices of Dab1 shRNA electroporated rats led to subtle behavioral changes. The main finding was again an amphetamine induced hyperactivity, which can be explained by a disruption of the dopamine dependent activity network. Through alternative positioning of the electroporation targeted neurons the intended connectivity of these cells was disrupted. Thereby, the mesocortical pathway of the dopaminergic system, where projections from the ventral tegmental area connecting with the cortex, are affected. This pathway plays a role in concentration, executive motor function and working memory. The fact, that in study II working memory function was only subtly affected might be due to the unilateral electroporation and demonstrates the resilience of the brain.

Overall, the behavioral investigations showed that small disturbances during brain development lead to behavioral impact in the adult animal. Not only the disruption of a specific gene but more likely a time limited disruption of developmental pathways might cause the subtle anatomical changes in the brains of affected people. This might complicate the search for mental illness biomarkers even more.

6 Conclusion

In utero electroporation was successfully established to generate animal models to study brain illnesses in a fast and animal saving way. Especially study II indicated that neurodevelopmental disturbances through IUE can have impact on animal behavior associated with chronic mental illnesses such as schizophrenia.

As a useful method to select for IUE success and accuracy in born animals, the *in vivo* bioluminescence imaging has been introduced in study I and further refined in the studies II and III. Thereby exclusively suitable animals could be provided for further analysis during adulthood. Furthermore, the possibility to track electroporated luciferase expression in old mice, already revealed in the living animal that expression of genes from induced plasmids is still ongoing in old ages of mice.

In study III the investigation of IUE to induce somatic mutations associated with Alzheimer's disease as a chronic brain disease has been studied. Expression of induced proteins was demonstrated up to an age of 1.5 years and therefore the suitability of this method to induce expression changes in a small amount of cells could be proven. This might be a possibility to investigate genetic mosaicism in the brain that has been proposed for sporadic forms of neurodegenerative diseases.

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