

From THE DEPARTMENT OF NEUROSCIENCE  
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**TOUCHING COGNITION:  
SOMATOSENSORY PROCESSING AND  
PLASTICITY DEFICITS IN MOUSE  
BARREL CORTEX**

Konrad Juczewski



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Cover image: an artistic summary of this thesis and a graphical representation of my scientific interests. Original picture obtained from Angelo Cordeschi was slightly modified for the purpose of this cover; picture title: *Human brain made with hands*.

# Touching cognition: somatosensory processing and plasticity deficits in mouse barrel cortex

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Konrad Juczewski**

*Principal Supervisor:*

Professor Doctor Patrik Krieger  
Ruhr University Bochum  
Department of Systems Neuroscience

*Co-supervisor(s):*

Professor David Lovinger  
National Institutes of Health  
National Institute on Alcohol Abuse and Alcoholism

Professor Kevin Fox  
Cardiff University  
School of Biosciences

Professor Sten Grillner  
Karolinska Institute  
Department of Neuroscience

Associate Professor Gilad Silberberg  
Karolinska Institute  
Department of Neuroscience

*Opponent:*

Assistant Professor Gül Dölen  
Johns Hopkins University  
Department of Neuroscience

*Examination Board:*

Docent Karima Chergui  
Karolinska Institute  
Department of Physiology and Pharmacology

Docent Christian Broberger  
Karolinska Institute  
Department of Neuroscience

Department Coordinator  
Åsa Konradsson-Geuken  
Uppsala University  
Department of Pharmaceutical Biosciences





*To my beloved wife Maria,  
the most tactile person I have ever met...*



## ABSTRACT

Senses together with thoughts and experience are key factors that shape our cognition. Touch is an important source of sensory information. Disturbances to the development of the somatosensory system have serious consequences for social behaviour and may lead to many neurodevelopmental disorders. In our studies we used three different mouse models of disease. DISC1-cc transgenic mouse with transient disruption of the DISC1 protein signalling was used in the project related to the pathophysiology of schizophrenia. DISC1 is a molecule implicated in psychiatric disorders, which was discovered in a large Scottish family whose several members over four generations exhibited schizophrenia, bipolar disorder and major depression. In the following projects we focused on the fragile-X-syndrome-related problems. We analysed *Fmr1* KO mouse with a knock-out gene *Fmr1* coding the FMR1 protein and the BC1 KO mouse with a knock out gene coding BC1 non-protein-coding RNA. *Fmr1* KO mouse constitutes the most popular mouse model of fragile X syndrome mimicking many of its phenotypes that occur in humans. In contrast, BC1 KO mouse was created to study a specific role of this molecule rather than mimic symptoms of the fragile X syndrome. Both the FMR1 protein and BC1 non-protein-coding RNA are involved in control of a protein translation process and they are found in similar molecular complexes side by side.

In DISC1-cc mice we expressed the truncated protein DISC1-cc for a controlled period of time at different points during the early postnatal development. Development is shaped by sensory experience, especially during phases known as critical periods. Disruption of experience in the critical period normally produces neurons that lack specificity for sensory experience in adulthood. We found that transient disruption of DISC1 signalling during a critical period of development produced neurons that lack plasticity in adulthood. Adult plasticity deficits may be associated with cognitive deficits and the delayed onset of psychiatric symptoms in late adolescence. In *Fmr1* KO mice, we focused on analysing somatosensory processing defects that lead to hypersensitivity to touch in fragile X syndrome patients. We showed neuronal mechanisms that appear to underlie hypersensitivity to somatosensory stimuli (whisker deflections) thus causing an altered behaviour observed in *Fmr1* KO mice. In further studies on BC1 KO mice, we characterized electrophysiological aspects of whisker-stimulation-evoked cortical responses and spontaneous activity of recorded neurons. We used similar recording protocols to the ones optimized by us for previous recordings in *Fmr1* KO mice therefore these experiments might be informative for future studies on specific roles and interactions between the FMR1 protein and the BC1 non-protein-coding RNA. Furthermore, our experiments on BC1 KO mice were a part of a larger project that provides a broader overview of the phenotypes represented in this mouse. To sum up, using the whisker somatosensory circuit as a model system we have obtained insight into potential disease mechanism involving sensory processing that could contribute to human brain disorders.



## LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers and manuscripts:

- I. Greenhill SD\*, **Juczewski K\***, de Haan AM, Seaton G, Fox K, Hardingham NR. NEURODEVELOPMENT. Adult cortical plasticity depends on an early postnatal critical period. Science 2015;349:424-7. (\*these authors contributed equally to this work).
- II. **Juczewski K**, von Richthofen H, Bagni C, Celikel T, Fisone G, Krieger P. Somatosensory map expansion and altered processing of tactile inputs in a mouse model of fragile X syndrome. Neurobiology of disease 2016;96:201-15.
- III. Briz V\*, Restivo L\*, Pasciuto E\*, **Juczewski K**, Mercaldo V, Baatsen P, Gunko NV, Borreca A, Girardi T, Luca R, Nys J, Lo AC, Poorthuis R, Mansevellder H, Fisone G, Ammassari-Teule M, Arckens L, Krieger P, Meredith R, Bagni C. The non-coding RNA BC1 regulates experience-dependent structural plasticity and learning. Manuscript submitted for publication. (\*these authors contributed equally to this work).

The manuscript of paper III could not be printed in the thesis book due to publishers' embargo for the manuscript to be published in the thesis prior to publication by them. The publisher has agreed for the manuscript to be available to the persons performing the examination. This solution, which is an exception from the standard procedures, has been approved by the Dean and Pro-Dean of Doctoral Education at Karolinska Institutet after discussions with the chairpersons of the Dissertation Committee and the Swedish Higher Education Authority (UKÄ).

Publications not included in the thesis:

Kupferschmidt DA, **Juczewski K**, Cui G, Lovinger DM. Parallel processing of discrete cortical inputs to striatum predicts skill learning. Manuscript submitted for publication.



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## LIST OF ABBREVIATIONS

AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	autism spectrum disorders
B6	mouse strain C57Bl/6J
BC	brain-specific cytoplasmic
BC1 RNA	BC1 non-protein-coding ribonucleic acid
DISC1	disrupted in schizophrenia 1
DMSO	dimethyl sulfoxide
EDP	experience-dependent plasticity
EPSPs	excitatory postsynaptic potentials
FMR1	fragile X mental retardation 1
FMRP	fragile X mental retardation protein (FMR1 protein)
FXS	fragile X syndrome
GABA	gamma-aminobutyric acid
GluR	glutamate receptor
GSK3 $\beta$	glycogen synthase kinase-3 beta
IPSPs	inhibitory postsynaptic potentials
KO	knock out
L2/3	layer 2/3
L4	layer 4
Lis1	lissencephaly-1
LTD	long-term depression
LTP	long-term potentiation
mGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
NMDA	N-methyl-D-aspartate
npc	non-protein-coding
Nudel	nuclear distribution protein nudE-like 1
P...	postnatal day ...
PBS	phosphate-buffer saline

PSD	postsynaptic density
PW	principal whisker
PW latency	PW response latency
PW response	PW response rate
RNA	ribonucleic acid
S1	somatosensory primary cortex
sEPSCs	spontaneous excitatory postsynaptic currents
SW	surrounding whiskers
SZ	schizophrenia
VDH	Vibrissae Dominance Histogram
WSI	Whisker Selectivity Index
WT	wild type
WVDI	Weighted Vibrissae Dominance Index
$\alpha$ -CaMKII	$\alpha$ -calmodulin kinase II

# 1 OVERVIEW

*No great mind has ever existed without a touch of madness – Aristotle*

This quotation from ancient times reminds us about two very interesting issues related to the human brain in the context of this dissertation. First, the distinction between great, outstanding minds and the ones that we consider mad and abnormal is oftentimes difficult or even impossible to make. Why is it so challenging? Simply, because what we consider great in terms of man's thinking relates to unusual, uncommon and unexpected – typical characteristics of human behaviour in mental disorders. Therefore, it is very important to carefully analyse these unusual phenotypes, to look at special conditions very closely and to identify borderlines for a medical intervention if necessary. Furthermore, these detailed studies are necessary for development of new, efficient treatments. In this thesis I will discuss projects related to cognitive problems observed in mental disorders such as schizophrenia (SZ) and autism spectrum disorders (ASD), with fragile X syndrome (FXS) as a particular example.

Another interesting aspect of the Aristotle's observation is the fact that "a touch of madness", a phrase used by him to describe a state of a mind, is a very intriguing way of putting it. In this metaphor "touch" is perceived as a very powerful sensory modality. It illustrates the intuition that something as complex as our mind can be changed by "touching". Not by seeing, not by hearing, but by touching. It might be interpreted as a verbal reflection of our understanding of touch as a more active and influential modality than other senses. Obviously, "madness" and "mind" are not persons, so they cannot touch each other in a physical way. But the metaphoric meaning of "a touch of madness" shows our respect for this sensory modality, even if on a subconscious level. Where may this image originate? The sense of touch is the first one that appears during human development (Linden, 2015). In early prenatal time, around eight weeks' gestation, formation of the somatosensory system begins. It is really amazing to imagine that the human foetus, only 1.5 centimetres long and weighing about a gram at that time, exhibits the first brain activity and has first tactile experiences at this age. Furthermore, touch seems to be more resistant to human aging as well. We lose our vision and our hearing much easier and much earlier than we lose our sense of touch. In our studies, we focused on somatosensory system function but we used it as a model system rather than a research subject per se. We wanted to learn more about abnormalities in tactile functioning evoked by genetic mutations occurring in the aforementioned mental disorders by analysing normal and genetically modified organisms.

Studying human disease mechanisms directly in humans would possibly be the best way to understand their pathophysiology. However, it is not always possible due to practical or moral reasons. Therefore, investigators have used cell cultures and other *in vitro* methods. Although they simplify complexities of a complete organism, they can be very informative when it comes to understanding of the basic physiological mechanisms at the molecular level. Furthermore, we also use model animals that we can manipulate experimentally to gain insight into interactions between the genes and environment in a complete animal. One of the best animal models is the modest mouse because of its small size, short generation time and the ease of genetic modification. Moreover, comparative genomic studies revealed that 99% of human genes have mouse homologues and that the gene order is highly conserved. Thus, it is not surprising that several human diseases caused by mutations are often mimicked by similar mutations in mice.

In this thesis, I shall present the original work performed in a search for disease mechanisms related to cognitive problems observed in SZ and FXS, considered to be neurodevelopmental disorders with a strong genetic background. A common definition says that cognition is the way we understand the world that surround us which is shaped by three key factors: senses, thoughts and experience. How our brain perceives, analyses, responds, learns and integrates sensory information to create thoughts and remember our experiences is a complex and still unresolved question. Therefore, as we tried to address it here, we chose a reductionist approach. We found a common denominator to all those brain activities, namely sensory processing. We narrowed it down to one important sensory system, a sense of touch, and we decided to study tactile experience and related brain activity in the mouse models of disease. In genetics, there are two main ways to learn about behaviour: “classical genetics” where we start from a phenotype trying to find a genetic cause for it; and “reverse genetics” where we alter a gene function trying to find its consequences. In our studies we used a mixed approach. Knowing human symptoms and a potential genetic cause, we tried to model it in a mouse to look for similar phenotypes. Then, we were analysing physiological changes in plasticity mechanisms (paper I) or processing and encoding of the tactile information (paper II and III) in the somatosensory system of these mouse models.

In later sections of this thesis, I shall introduce to the reader basic information from the field of neuroscience necessary to understand the context of our experimental work. I shall discuss the somatosensory whisker system in a mouse with detailed focus on its main components, the whisker and the barrel. Then, I shall present definitions crucial to understand the results obtained, namely: receptive fields, sensory maps and plasticity. Finally, I shall present a

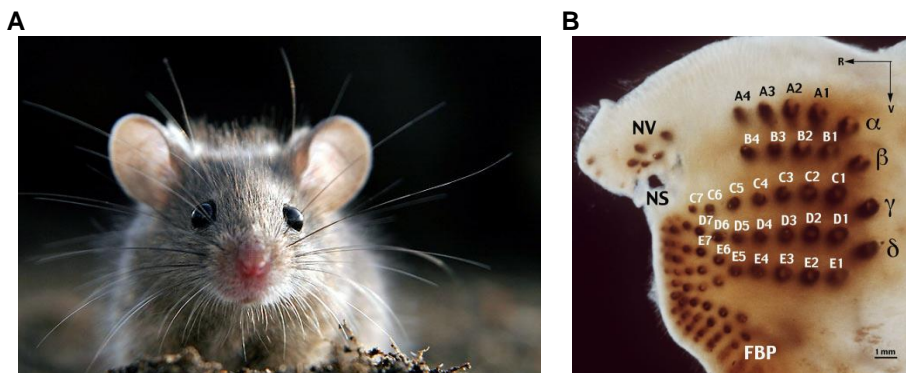
general perspective on pathophysiology of SZ focusing in particular on the *DISC1* gene and pathophysiology of FXS focusing in particular on the *FMR1* gene and the BC1 non-protein-coding (npc) ribonucleic acid (RNA). Information about those diseases (SZ and FXS) and all three genes (*DISC1*, *FMR1* and *BC1*) is relevant because in our three projects we performed experiments on transgenic animals, SZ-related (project 1) and FXS-related (project 2 and 3) mouse models, which have modifications in one of these genes. The introduction in each section shall be followed by a short explanation of the main aims for each project. Next, the methods used in all three papers shall be discussed together because there are many similarities between them. Furthermore, they shall provide a good platform for direct comparisons of those projects and the results obtained. In the final part of the thesis experimental results shall be presented in light of the work of other researchers. Additionally, some direct comparisons between projects shall be drawn. At the end, concluding remarks shall be presented to discuss wider implications of the projects collected in this thesis.



## 2 INTRODUCTION

### 2.1 Whisker somatosensory system in mouse

Mice – similarly to all rodents, cats, marsupials and a few other species – have facial hair called whiskers or vibrissae (Fox, 2008). Rodents' whiskers are very well organized in space and this is consistent among individual animals (Fig. 1). This property was extensively studied for the first time by a curious medical student, Thomas A. Woolsey, during his fourth year “electives” at The Johns Hopkins University (for review, see Woolsey, 2016). It is true that some researchers, e. g. Lorente de No or Rose, noticed “the structural peculiarities of the region” in the somatosensory primary cortex (S1) using Nissl's staining already in 1920s (Woolsey and Van der Loos, 1970). However, it was only in 1970s that Woolsey's curiosity combined with a very efficient new staining method developed by his research mentor at the time – Hendrik Van der Loos – allowed them to understand the basic anatomical structure of this region relating it to the sense of touch. In their article Woolsey and Van der Loos (1970) described a very special feature of the mouse's facial hair, the fact that each of the whiskers has a distinct representation place a “barrel” because of its characteristic three-dimensional shape visible on histological sections in the cortical layer 4 (L4). All of the barrels are in the area of S1 called the barrel cortex or barrel field and they are organised throughout cortical layers in cortical columns. The barrel field contains posteromedial barrel subfield (PSMBF) that is a highly consistent region corresponding to the large caudal whiskers (macrowhiskers). In this area the barrels are the



**Fig. 1 Organisation of the whisker system in rodents.** (A) A mouse with its whiskers visible on the snout. (B) Spatial organization of the macrowhiskers of the rat which is the same also in the mouse. Image courtesy of Daniel J. Simons, University of Pittsburgh School of Medicine.

largest, the most elliptical in shape and have striking topographical organisation. Macrowhiskers consistently present on a mouse snout, are placed in five rows (A, B, C, D, and E), in four to seven arcs (1-7) and with four whiskers in-between the rows ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ). The same organization of barrel columns is noticeable in the barrel cortex of the contralateral hemisphere.

In the barrel cortex, six layers can be distinguished based on different cell density and variable cell types present in specific layers. Characteristic shapes of barrels are clearly visible only in L4, the input layer where the thalamus sends its afferents. The thalamus receives somatosensory projections from the trigeminal nucleus of the brain stem, which has a direct link with a mouse snout through the trigeminal nerve. Trigeminal nerves are directly connected to the whisker follicles on the mouse snout. Every whisker has its separate follicle, a dedicated place in the skin where it is attached to the snout. Follicles have their own sinuses and many mechano-gated receptors that convert mechanical movements of the whiskers into a chemical gradient, and then, an electrical signal that can be transmitted through the trigeminal nerves further up the somatosensory pathway. When a whisker is in motion, its sinus immediately fills with blood that makes the whisker more rigid and presses mechano-receptors against its base. In this way the whisker-barrel system becomes extremely sensitive even to very small vibrations caused by a whisker movement. The basic organization of the whisker-barrel system in rodents has been summarized in several reviews, for instance in Petersen (2007), Brecht (2007), Diamond et al. (2008) or, more recently, in Diamond and Arabzadeh (2013) and in Feldmeyer et al. (2013).

## **2.2 Whiskers and whisking**

Under the microscope, whiskers look like thin conical rods attached to mechanosensory receptors at their base (Birdwell et al., 2007; Sofroniew and Svoboda, 2015). The conical shape of whiskers thousand times larger at the tip than at the base, gives them unique flexibility which is very useful for active tactile sensation process (Hires et al., 2013). Rodents move their whiskers spontaneously most of the time because it helps them in spatial localization, recognition of objects in their proximity and guides their locomotion (Brecht et al., 1997; Knutsen et al., 2006; Vincent, 1912). This “whisking” is driven by pattern generators and usually occurs in “bouts” of one to many whisker movements (Berg and Kleinfeld, 2003; Gao et al., 2001). Mice usually “whisk” at higher frequencies than rats that is up to 25 Hz (Jin et al., 2004). Furthermore, whisking rate is modified by the environment (Mitchinson et al., 2007), although independently from direct sensory feedback (Gao et al.,



2001). Whisking at higher frequencies occurs during “active touch” (exploration), e.g. during palpation of objects, as reported by Berg and Kleinfeld (2003). Interestingly, Knutsen et al. (2006) showed that whisking frequency in rats does not affect performance directly but the total whisking spectral power is strongly correlated with performance in a detection task: the higher the spectral power, the lower the detection threshold. Whisker movement dynamics change not only at the beginning and at the end of the contact with an object but also when the whisker is moving along its surface. Apart from large whisking movements used for object distinction and localization, there are also smaller vibrations (micromotions) of the whisker. These micromotions occur when the animal is exploring the object and whiskers are used to learn fine details about its surface, e.g. texture characteristics (Lottem and Azouz, 2008). Although there is no consensus as to the detailed translation mechanisms for information collected by whisker movements, whisking behaviour is thought to be a part of a specialized sensory encoding strategy.

### **2.3 Barrels and the barrel column**

Barrel cortex is well-organized in both dimensions, horizontally – with cortical layers – but also vertically with cortical columns (barrel columns). Mountcastle (1957) in his pioneering work on the cat somatosensory system observed that some neurons localized along different cortical layers had similar response properties to the applied stimuli. These observations led to the first description of cortical columns and inspired future work on the cat visual cortex. Next Hubel and Wiesel demonstrated that columnar organization of the neocortex is universal for different sensory areas by describing for the first time ocular dominance columns (Hubel and Wiesel, 1962; Hubel and Wiesel, 1963). A few years later Woolsey and Van der Loos (1970) observed cortical columns in the mouse somatosensory system. They called them “barrel columns” because of their barrel-like three-dimensional shape visible in the sectioned brain. The barrel structure defined in L4 was visible due to aggregations of somata of neurons, mostly spiny pyramidal cells, that were organized around the “hollow” centre of the barrel (Woolsey et al., 1975). Barrels were separated by narrow “septa” (Woolsey and Van der Loos, 1970) with potentially distinctive microcircuits. Differential functions of barrel and septal circuits are not yet fully defined, and this is one of the topics of exciting ongoing investigations (Alloway, 2008).

Each barrel column contains two main types of neurons: excitatory principal neurons and inhibitory interneurons (as reviewed in Schubert et al., (2007). Detailed anatomy of the barrel column had been much more extensively studied in rats than in mice. Nevertheless, the

general proportion between excitatory and inhibitory cells is strikingly similar in the two species. In rats about 88-89% of the 19,000 neurons are excitatory cells, the rest being the inhibitory ones (Meyer et al., 2011; Meyer et al., 2010). In mice 11% of 6,500 neurons in the C2 barrel column are inhibitory, a fraction very similar to the rat barrel cortex (Lefort et al., 2009). The excitatory neurons, also called principal neurons due to the aforementioned proportions, use L-glutamate as the major neurotransmitter and they are mostly projection-type of neurons. In contrast the inhibitory interneurons are called non-principal and they are “local-circuit” or “microcircuit” cells since their axons mainly stay within one column or even within a few layers of a column. All interneurons use gamma-aminobutyric acid (GABA) as their main neurotransmitter and all of them fulfil two basic tasks of perisomatic or dendritic inhibition (Schubert et al., 2007). Nevertheless, interneurons constitute a largely heterogeneous group with up to 20 different types depending on the identification methods (for detailed characterization, see Markram et al. (2004)). On the other hand, excitatory cells constitute a much more homogenous group with 3 major classes of neurons: spiny stellate, star pyramidal and pyramidal cells (for detailed characterization, see Feldmeyer (2012)). On top of this division, we can distinguish regular-spiking and intrinsically burst-spiking cells based on their electrophysiological activity (McCormick et al., 1985). In all our projects we chose to focus on the excitatory cells due to their relative homogeneity as well as their projection characteristics important for the assessment of changes in the receptive fields of neurons discussed below.

## **2.4 Receptive fields, sensory maps and plasticity**

Initial anatomical findings about the barrel cortex structure led to a hypothesis that each barrel is responsible for processing tactile information originating from the corresponding whisker on the other side of the snout (Woolsey and Van der Loos, 1970). However, subsequent studies showed that cortical processing of information from the whisker is far more complex (Armstrong-James and Fox, 1987; Simons, 1978). On the one hand, there is evidence that mechanical movement of the main whisker representing certain barrel column evokes the largest and the fastest response in the cells of this cortical column. On the other hand, the concept of exclusive one-to-one columnar representation was supplemented by the observation of smaller and delayed cortical responses in the adjacent barrel columns. Taking into account the complexity of tactile information collected by rodent whiskers, it should not be surprising that a single whisker and a single barrel cannot encode any object alone (Hutson and Masterton, 1986). Rodents are able to distinguish different objects by their texture (Carvell and Simons, 1995), shape (Harvey et al., 2001), and even width of an experimental

alley that they need to choose in a behavioural task (Krupa et al., 2001). Thus, as suggested by Schubert et al. (2007), all the somatosensory information transmitted by the set of whiskers touching certain objects should be somehow coded within neuronal circuitry. One of the phenomena observed in the neural circuit that helps to solve this issue is a receptive field of a neuron, the part of the external sensory surface whose information is transferred to this neuron. Possibly every sensory neuron has its specific receptive field. Receptive fields of different neurons may overlap and their general characterization differs between cortical layers. It seems that excitatory cells of L4 have the smallest receptive fields, almost limited to a single whisker. In contrast, layer 2/3 (L2/3) neurons are strongly involved in trans-columnar interactions that result in larger receptive fields. Neuronal receptive fields describe the basis for stimulation-evoked cortical activity, which is crucial for the brain's ability to adapt to the environment. In the Fragile X and BC1 Projects, we studied basic properties of whisker-stimulation-evoked neuronal activity but we also focused on neuronal receptive fields potentially affected by genetically induced mutation.

Looking at the cortical organization in a broader perspective, horizontal and vertical organization of the somatosensory cortex results in the somatotopic maps also called neural maps. While a neuronal receptive field is a definition describing external space which is directly receiving external stimulations, neural maps constitute a cortical representation of this external space. In short, they are “representation of one's own body”. Sensory maps are characteristic for all mammals and different sensory modalities have their dedicated cortical representation (Marshall and Meltzoff, 2015). They had been identified as areas performing a specific information processing function (for instance texture maps, colour maps) and they can respond to a single stimulation from the external environment. Tactile information is processed in the somatosensory barrel cortex that constitutes somatotopic maps related to the whiskers. Tactile experience shapes the somatosensory maps. Change in response to experience and use is a fundamental property of the brain and it is called plasticity. Plasticity allows the brain to learn and remember patterns of the sensory world, to refine movements, to predict and obtain reward, and to recover after injury (Feldman, 2009). In short, plasticity can be described as permanent changes of structure elicited by external signals and constraints modulated by one's physiology and internal environment. Now we know that remodelling of brain connectivity takes place for the entire lifetime, however, a scale of this process and dominating plasticity mechanisms are changing as a function of time (for review, see Merzenich et al. (2014)). Initially, during the early postnatal development all inputs are involved in processes of the competitive plasticity. This flexibility is reduced with age and

becomes more specific. In adult organisms plasticity mechanisms are regulated by behavioural context and outcome.

Barrel cortex of the rodent has proved to be a fruitful model for investigating mechanisms of map plasticity in the somatosensory barrel cortex due to its clear structure and superficial location (for review, see Petersen (2007)). In the barrel cortex changes induced by sensory experience are called experience-dependent plasticity (EDP) and can occur as a result of sensory deprivation (e.g. Fox, 1992) as well as sensory enrichment (e.g. Megevand et al. (2009)). EDP is most often used to describe changes of cortical somatotopic maps called map plasticity. A possible mechanism to explain map plasticity is synaptic plasticity that is described as long-term changes of synaptic weight elicited by presynaptic and postsynaptic activity. Synaptic plasticity can be observed *in vivo* as well as *in vitro*. The historical protocol consists of high frequency presynaptic stimulations which can induce long-term potentiation (LTP) or of low frequency stimulations which can induce long-term depression (LTD) (for review, see Bliss and Cooke (2011)). Another type of protocols that we used in the DISC1 Project consists of pairing presynaptic and postsynaptic responses to evoke LTP and postsynaptic and presynaptic responses to evoke LTD. One of the molecular mechanisms for plasticity especially relevant to the projects presented in this thesis is related to  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors' dynamics. Trafficking and insertion of AMPA receptors into plasma membrane is often related to LTP expression, whereas AMPA receptors' internalization and their loss is related to LTD expression (for review, see Malenka and Bear (2004)). We studied EDP, LTD and LTP mechanisms in more detail in the DISC1 Project that was related to pathophysiology of SZ.

## **2.5 Schizophrenia and the *DISC1* gene**

SZ is predominantly defined by observed signs of psychosis. Nevertheless, it is a syndrome with many signs and symptoms of unknown aetiology as reviewed by Insel (2010). Despite the fact that people have been interested in psychosis since ancient times (for the history review, see Adityanjee et al. (1999)), we still lack basic understanding of its pathophysiology and, thus, its prevention or treatment is very limited. Ancient descriptions referring to conditions similar to psychosis described affected person as “nude, filthy, confused and lacking self-control”. Other ancient documents described catatonia and paranoid delusions. In later documents coming from the times before the nineteenth century, we can find diagnoses of mania or melancholia discussing bizarre, grandiose and persecutory delusions; visual and auditory hallucinations; disorder thoughts; inappropriate affect; and social isolation

(Jablensky, 1986). Eventually, in the late nineteenth century, a German psychiatrist, Emil Kraepelin, defined “dementia praecox” or premature dementia distinguishing it from the insanity of tertiary syphilis and the cyclic, non-deteriorating psychosis of manic-depressive illness. In fact, “premature dementia” was a descriptive name pointing towards the main visible symptom of the described disease – cognitive dysfunction. As of today, cognitive deficits are not only considered to be a key feature of SZ pathophysiology but also a primary cause of long-term disability (Javitt, 2009b). Unlike in the case of positive symptoms that can be pharmacologically modulated to a certain extent, we lack treatment and basic understanding of the mechanisms underlying cognitive symptoms. Nevertheless, in contrast to negative symptoms “undruggable” due to their potentially anatomical aetiology, the cognitive symptoms may have greater potential for treatment if we look at them from the sensory processing deficits’ perspective. Human studies with event-related potentials as well as functional resonance imaging techniques revealed interesting changes in visual and auditory modalities (for review, see Javitt (2009a)). Most of these sensory-modality-related studies focused on the so-called “gating” paradigms that test the neural response to the second stimulus in a pair as compared with the first one, hence they mostly examine adaptation mechanisms. Somatosensory system is largely neglected in this study and limited data available indicates rather inconsistent findings as reviewed in Andrade et al. (2016). Therefore, research leading towards establishing new tactile-modality-related endophenotypic markers is in high demand. Indeed, studies on sensory regions can breakdown cognitive problems to specific disruptions in different sensory modalities. Yet, an impairment of the early sensory perception may have severe impact on information available for subsequent processing. Moreover, some data from SZ patients suggest reduction in sensory adaptation that may result from impairments in sensory plasticity (Andrade et al., 2016). Therefore, our studies on plasticity processes in the SZ-related animal model, a DISC1-cc mouse, should be also seen in this perspective.

Disrupted in schizophrenia 1 (DISC1), the gene that codes for the DISC1 protein, is disrupted by a balanced translocation (1q42; 11q14.3) that cosegregates with SZ, major depression, and bipolar disorder. Association between this autosomal translocation was discovered by St Clair et al. (1990) in a large Scottish family. Ten years later, Millar et al. (2000) found a specific locus of the affected genes, a protein-coding gene DISC1 and an antisense npcRNA gene DISC2. Further studies showed that an overall expression pattern of DISC1 is conserved across the species and is mainly expressed in the neurons of various brain areas, including the olfactory bulb, cortex, hippocampus, hypothalamus, cerebellum and brain stem (Schurov et al., 2004). Interestingly, DISC1 protein was distributed as uneven clusters in all the

mentioned regions of the mouse brain, which corresponded to a punctate intracellular distribution found in the primate and human brain. Schurov et al. (2004) also showed that DISC1 is specifically expressed in neurons and that it is largely absent from astroglia. Nevertheless, more recent studies suggested DISC1 expression in multiple classes of glial cells, also in both rodent and human tissue, including oligodendrocytes as well as astrocytes (Randall et al., 2014). DISC1 was found to co-localize with the glutamate transporter (excitatory amino acid carrier 1, EAAC1) expressed in glutamatergic neurons as well as with glutamate decarboxylase (GAD) expressed in GABAergic neurons (Schurov et al., 2004), which suggested DISC1 localization in both types of neurons and might partially explain the potential for multiple roles of DISC1. The full-length human DISC1 protein comprises of 854 amino acids and can be divided into an N-terminal region, referred to as the globular “head” domain, and a C-terminal region containing coiled-coil domains (Millar et al., 2001). Coiled-coil domains make the DISC1 protein an attractive binding partner for several proteins, which had been extensively studied with the protein-protein interaction analysis (Camargo et al., 2007) and other types of meta-analysis (Hennah and Porteous, 2009). It appeared that DISC1 interacts with surprisingly large numbers of signalling molecules and may affect diverse aspects of neuronal development, including corticogenesis, especially radial neuronal migration and dendritic arborisation (interaction with lissencephaly-1 (Lis1), nuclear distribution protein nudeE-like 1 (NDEL1/Nudel), nuclear distribution protein nudeE homolog 1 (NDE1) and pericentriolar material 1 (PCM1)), or determination of proliferation and fate of neural progenitors (interaction with glycogen synthase kinase-3 beta, GSK3 $\beta$ ) (for review, see Brandon et al., 2009; Jaaro-Peled et al., 2009; Porteous et al., 2011). In our studies we examined whether the DISC1 protein plays any important role in synaptic plasticity. We also analysed spine morphology in a DISC1-cc mouse that gave us some information about DISC1 role in the spine formation mechanisms.

## **2.6 Fragile X syndrome, FMR1 protein and BC1 non-protein-coding RNA**

FXS belongs to the autism spectrum and represents the most common cause of inherited intellectual disability (Bagni and Oostra, 2013; Dolen and Bear, 2009; Tranfaglia, 2011). According to conservative epidemiological studies, FXS occurs in about 1:2500-5000 males and 1:7000-8000 females (Coffee et al., 2009). Its symptoms appear to have monogenic origin in the mutation of a fragile X mental retardation 1 (FMR1) gene that leads to expansion of a non-coding CGG trinucleotide in the 5'-untranslated region (Contractor et al., 2015). Subsequent hypermethylation results in transcriptional silencing, and partial or full loss of expression of the fragile X mental retardation protein (FMR1 protein or FMRP). In the

general population there are 5-44 CGG repeats, in the fragile X premutation 55-200 and above 200 in the fragile X full-mutation (Foote et al., 2016; Hagerman et al., 2014). Individuals with 45-55 CGG repeats are considered to be the "grey zone" and they are at an increased risk of fragile X premutation-related diseases like fragile-X-associated tremor/ataxia syndrome (FXTAS) or fragile-X-associated premature ovarian insufficiency (FXPOI). Although it has been shown in knock-in mouse lines with increased CGG repeats that FMRP levels depend on CGG repeat length (Hunsaker et al., 2012), carriers with premutation exhibit potentially toxic increase in FMR1 messenger RNA (mRNA) level but usually only slightly reduced FMRP level (Hagerman et al., 2014). Full mutation in males always results in developing a wide variety of FXS symptoms, from a range of anatomical changes, like macroorchidism, elongated face or everted ears, through sensory perception and integration problems (e.g. sensory hypersensitivity), to attention deficits, anxiety and cognitive dysfunction (Ferron, 2016; Hagerman et al., 2014; Miller et al., 1999). Although CGG repeat mutation in the *FMR1* gene remains the main reason for development of the FXS pathologies, there are a few reports presenting point mutations or deletions in the FMR1 locus causing similar FXS symptoms, further proving the crucial role of the *FMR1* gene dysfunction in this disease (De Boulle et al., 1993; Feng et al., 1997; Myrick et al., 2015; Penagarikano et al., 2007).

There have been a few milestones in identifying FXS as a genetic disease. Martin and Bell (1943) shared their clinical observation of mental deficiency that may be potentially explained as "the manifestation of a sex-linked gene". Over the next decades it had been confirmed that this form of mental retardation is associated with genetic mutation (Penagarikano et al., 2007), initially described as an unusual secondary constriction at the end of the long arm of the X chromosome (Lubs, 1969), then localized specifically on band Xq27.3 (Harrison et al., 1983; Sutherland, 1979a; Sutherland, 1979b). Finally, a major cause of FXS was discovered, a mutation that silenced a single gene (FMR1) on the X chromosome (Pieretti et al., 1991; Verkerk et al., 1991) and a mouse model of FXS (Fmr1 knock out (KO) mouse) was generated shortly afterwards (Consortium, 1994). The murine homolog gene (Fmr1) has high sequence (95%) and high amino acid identity (97%) with its human counterpart and a similar expression pattern (Ashley et al., 1993). The product of its expression, the FMRP protein, although widely expressed throughout the body, is enriched in neurons and testes (Kazdoba et al., 2014). FMRP can be found in the cell body, mainly in dendrites and synapses, since its expression is mostly cytoplasmic but some FMRP is capable of shuttling between cytoplasm and nucleus (Bhakar et al., 2012). Although the preferential areas of FMRP operation are dendritic spines, growing evidence suggests its important role in

axons as well (Ferron, 2016). Thus, an FMRP role in controlling synaptic transmission through both pre- and postsynaptic actions seems to be possible (Centonze et al., 2008). FMRP is associated with polyribosomes and it is an RNA binding protein (Darnell et al., 2011). Its function is not fully understood yet but it certainly plays a role in the trafficking of specific mRNAs to translation sites as well as in the stalling of their translation (Ferron, 2016), so it is also called a “translation brake”. The FMRP role in translation is of particular interest because new protein synthesis is required for plasticity processes and plasticity is thought to be a crucial phenomenon underlying memory (Bear and Malenka, 1994; Malenka and Bear, 2004). Finally, FMRP had been shown to interact directly with several ion channels including the sodium-activated potassium channel Slack, the large conductance Ca-activated potassium big potassium (BK) channel and the N-type voltage-gated calcium channels (Ferron, 2016). The functional meaning of these interactions is not clear, however. It may be another way of participating in activity-dependent protein synthesis.

FMRP together with fragile X mental retardation syndrome-related protein 1 and protein 2 (FXR1P and FXR2P), proteins expressed by FMR1 paralogues FXR1 and FXR2, constitute the FMR1 RNA-binding protein family (Ascano et al., 2012). FMRP binds about 4% of mRNA in the mammalian brain and it has been suggested that these interactions are crucial for its regulatory function over protein translation (Ascano et al., 2012; Ashley et al., 1993; Bassell and Warren, 2008). In addition to mRNAs that code for proteins, other types of RNAs have been distinguished, including ribosomal RNAs, transfer RNAs and other RNA molecules that are part of regulatory mechanisms in many cellular processes (Iacoangeli et al., 2010). The latter group is generally referred to as npcRNAs and, although npcRNAs do not encode specific amino acid sequences and they are untranslated, they carry codes (Brosius and Tiedge, 2004). Brain-specific Cytoplasmic (BC) RNAs, one of the example of regulatory npcRNAs, were identified in rodents in 1980s as reviewed in Iacoangeli et al. (2010), even before the FMRP. Nevertheless, research on the role of the major BC RNA representative, BC1 npcRNA (BC1 RNA), had been largely absent until generation of the BC1 KO mouse by Skryabin et al. (2003). In the meantime, BC200, a small cytoplasmic RNA of 200-nucleotides length, was identified as a human homolog (Tiedge et al., 1993). Furthermore, detailed gene mapping of the gene coding BC1 RNA was done as well (Taylor et al., 1997). The following years brought us much more information about this fascinating molecule pointing towards its important regulatory role in translation processes as well as its specific localization in synaptodendritic domains (Iacoangeli and Tiedge, 2013). Moreover, BC1 RNA was found to be a part of the same ribonucleoprotein complex as FMRP that led to questions about their interactions (Johnstone et al., 2011). Two main ideas appeared in the



literature referring to FMRP-BC1 RNA interactions, one is in favour of direct and specific interaction between the two molecules (Bagni, 2008; Centonze et al., 2007; Zalfa et al., 2005; Zalfa et al., 2003), another one states that their interaction is rather indirect or at least non-specific (Iacoangeli et al., 2008a; Iacoangeli et al., 2008b). Because both FMRP and BC1 RNA are thought to regulate protein synthesis by repressing their translation at the synapse (Bhakar et al., 2012; Iacoangeli and Tiedge, 2013; Kondrashov et al., 2005; Wang et al., 2005; Wang et al., 2002), solving the problem of their interaction may shed some light on the molecular basis of FXS and associated disorders. On the other hand, functional interaction of FMRP and BC1 RNA was suggested in studies on BC1 KO mice lacking BC1 RNA that showed increased neuronal excitability and several other apparent commonalities between phenotypes of this mouse model and *Fmr1* KO mouse (Chuang et al., 2005; Lewejohann et al., 2004; Skryabin et al., 2003; Yan et al., 2004; Zhong et al., 2009; Zhong et al., 2010). Therefore, the combination of both molecular and functional approaches in studies on BC1 KO along with *Fmr1* KO mice should help us in our understanding their functioning.



### 3 AIMS

In this thesis three projects with different aims are presented.

- I. Investigation of cortical plasticity changes in DISC1-cc mouse model of SZ after a short term activation of DISC1-cc protein during early postnatal development (paper I, referred as the “DISC1 Project”).
- II. Electrophysiological and behavioural characterization of somatosensory processing defects in *Fmr1* KO mouse, an animal model of FXS that lacks FMR1 protein (paper II, referred as the “Fragile X Project”).
- III. Identification of roles of BC1 RNA in cortical synaptic physiology and structural plasticity using another FXS animal model, BC1 KO mouse, that lacks BC1 RNA (paper III, referred as the “BC1 Project”).



## 4 METHODS

Detailed description of methodology for each project is presented in the specific articles included at the end of this thesis. Nevertheless, this section is written to give an overview of the experimental design and explanation of some technical considerations that arose during the projects' development. All experiments were approved by the local ethical committees in the countries where they were performed according to the institutional guidelines that are in compliance with national and international laws and policies.

### 4.1 Animal models

#### 4.1.1 DISC1-cc mouse

To study the role of the DISC1 protein in neuronal plasticity processes we chose the DISC1-cc mouse model developed in the Alcino Silva's lab (Li et al., 2007). This is an inducible and reversible transgenic system, where by a single tamoxifen injection a truncated version (isomer) of the full-length DISC1 protein, DISC1-cc protein, can be expressed for a fixed period between 6 to 48 hours after the injection. The DISC1-cc isomer is expressed only under control of the  $\alpha$ -calmodulin kinase II ( $\alpha$ -CaMKII) promoter that is restricted to primary neurons of the forebrain (Mayford et al., 1995). The DISC1-cc isomer spans residues 671-852, a C-terminal portion of the full-length DISC1 protein, and it corresponds to the one produced in the Scottish family as a result of DISC1 mutation (Brandon and Sawa, 2011). The DISC1-cc mouse is one of several DISC1 models constructed to address different aspects of DISC1 mutation.

DISC1 models can be divided into three main categories: haploinsufficiency, point mutation, and transgenic models (for review, see Brandon and Sawa, 2011; Cash-Padgett and Jaaro-Peled, 2013; Jaaro-Peled, 2009; Johnstone et al., 2011; Tomoda et al., 2016). Haploinsufficiency systems were constructed to mimic naturally occurring DISC1 mutation based on DISC1 loss-of-function paradigm. DISC1 mutation is a consequence of a balanced chromosomal translocation (1q42; 11q14.3) that disrupts *DISC1* gene at intron 8 (Blackwood et al., 2001). Despite the fact that *DISC1* gene on the other chromosome stays intact, this disruption may lead to loss-of-function due to potentially reduced expression of full-length DISC1 protein. There are three haploinsufficiency systems currently in use and in each of them different exons in *Disc1* gene are modified (Koike et al., 2006; Kuroda et al., 2011; Shahani et al., 2015).

The second method is based on screening for point mutations in the mouse after mutagenizing it with N-ethyl-N-nitrosourea (for details on ENU method see Sakuraba et al. (2005)). Clapcote et al. (2007) used the ENU method to produce two DISC1 mouse models: Q31L and L100P. These models indicated that the ENU method may be also helpful in studying DISC1 mutation when focusing on specific changes in DNA and their potential contribution to this mutation.

The third approach harnesses transgenic methods to construct models of human DISC1 breakpoint mutation. In these models different dominant-negative isoforms of DISC1 are expressed in mouse brains. Sawa and co-workers (Hikida et al., 2007) generated transgenic lines postnatally expressing a human truncated DISC1 protein, amino acids 1-597, with the specific postnatal expression predominantly in forebrain neurons. Pletnikov et al. (2008) created another transgenic line using a Tet-off double transgenic system with doxycycline-dependent expression of human-derived mutant DISC1 (Clontech, Palo Alto, CA, USA), where expression-time can be partially-controlled via a doxycycline diet. Shen et al. (2008) in their model used mouse transgenes that are known to be more efficiently expressed instead of cDNA-based constructs. They also focused on the N-terminal portion of DISC1 protein since the protein in this transgenic animal contained exons 1-9. The model used in our studies (DISC1-cc mouse) also belongs to the category of transgenic systems, however, it was constructed with different aims. It does not try to imitate human DISC1 mutation in a mouse like other transgenic models. It is rather a tool to address questions about the specific role of a truncated, missing part of the DISC1 protein, the C-terminal portion. Furthermore, this is the only available system with the short-term transgene activation in a time-controlled manner, which is crucial for neurodevelopmental studies such as ours.

#### **4.1.2 *Fmr1* KO and BC1 KO mice**

In our projects related to FXS we used two different genetically modified mouse lines – *Fmr1* KO and BC1 KO. The *Fmr1* KO mouse model was generated by the Dutch-Belgian Fragile X Consortium in 1994 (Consortium, 1994) shortly after discovery of the *FMRI* gene mutation prevalence in FXS human patients. It was created for use in search for the FMR1 protein's physiological function entirely unknown at that time. Initial study revealed suitability of *Fmr1* KO mice as an animal model of FXS – mutants lacked normal FMR1 protein, showed macroorchidism, hyperactivity, and partial learning deficits in the Morris water maze task. Over many years further studies confirmed similarities with FXS human symptoms regarding anatomical, physiological and behavioural differences as most recently

reviewed by Kazdoba et al. (2014) or Santos et al. (2014). The first *Fmr1* KO mice were generated by injecting embryonic stem cells into wild type (WT) mice of the C57Bl/6J (B6) strain. The offspring were backcrossed within the same B6 strain (Consortium, 1994). Many other strains have now been used as background strains for breeding of the *Fmr1* KO mouse line, leading to variety of differences in results observed in FXS-related studies. These differences are noticeable not only in severity of symptoms but also in the direction of changes revealed, mostly observed in behavioural paradigms. Therefore, Spencer et al. (2011) compared directly *Fmr1* KO mice bred with 6 different background strains. Their results indicated that genetic background clearly affects almost all behavioural measures they generated. This status quo may lead to a conclusion that genetic background may be even more important in shaping the final phenotype than the actual mutation. Nevertheless, all tested mutants shared several common symptoms as well, namely hyperactivity, lower anxiety levels, abnormal sensory and social responses. Many of those changes were previously reversed in *Fmr1* KO mice on the B6 background strain by expressing a transgenic FMR1 protein (Spencer et al., 2011). Furthermore, in a direct comparison of *Fmr1* KO mice of B6 and FVB backgrounds (Pietropaolo et al., 2011), both strains appear to be quite similar in tested phenotypes with mild differences suggesting that B6 background may be more appropriate for studying autistic-like symptoms (observed changes were more similar to human phenotypes in B6 background). As the Spencer et al. (2011) study indicated that many behavioural phenotypes differed between genetically modified mice depending on their background strain, one could potentially select a background with more robust differences in a certain phenotype to improve one's examine that phenotype. Unfortunately, no tactile-related studies were performed in *Fmr1* KO mice at the time we started our project, so we could not use this advantage. Therefore, in our project we chose to breed our mutant mice of the most popular original B6 background strain.

Referring to BC1 KO mice, we used the only available mutant line that was originally generated by Skryabin et al. (2003) and chosen from initially tested lines. They were established by breeding BC1-deficient mice, male chimeras (129Sv strain) and non-mutant females (B6 strain), to produce heterozygous mice subsequently interbred to BC1-/- homozygosity. Male chimeras were established from three independent mutant embryonic cell lines derived from 129Sv non-inbred strain. In this way, the generated mutant line featured a heterozygous genetic background with different relative contributions of alleles from 129Sv and B6 (about 50% contribution of each). In our study we used the same two background strains for our mutant breeding, either homogenous B6 or mixed 129Sv-B6. Using non-inbred background strains helps to avoid random mutations and confounding

inbreeding effects. BC1 KO mice when created in the original study had been initially characterized in a series of exploration and spatial memory tasks in which they revealed reduced exploration, increased anxiety levels and normal spatial memory (Lewejohann et al., 2004). Because further studies revealed that BC1 RNA potentially plays a role similar to FMR1 protein in regulation of translation (reviewed in Iacoangeli et al. (2010) or Iacoangeli and Tiedge (2013)), BC1 KO mice become an interesting model to study FXS pathologies. Electrophysiological studies in the hippocampus (Zhong et al., 2009; Zhong et al., 2010) and in the striatum (Centonze et al., 2008; Centonze et al., 2007; Maccarrone et al., 2010) confirmed the validity of this model. Interestingly, a more recently-developed BC1 KO mouse line was successfully used as a recipient background in creating transgenic mice. Robeck et al. (2016) generated a series of BC1 RNA variants and expressed them in transgenic mice to study structure and function of BC1 RNA in more detail.

## **4.2 *In vivo* electrophysiology**

### **4.2.1 Recording techniques**

Electrophysiological recordings from anaesthetised animals were used to determine intrinsic properties of specific neuronal populations. In all projects we used urethane anaesthesia (about 70% of the maximum dosage of 1.0 or 1.5 g/kg) with supplemental doses when anaesthesia level was diminishing (10% of the maximum dosage). We chose urethane carefully considering its advantages over other available drugs. Urethane is the only stable, long-lasting anaesthesia that affects several receptors in the brain in a balanced manner (Hara and Harris, 2002). Recordings were collected at a similar medium depth of anaesthesia that was monitored regularly throughout the experiment by testing reflexes and observing spontaneous firing rate of neurons. We aimed to record at the level corresponding to stage 3-4 sleep, where slow oscillations in the delta range (around 5 Hz) are present and spindle waves are absent (Armstrong-James and Fox, 1988; Friedberg et al., 1999) to avoid variability in brain activity caused by varying anaesthesia depth (Fox, 1992). Single neuron activity was recorded extracellularly from L2/3 (depth 100-350  $\mu\text{m}$ ) and L4 (depth 350-500  $\mu\text{m}$ ) of the somatosensory barrel cortex. We distinguished the boards of layers using standard depths described earlier in the barrel cortex literature (for review, see Fox (2008)) and confirmed by Groh et al. (2010) more recently with the use of modern immunohistochemistry techniques (GAD67 and NeuN staining). We used two different types of electrode for extracellular recordings. In the DISC1 Project, all neurons were recorded extracellularly using single barrel carbon fibre microelectrodes made as previously described (Armstrong-James et al.,



1980). This type of electrode was chosen due to their low impedance (typically between 200 k $\Omega$  and 2 M $\Omega$ ) and their thin tip – both characteristics crucial for achieving good recordings of cortical plasticity. In the Fragile X Project and the BC1 Project we used juxta-cellular recordings performed with electrodes pulled from borosilicate filamented glass (resistance 4 to 8 M $\Omega$ ) and filled with a standard salt solution corresponding to the extracellular space under resting conditions. Using this method helped us to achieve a stable, isolated, single-neuron response recorded over an extended period (sometimes even longer than 90 minutes), necessary for the stimulation protocols that we used to study somatosensory processing. In all projects we only focused on excitatory cells, distinguishing them by the spike-waveform analysis, a standard practice that has been used since the 1980s (e.g. see Armstrong-James and Fox, 1987; Bortone et al., 2014; Bruno and Simons, 2002; Denman and Contreras, 2015; Niell and Stryker 2008).

#### **4.2.2 Whisker stimulation**

Whiskers were stimulated using a computer-controlled piezo-electric stimulator in all discussed projects. We chose this method to have the ability to move a single whisker at a time. Furthermore, we were able to control all detailed parameters of the whisker movement including stimulation pulse duration, rise/fall time of the piezo or piezo-deflection amplitude. Whiskers were initially cut to a similar length to ensure equal movements when stimulated and they were moved by a glass capillary glued to a piezo-electric wafer. The glass capillary tip was placed in loose contact with the whisker and it was moved in a dorso-ventral direction. Alternative whisker stimulation method, an air-puff stimulation, gives the opportunity to move whiskers in the antero-posterior direction in a manner resembling natural whisker movements much better than the piezo-stimulation. However, with the air-puff stimulation the stream of high-pressure air delivered through a small tube usually placed in the front of the whisker pad moves many whiskers together at the same time. This lack of control over the individual whisker movements makes this method useful for different types of tactile-related studies asking questions about somatosensory processing in a more general terms, for instance the process of multisensory integration in the striato-cortical circuits (Reig and Silberberg, 2014; Reig and Silberberg, 2016).

Despite the fact that a piezo-stimulation was the best method for our purpose, it has some downsides that we took into account. The piezo-electric stimulator is activated by an electric square pulse with certain parameters that evokes an ON- and OFF-movement but it evokes ringing at the end of a stimulation as well (a result of the piezo stiffness). However, measured

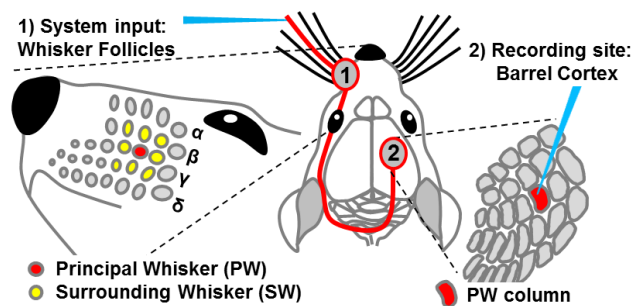
ringing frequency is 30 Hz and it is above the typical frequency of mouse whisker movements previously described to be in a range of 1-20 Hz (Cao et al., 2012; Carvell and Simons, 1996; Sofroniew et al., 2014; Sofroniew and Svoboda, 2015; Voigts et al., 2008). However, we used the same stimulation set up for genetically modified mice and their WT littermates, thus our data should be consistent. Another possible concern is the single deflection angle that we used (stimulation in a dorso-ventral direction). This stimulation direction may not be the most optimal angle for either principal or surrounding whiskers, especially given the fact that some neurons in the barrel cortex show directional selectivity, as was shown in many studies in rats (Bruno et al., 2003; Kremer et al., 2011; Lee and Simons, 2004; Wilent and Contreras, 2005) and also, more recently, in mice (van der Bourg et al., 2016). Stimulating a “sub-optimal” direction theoretically may affect our PW-D1 or PW-SW comparisons under the assumption that PW and SWs have different tuning curves only in the genetically modified animals but not in WT. If true, it would be an interesting finding, however, there is no evidence for such a scenario. Additionally, Kida et al. (2005) showed in WT mice that a directional tuning curve is similar between PW and SWs. Therefore, in our cell-sampling procedure at first we manually moved different whiskers with a small wooden stick. Next, when a responding cell was found, we localized a PW and moved it in various directions. Finally, we proceeded with the recording and the piezo-stimulation protocol only when the cell was clearly responding to the movements in the dorso-ventral direction.

### **4.2.3 Whisker stimulation-evoked response**

In all projects, we recorded whisker-stimulation-evoked responses to mechanical movements of the principal whisker (PW) or adjacent surrounding whiskers (SW) (Fig. 2). This distinction was based on a widely approved concept that tactile stimulation of each individual whisker evokes localized activation of the cortical area corresponding to this whisker (Feldman and Brecht, 2005). Because of the columnar organization of the barrel cortex, the strongest stimulation-evoked-response (the greatest number of action potentials) with the shortest latency (the shortest delay between the stimulation and the response) is usually observed in one particular barrel column connected to the moved whisker (Armstrong-James and Fox, 1987). This column is called the PW column and, by analogy, the corresponding whisker is called the PW. All the whiskers in the immediate surrounding are called SWs may also evoke a response in the PW column because of the intercolumnar projections but, normally, this response is smaller and has a longer latency (Armstrong-James et al., 1992; Feldmeyer et al., 2002; Laaris and Keller, 2002; Schubert et al., 2003).

Direct comparison between the PW and SW response parameters is a very useful method to study basic properties of somatosensory processing. We can compare PW and SW responses separately to gain information about the processing within specific whisker-barrel pathways or we can learn about the cortical spread of excitation and somatosensory maps by comparing PW and SW response ratios. Knowing typical response parameters for specific cortical layers in WT mice in normal unchanged conditions, we can manipulate these conditions in various ways to learn how the neural system will respond to these changes. One such manipulation is generating genetically modified animals lacking certain genes or proteins. These animals can serve as a great tool in studying neural processing by mimicking pathological conditions observed in various pathological conditions. Genetically modified animals were used in all projects and we compared their cortical responses with responses recorded in their WT littermates.

Another manipulation used for the *in vivo* electrophysiological experiments was whisker removal called whisker deprivation applied temporarily for a certain period. We used whisker deprivation in the DISC1 Project to check whether the *DISC1* gene plays any role in plasticity processes. In our protocol we removed unilaterally all the whiskers sparing only one whisker (D1) on this side of the snout. This protocol was applied for several consecutive days and it evoked permanent changes in the somatosensory map of the barrel cortex even in the adult animals (Fox et al., 1996; Li et al., 1995). The area corresponding to the spared D1 whisker enlarges to involve also the barrels adjacent to the D1 barrel and the area corresponding to the removed whiskers shrinks. Obviously, this change has its consequences for responses recorded in the barrels corresponding to the removed and spared whiskers. To



**Fig. 2 Scheme illustrating electrophysiological experiments on the whisker system.** Whiskers were mechanically moved with a piezo-electric stimulator one at a time. At the same time, single cell recordings were performed in the barrel cortex. Note that recordings were always collected from the barrel column corresponding to the principle whisker (PW) called, by analogy, the PW column.

capture these differences two indexes were designed in the Fox's laboratory based on the similar approach used by Ramoa et al. (1988) for quantitative summary of ocular deprivation experiments. In the Vibrissae Dominance Histogram (VDH), the D1 response was compared directly to the PW response for each recorded cell ( $F = D1 / (D1 + PW)$ ) and the index value was assigned to this cell. Subsequently, these values could be presented on the histograms by comparing the percentage of cells with certain VDH indexes. VDH F values were easily adapted for a graphical representation of collected data, however, to compare them statistically another measurement was designed. The Weighted Vibrissae Dominance Index (WVDI) for each animal was calculated basing on the previously calculated VDH F values:  $WVDI = (0F0 + 1F1 + 2F2 + \dots + 9F9) / 9N$  where, F0 is the number of cells in the 0.000-0.099 band; 0.100-0.199 band etc. and N is the total number of cells in a particular sample. Because this measurement creates a single number for each subject that can be averaged along with all the subjects within a group, the results from a single animal cannot bias the study. Also, because both VDH and WVDH are based on a direct comparison between D1 and PW response, response changes due to anaesthesia are compensated for.

In the Fragile X Project we studied somatosensory processing in the genetically modified mice and the main goal was to test whether a genetic mutation causes any changes in the processing of tactile information. Similar to the DISC1 project, we expected some changes in the cortical spread of excitation, however, this time potentially more subtle and without any specific localization like the one observed in the case of D1 column after whisker deprivation. We had been searching for a clear visual representation of the somatosensory map that could help us to notice potential changes in the spread of excitation. Inspired by the Fox's laboratory solution for the PW-D1 whisker response comparison, in the Krieger's laboratory we came up with the idea of normalizing the SW response to the PW response and using these normalized values for the graphical representation. In this way we were able to extract even subtle differences between the PW and SW response levels compensating for "the cell effect" or "the animal effect", the effect where a few different recordings would mask potential changes. Additionally because, even in pathological conditions, SW stimulation was not expected to evoke a greater response than the PW stimulation, normalized response values were on a limited scale between '0' and '1' that helped us in designing a new parameter called the Whisker Selectivity Index (WSI). The WSI was created for a quantitative representation of our data and it was calculated as  $WSI = 1 - (SW / PW)$ . By the "whisker selectivity" we understood the animal's ability to distinguish between deflections of different whiskers. We thought that if the response rate is an important part of encoding of tactile information, a change in the relative PW and SW response rates may affect an

animal's ability to differentiate between different whisker movements. Reduced whisker selectivity was represented by lower index values, normal selectivity by higher ones. Finally, we were also interested in the background activity of neural circuit knowing that stimulation-evoked cortical responses may be affected by the spontaneous activity of the neural circuit (Sachdev et al., 2004). We recorded regular spontaneous activity before starting any whisker stimulation. However, we also came up with the idea that the background activity recorded during the whisker-stimulation train may more closely reflect the activity in the system during the actual sensory processing (Sachdev et al., 2004). Moreover, sub-threshold activity changes during whisker deflections may affect stimulation-evoked responses (Moore and Nelson, 1998). Therefore, we recorded activity in-between the stimulations during ongoing stimulation trains and we called it the Inter-Stimulus Activity. In the BC1 Project we based our cortical activity analysis on the same measurements as in the Fragile X Project.

#### **4.3 Histology and immunohistochemistry**

In all projects after each experiment the animal was deeply anaesthetised with a lethal overdose of euthatal (pentobarbital sodium) or a urethane/acepromazine mix, perfused with 0.1 M phosphate-buffer saline (PBS) followed by 4% paraformaldehyde and the mouse brain was carefully removed from the skull. In the case of the DISC1 Project, after the perfusion the recorded hemisphere's cortex was dissected and flattened between two glass slides as previously described (Strominger and Woolsey, 1987), postfixated with sucrose formaldehyde and sucrose PBS, sectioned on a freezing microtome and stained for cytochrome oxidase following the protocol by Wong-Riley (1979). At the end of each recording penetration small electrical lesions were made at the estimated depth of 350  $\mu\text{m}$ . After the cytochrome oxidase staining, it was possible to confirm in which barrel each cell was recorded by correlating lesions with histology using a camera lucida system combined with light microscopy. In the Fragile X Project, fixed brains were sliced using a microtome, stained for cytochrome oxidase and biocytin development as previously described (Krieger, 2009). Biocytin was added to the recording pipette solution and it was used to fill recorded cells at the end of each recording according to the microelectroporation protocol of Pinault (1996). Depth and location of the recorded cell was accurately verified during the experiments and the histology analysis was an additional control. Due to time limits only a few brains were sectioned and fully stained but in every analysed case recorded cell's localization was very accurate.

#### 4.4 Gap-crossing task

In the Fragile X Project our goal in behavioural experiments was to specifically test how *Fmr1* KO mice use tactile whisker information to solve a sensory-motor task. Because tactile processing in the whisker system is rather a challenging topic for behavioural studies, there is a limited choice of available behavioural paradigms. Therefore, after careful consideration of the available literature, we decided to use a whisker-dependent paradigm called the gap-crossing task (Celikel and Sakmann, 2007; Harris et al., 1999; Hutson and Masterton, 1986). In this task a mouse is placed on one of two elevated platforms placed next to each other with a gap in-between them. Motion sensors were installed at the beginning and at the end of each platform to record locomotor activity in these areas. In time-fixed sessions repeated on a daily basis, a tested mouse is supposed to explore its new surroundings and learn to cross the gap. Once the mouse has learnt how to cross the gap, the gap-distance is increased, usually depending on the number of crossings performed (successful trials). Our protocol was based on successful trials as well, however, we changed the gap-distance in a pseudo-random manner, which allowed the mice to learn to jump over increasingly greater gaps and maintain a degree of unpredictability at the same time. Reinforcing this “feel-before-jumping” phase might be important to maintain the animal’s interest in continuing the task. In the past, to achieve the same result, Hutson and Masterton (1986) widened the gap to a larger-than-a-whisker-reachable distance. All tests were performed in a dark room with a red light to ensure that a mouse’s decision about crossing the gap was based purely on tactile information (rodents do not see the red light). Furthermore, we tested whether sensory input from the whiskers had been crucial for this decision making. We increased the gap to a larger-than-a-whisker-reachable distance and it turned out that none of the mice attempted to cross this large gap. Finally, it is known from previous studies that touching a platform is a crucial prerequisite for attempting to cross the gap because this is the way a mouse collect the information necessary to judge the distance between the platforms in a dark room (Celikel and Sakmann, 2007). Also, Hutson and Masterton (1986) confirmed whisker-dependence of this task using blinded or entirely whisker-deprived rats.

In the past, the gap-crossing task had been used to test general sensorimotor exploratory behaviour (Hutson and Masterton, 1986). Nowadays, by means of modern technology, it is possible to analyse whisker-kinematics at the same time (Voigts et al., 2008). We mounted a high-resolution infrared video camera on top of the gap and recorded whisking behaviour during the gap-crossing attempts. Optimal analysis of simultaneous multiple whisker movements demanded multiple cameras to achieve a clear 3D picture of each whisker

(Voigts et al., 2008). Because our system had only a single camera, we decided to test whisker-deprived animals with a single whisker on each side of the snout to overcome these problems. It has been shown by Celikel and Sakmann (2007) that mice with single whiskers perform the gap-crossing task as well as the mice with multiple whiskers, with the only difference being a faster response time in the multiple-whisker mice. Moreover, they suggested a potential redundancy of the information collected by multiple-whiskers in this task since the surface, structure, and the formation of the edge of the platforms were all uniform. In line with their argument, in a more complex horizontal object localization task, Knutsen et al. (2006) showed that a complete set of whiskers is unnecessary for the task performance itself, however, rats had to have more than one whisker on each side of the snout to accomplish the task. In fact, following the learning period, in later phases of the task, whisker-trimming even improved rats' efficiency in task performance.

Spontaneous gap-crossing task is a simple whisker-dependent behavioural paradigm and it is sufficient that mice detect a platform without any additional complications like texture discriminating or working to receive a reward. It is true that it seems to be a very crude paradigm with an all-or-none response to detect whether the second platform is there. However, rather than being a crude measure of tactile processing, we thought that this simplicity gives us an opportunity to show a clear difference in cortical computation related to supposedly different whisking phenotypes in *Fmr1* KO mice. FXS mouse model had been characterized for many different behavioural phenotypes (for review, see Kazdoba et al. (2014)) but information about any tactile deficits in this animal was not available back in 2010 at the time when our behavioural experiments were started. Therefore, our aim was to check whether there is any difference in general haptic behaviour of *Fmr1* KO mice. This behavioural test shows how animals use tactile information for decision-making. We did not use reward in the gap-crossing task and we did not choose more complex whisker-dependent paradigms like fear-conditioning with a cue conditioned to a whisker deflection. These tasks would strongly involve other brain areas, such as prefrontal cortex, basal ganglia or amygdala. This would make the interpretation of tactile phenotype more difficult and possibly even unclear. Furthermore, it would test a set of other behavioural deficits, not necessarily the one we aimed at investigating. Therefore, we believe that we chose the best possible method currently available to inquire into our questions on somatosensory processing.

## 4.5 Other methods used in discussed projects

### 4.5.1 *In vitro* electrophysiology

The brain slice preparation was used to characterize pyramidal neurons using intracellular recordings performed with glass pipettes pulled from standard borosilicate capillary glass tubing and filled with intracellular solution and biocytin. Slices containing barrel cortex (300-400  $\mu\text{m}$  thick) were made from mice cortex using a vibrating microtome, maintained in a submersion chamber in artificial cerebrospinal fluid bubbled with 5% CO<sub>2</sub>-95% O<sub>2</sub> and kept at a room temperature. For recordings, slices were placed in the recording chamber under a microscope. Pyramidal neurons were chosen in L2/3 of the somatosensory barrel cortex based on morphology and basic active/passive properties (e.g. input resistance, resting membrane potential or spike half-width). The identity of neurons as pyramidal was subsequently confirmed by histological processing, including staining for cytochrome oxidase and biocytin development (Horikawa and Armstrong, 1988). In the DISC1 Project, following the initial electrophysiological characterization, excitatory postsynaptic potentials (EPSPs) were evoked by a monopolar stimulating electrode. Recordings of trains of EPSPs were used for quantification of short-term plasticity evoked by this stimulation. Subsequently, long-term plasticity protocols were applied where post-synaptic neurons were subjected to a paired pre- and postsynaptic spiking protocol to evoke LTP experiments and a paired post- and presynaptic spiking protocol to induce LTD. Also, AMPA to NMDA ratios of evoked EPSPs (Feldmeyer et al., 1999) and the NR2B component of the NMDA-mediated postsynaptic potentials (Bird et al., 2015) were obtained pharmacologically. Moreover, inhibitory postsynaptic potentials (IPSPs) were obtained using pharmacological methods (Hajos et al., 2000). Additionally, recordings measuring the incidence of silent synapses were made as previously described (Hardingham and Fox, 2006). At the end of the recordings, biocytin was deposited inside the recorded cell using current injection. Finally, anatomical reconstructions and analysis of recorded neurons were undertaken according to the methods described previously (Hardingham et al., 2011). In the BC1 Project, after the initial electrophysiological characterization, spontaneous excitatory postsynaptic currents (sEPSCs) were recorded to verify whether the observed alterations in spine number and morphology correlated with concomitant changes in synaptic function. Similarly to the DISC1 Project, recorded neurons were filled with biocytin and stained for cytochrome oxidase and biocytin development. Nevertheless, they were not reconstructed since very detailed structural analysis of potential structural changes was performed with more extensive microscopy studies.



#### **4.5.2 Anatomy and morphology**

In both the DISC1 Project and the BC1 Project, a variety of imaging techniques was used to screen pyramidal cells of genetically modified mice for anatomical changes. In DISC1-cc mice dendritic branching process as well as the proportion of spines with different head types (mushroom, thin, stubby spines, and filopodia) were measured in slices prepared from brains at different stages of development. Initial localization of cells after fixation (paraformaldehyde) and incubation (PBS with Triton X-100 and streptavidin Alexa Fluor 488 conjugate) was performed with fluorescence microscope imaging subsequently followed by 2-photon microscope imaging and z-stack analysis. In the BC1 Project dendritic complexity and spine morphology were also analysed, however, only in adolescent animals. Initial neuronal identification was performed with a light microscope under low magnification in coronal sections stained with Golgi staining. Then a series of sequential photomicrographs was taken under higher magnification to create a stack of sequential images for spine counting and spine head analysis (size-categorized as either large or small). Electron microscopy of high-pressure frozen sections was used for further detailed analysis of spine morphology focusing on the postsynaptic density (PSD) and active zone length and the general size of the spine head. Moreover, in the BC1 Project primary cell morphology and molecular analysis was performed on neuronal cultures and with the use of a transfection technique.

#### **4.5.3 Behavioural analysis in BC1 KO mice**

Since their first generation in 1994, *Fmr1* KO mice were tested in many different behavioural paradigms and they were proven to represent various symptoms that could be related to problems observed in human FXS patients (see e.g. Spencer et al. (2011)). Thus, in the Fragile X Project, we could focus on a very specific behavioural paradigm aimed at a characterization of haptic behaviour and somatosensory-dependent learning that was not described in the previous studies. On the other hand, BC1 KO mice were created a decade later and have not been studied as extensively as *Fmr1* KOs, especially in terms of behaviour. Therefore, in the BC1 Project several behavioural tests were chosen to prepare a general behavioural profile of BC1 KO mice. Novel object recognition and social novelty tasks were used to test their ability to discriminate novelty. The social dominance task tested their normal social hierarchic behaviour. Two tasks were used to assess stereotypic and compulsive behaviours and anxiety, namely self-grooming and marble burying. Additionally, anxiety-like behaviour and locomotor activity was checked in the simple open-field test. Finally, analysis of nest building performance tested a natural home-cage behaviour. All

mentioned tests gave us a diverse behavioural profile of BC1 KO mice that we could relate to the phenotypes commonly observed in similar behavioural tests in animal models of FXS/ASD (Pasciuto et al., 2015; Santos et al., 2014).

#### **4.5.4 Molecular biology and immunohistochemistry**

In the DISC1 Project, NeuN immunostaining was used for cell density characterization. In the BC1 Project, possible alterations in glutamate receptor (GluR) expression were measured by subcellular fractionation and immunoblotting. GluR subunits and associated scaffold proteins in PSD-enriched preparations from the cortex were analysed in this preparation. Local protein translation levels were tested with the use of metabolic labelling of cortical synaptoneurosome. Possible modifications in the actin cytoskeleton were studied by determining relative amounts of F-actin and G-actin using the F/G-Actin assay. Basal protein synthesis in neurons from cell cultures was tested with the SunSET assay followed by Western blotting. Finally, CO staining was also used to assess changes in cortical activity as previously described by (Wong-Riley, 1979).

## 5 RESULTS AND DISCUSSION

In all our projects we addressed questions related to perception, processing, coding and storing of somatosensory information, although with different focus. In the DISC1 Project we used whisker deprivation protocol to evoke experience-dependent plasticity changes in the barrel cortex recorded using electrophysiology in the anaesthetised mouse *in vivo* and in slice preparation. *In vivo* we showed differences in experience-dependent plasticity, analysing whisker-stimulation-evoked responses in adult DISC1-cc mice and their wild-type littermates. In slice preparation we described specific mechanisms for these changes using LTP/LTD electric-stimulation protocols. Additionally, we compared neurons' morphology in consecutive developmental windows using two-photon microscopy. We focused on dendritic branching and spine formation, features crucial to establish a systemic frame for correct neural circuit functioning. In the Fragile X Project we tried to understand changes in the physiology of cortical neurons and differences in behaviour of adult *Fmr1* KO mice that potentially developed incorrect neural circuits due to the lack of FMR1 protein. Initially, in electrophysiological recordings, we showed changes in somatosensory cortical maps as well as disruptions in encoding of tactile information in this mouse model. Similarly to DISC1 project, we used a mechanical movement of the whisker as a main stimulation-source for responses recorded from the barrel cortex. Then, we studied behaviour in a gap-crossing task to compare general performance as well as learning and whisking dynamics of *Fmr1* KO mice and their wild-type littermates. Finally, in the BC1 Project by means of electrophysiology, immunohistochemistry, molecular biology and behavioural techniques, we undertook an attempt to further understand multiple roles of BC1 RNA. The BC1 Project was immediately related to the Fragile X Project because both FMR1 protein and BC1 RNA had been found together and potentially interact in the same molecular complex important for FXS pathology. Because the absence/malfunction of FMR1 protein is one of the key features of this disease at the molecular level, the role and function of its partner BC1 RNA, became an important question.

### 5.1 THE DISC1 PROJECT (PAPER I)

Li et al. (2007) in their article described DISC1-cc transgenic mouse for the first time. This was a new mouse model to study *DISC1* gene function. In this mouse, they were able to selectively activate DISC1-cc protein in primary neurons of the forebrain by a single subcutaneous injection of tamoxifen. They showed that DISC1-cc expression was restricted to a very specific time window, 6 to 48 hours after the injection. They also showed that

DISC1-cc binds to Nudel and Lis1 proteins, natural binding partners of endogenous DISC1 protein, in the same time-restricted manner. Finally, they found that levels of endogenous DISC1 protein in DISC1/Nudel complexes were reduced in animals with activated DISC1-cc protein, suggesting a dominant-negative mechanism where DISC1-cc protein replaces endogenous DISC1 in its interactions. Further behavioural tests revealed that DISC1-cc mice exhibit several schizotypic behaviours similar to SZ human symptoms (Harvey et al., 1996) such as depressive-like traits, abnormal spatial working memory and social withdrawal. Moreover, they performed additional electrophysiological and anatomical experiments that showed reduced synaptic transmission consistent with reduction in dendritic complexity. Knowing the results mentioned and having access to such an exciting inducible and reversible transgenic system, we decided to use it for studies on plasticity mechanisms, the main topic in Kevin Fox's laboratory.

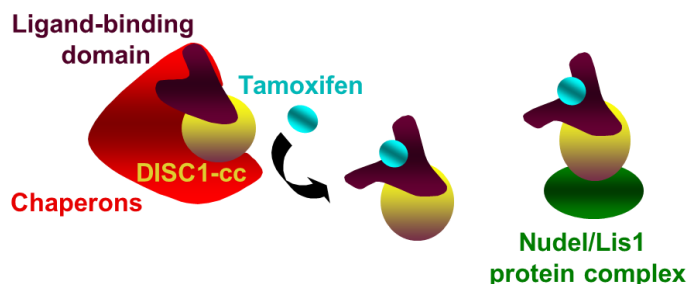
### **5.1.1 Experience-dependent plasticity, experimental preparation**

Feinberg, Weinberger and Murray already in the 1980s and the 1990s presented a neurodevelopmental concept of SZ pathophysiology (Feinberg, 1982; Murray et al., 1991; Weinberger, 1987). They suggested that a “fixed brain lesion” may affect “maturational events” that occur later in life. This means that a brain malfunction early in life, during brain development, may evoke changes in functioning of the adult brain circuits. In line with this perspective, we started the DISC1 Project with a set of experiments designed to test whether DISC1 protein interactions during early development are critical for EDP observed later in life. The EDP mechanisms are crucial processes that underlie sensory perception and integration in the brain that supposedly shape cognition throughout life. Our EDP experiments consisted of three important steps: the activation of the DISC1-cc mutated protein early in development, whisker-deprivation in late adolescence and recordings of the stimulation-evoked neural activity in adulthood. These steps corresponded with the possible dynamics of SZ pathology: an early developmental disruption, a late adolescent onset of psychosis and adulthood with a full-spectrum of SZ symptoms.

At first, mice used in the EDP experiments were intraperitoneally injected with tamoxifen at postnatal day 7 (P7) to activate the DISC1-cc protein. DISC1-cc is fused to a mutant oestrogen receptor ligand-binding domain that binds to tamoxifen instead of its natural ligand, oestrogen. The transgenic protein complex is inactive without tamoxifen (inducer) because it is sequestered by the heat-shock chaperone proteins. Tamoxifen injection evokes a conformational switch in the transgenic complex and the DISC1-cc protein is freed from the

chaperones and becomes functional for a short time. Once tamoxifen is metabolized, DISC1-cc becomes inactive again. DISC1-cc activation is shown in Fig. 3. For more details on the transgene construction and activation see Kida et al. (2002). We chose P7 for DISC1-cc activation because at this time all neurons in the barrel cortex are in their final destination. Neurogenesis and neural migration, major cytoarchitectural events, are already finished and a frame for the somatosensory maps is established. Furthermore, neurons are growing their dendrites (dendritic branching) and intensively forming synaptic connections (synaptogenesis) at this stage. Sensory experience stimulates and shapes both dendritic branching and synaptogenesis, which are crucial processes for a proper neurodevelopment. Finally, a P7 activation-time was used also in the original studies on the DISC1-cc mouse performed by Li et al. (2007). Thus, using the same injection time gave us the opportunity for an immediate comparison with their studies to obtain a more completed picture of our results. The DISC1-cc transgene contains  $\alpha$ -CaMKII promoter, therefore the DISC1-cc protein is expressed in the primary neurons of the forebrain only. Analysis with the western blot confirmed that the DISC1-cc protein is expressed in the cortex, hippocampus, striatum and cerebellum of the DISC1-cc mouse (Li et al., 2007). The DISC1-cc protein corresponds to the C-terminal portion of endogenous DISC1, the portion that possess sites interacting with Nudel and Lis1 proteins (Brandon et al., 2004; Kamiya et al., 2006). Because the DISC1-cc protein potentially acts in a dominant-negative manner to endogenous DISC1, activation of DISC1-cc temporarily disrupts regular interactions between DISC1, Nudel and Lis1.

Then, we used a standard 18-days-whisker-deprivation protocol immediately preceding our recordings from the tested mice. Deprivation was performed during the late adolescence/early adulthood, a typical onset time of the SZ symptoms visible in humans. In this protocol we

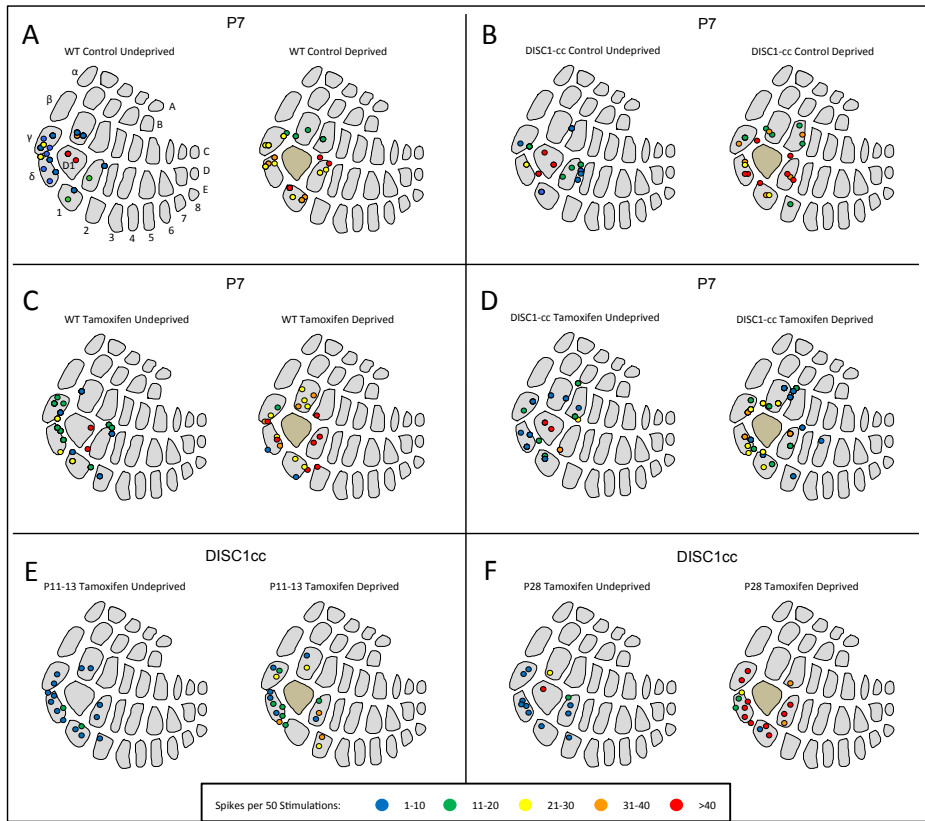


**Fig. 3 DISC1-cc protein activation mechanism.** DISC1-cc complex is sequestered with heat-shock chaperone proteins (chaperons). DISC1-cc protein is freed from the chaperons and becomes active for a limited time (6-48 hours after tamoxifen injection) once tamoxifen has bound to the ligand binding domain that is a part of this complex. DISC1-cc contains a fragment that is responsible for interactions with proteins Nudel and Lis1.

removed all but one whisker (D1) from one side of the mouse snout for a period of 18 days to invoke cortical plasticity. Whiskers were gently pulled from the follicle by applying slow, steady tension, a technique that had been found not to affect a whisker's innervation (Li et al., 1995). Eighteen days of deprivation were followed by a 6 to 10 days of regrowth period to allow whiskers to grow to a size suitable for a piezo-stimulation. In normal conditions, this protocol was proved optimal to evoke measurable plasticity changes, especially potentiation and expansion of neuronal receptive fields corresponding to the spared whisker (Fox, 1992; Fox et al., 1996; Glazewski and Fox, 1996). EDP changes evoked by whisker deprivation are a reliable measurement of nervous system ability to adapt to the environment.

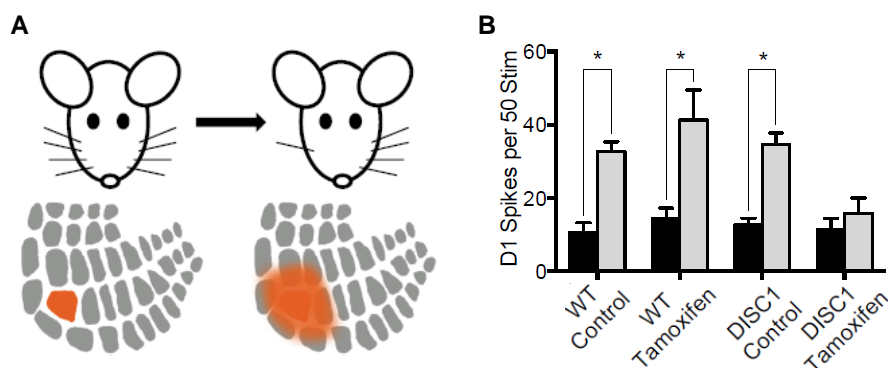
### **5.1.2 Lack of experience-dependent plasticity in the DISC1-cc mice**

In the third step of our EDP experiments we collected recordings from L4 and L2/3 of the barrel cortex of adult mice with carbon fibre microelectrodes. Single cell whisker-stimulation-evoked responses were recorded from the excitatory principal neurons in the barrel columns neighbouring to the D1 barrel column (D1 column). The D1 column is the one corresponding to the D1 whisker spared during the deprivation protocol. Using a piezo-electric stimulator, we mechanically moved PW and first-order adjacent SWs. We moved whiskers one at a time with single upward deflections of 10-ms duration, 200- $\mu$ m amplitude, and we repeated this stimulation 50 times. Then, we counted a number of stimulation-evoked spikes per 50 stimuli for D1 whisker response (D1 response) and compared between the tested groups (Fig. 4). In normal conditions, a deprivation protocol applied in the adolescent/adult WT mice does not change the D1 response rate at the level of L4 but it does affect a response to D1 whisker stimulation in L2/3. It increases a D1 response rate recorded in neurons surrounding the D1 column. This increase in whisker-stimulation-evoked spiking reveals changes in receptive fields of these neurons, an immediate result of the deprivation protocol (Fig. 5A). A cortical domain of the spared D1 whisker expands into the deprived barrels surrounding the D1 column resulting in the potentiation of the D1 response which is observed in the somatosensory maps. Indeed, after the deprivation, the D1 response was increased in L2/3 of all the control groups: WT Control and DISC1 Control that received a vehicle (corn oil) injection only, and WT Tamoxifen that received a tamoxifen injection at P7 (Fig. 5B). Interestingly though, there was no such change in DISC1 Tamoxifen group where DISC1-cc protein had been temporarily activated at P7 by tamoxifen injection. This absence of a deprivation-induced potentiation in the somatosensory maps is a sign of disrupted plasticity mechanisms in this group.



**Fig. 4 Average spike rate of recorded cells presented on the penetration maps.** Position of each penetration is shown by a circle. The colour coding represents the average response rate for D1 whisker stimulation calculated for cells located in that penetration. In undeprived groups there was a greater proportion of cells with less than 20 spikes per stimulation train (blue and green circles). In deprived control groups (A, B, C) and a deprived DISC1-cc group with tamoxifen injection at P28 (F) this proportion was shifted towards higher response rate (yellow, orange and red circles). In other DISC1-cc deprived groups this shift was totally absent in P7-tamoxifen-injected group (D) and noticeably less prominent in P11-tamoxifen-injected group (E).

We also directly compared the D1 and the PW response rate (PW response) using VDH index where we measured the extent of the PW response dominance over the D1 response. We assigned VDH index to every neuron, grouped them into bins with similar dominance levels, and plotted them on histograms for clear graphical representation. In normal WT animals distribution is skewed to the lower values on the left of the histogram, indicating that the PW stimulation evokes a greater response than the D1 stimulation (the PW response dominance). In the deprived animals the responsiveness of the cells to the spared D1 input increases in relation to the deprived PW input, which is reflected in a right-shift of the VDH distribution



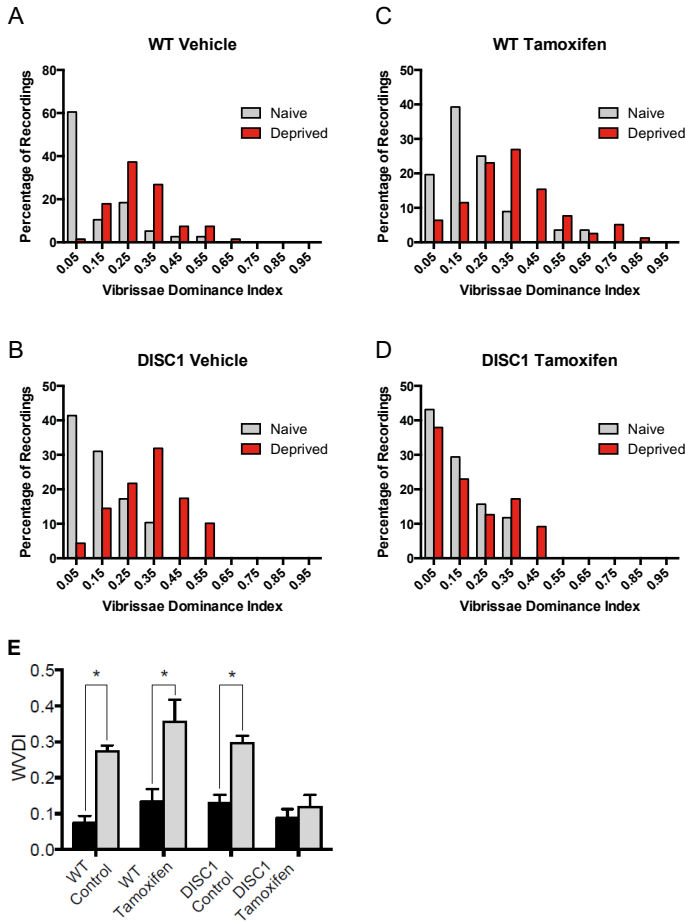
**Fig. 5 Expansion of a cortical area responding to D1 whisker movement.** (A) Scheme illustrating unilateral whisker deprivation (all except D1 whisker). An orange area corresponds to whisker-deprivation-induced expansion of spared whisker domain (D1 whisker domain). (B) Spared whisker domain increased with deprivation (grey bars) in all groups except for DISC1 Tamoxifen group in which DISC1-cc mice were injected with tamoxifen at P7 (\* $p < 0.0001$ ).

(weakening of the PW dominance). Again, this whisker-deprivation-induced change was observed in all the control groups but the PW dominance did not change in the DISC1 Tamoxifen group (Fig. 6A-D). In addition, WVDI was calculated for each animal and then it was averaged for each group to quantify changes observed in the VDH distribution (Fig. 6E). The WVDI value oscillates between “0” and “1”, where “0” corresponds to total dominance of the PW response and “1” to total dominance of the D1 response. This analysis statistically confirmed significance of the results showed in the VDH graphs. Lack of a change in the dominance histograms observed in DISC1-cc mice revealed problems with functional adaptation to change in the whisker input, a direct consequence of disrupted EDP mechanisms in the somatosensory barrel cortex.

### 5.1.3 Control for the experience-dependent plasticity experiments

At the beginning of the DISC1 Project, we used dimethyl sulfoxide (DMSO) as a vehicle for tamoxifen delivery. This solvent was suggested to us due to its high solving efficiency. Nevertheless, from our initial experiments we learnt that DMSO injection at P7 not only increased mortality rate among the injected mice, but also, affected neuronal response in our *in vivo* recordings from anaesthetized mice. These findings were in line with some DMSO studies performed with *in vitro* electrophysiology on brain slices obtained from lampreys (Tsuyetlynska et al., 2005) as well as obtained from mice (Tamagnini et al., 2014). Additionally, Kelava et al. (2011) reviewed in more detail biological actions of different drug





**Fig. 6 Whisker-deprivation-induced changes in the Vibrissae Dominance Histograms.** (A, B, C, D) Vibrissae Dominance Histograms (VDHs) are shown in pairs, undeprived (naive) versus deprived animals (respectively, grey and red bars). The D1 response was directly compared to the PW response for each recorded cell and the F value was assigned ( $F = D1 / (D1 + PW)$ ). Then, cells were divided in bins depending on their F value and the percentage of cells in each bin was calculated. In all groups except for a DISC1 Tamoxifen group (DISC1-cc mice injected with tamoxifen at P7) the skews of distribution in the deprived animals were right-shifted towards higher F values (D1 dominance over PW). In the DISC1 Tamoxifen group deprivation did not cause any change in VDH distribution. (E) The Weighted Vibrissae Dominance Indices (WVDIs) calculated as an average of VDI for each group separately (see Methods) are presented in pairs, undeprived (black bars) versus deprived (grey bars) animals. WVDI increased with deprivation in all groups except for the DISC1 Tamoxifen group where DISC1-cc mice were injected with tamoxifen at P7 (\* $p < 0.0005$ ).

solvents including DMSO. They suggested that DMSO induces significant changes in electrophysiological properties of neurons. Once we found out that DMSO was not the best solvent for our purpose, we tested the corn oil and the peanut oil as a potential solvent replacement. Both vehicles appeared to be harmless and they did not affect a cortical response in any visible way. Therefore, we chose corn oil for our further experiments where we had to deliver tamoxifen. We optimized solving protocol in our laboratory since the corn

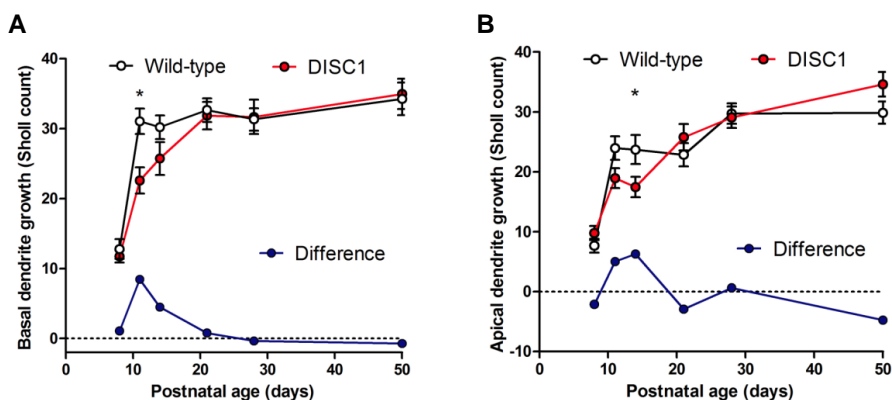
oil was not a popular tamoxifen solvent at the time when we began our project hence there were no standard procedures either. Nonetheless, with time, corn oil has become a popular and preferable vehicle for tamoxifen delivery.

Referring to the control groups in the EDP experiments that were discussed in the previous section, normal plasticity observed in WT mice receiving tamoxifen (WT Tamoxifen group) proved that tamoxifen interacted with a transgenic protein complex only. It did not affect cortical response. Moreover, tamoxifen injections did not perturb oestrogen signalling either because a mutated ligand-binding domain, a part of DISC1-cc transgenic protein complex, does not bind oestrogen (it binds tamoxifen exclusively). On the other hand, normal plasticity in the DISC1 Control group receiving a vehicle only (corn oil) revealed that a DISC1-cc protein complex was inactive. Therefore, it could not affect neuronal physiology without tamoxifen induction. Finally, in additional control experiments, we tested DISC1-cc mice with different background strains. In the original studies we bred DISC1-cc mice to C57Bl/6J strain (Harlan, Labs, UK) but in the background strain control experiments we used C57Bl/6N (Taconic, Ry, Denmark) instead. The results were the same for both genotypes, so we concluded that a genetic background of mutant mice used in our studies could not have biased our results.

The EDP experiments with P7-9 activation of the DISC1-cc protein revealed that normal functioning of endogenous DISC1 might be especially important during this early developmental time window that can be called a “critical period”. Our “critical period” hypothesis appeared to be in line with data showing that the highest expression of DISC1 occurs during early development and gradually decreases later in life (Austin et al., 2004; Nakata et al., 2009; Schurov et al., 2004). It may be true if we assume that DISC1 protein expression level corresponds directly to its activity represented by protein-protein interactions. We further tested whether the early “critical period” around P7 was crucial for DISC1 plasticity function. In the following experiments we transiently disrupted DISC1 interactions also later in development. In one group we injected tamoxifen at P13 (time corresponding to the late postnatal development). We found that in these animals EDP in the adulthood was significantly reduced but to a much lesser extent than in the P7 injected animals. In another group, we activated DISC1-cc at P28 (time corresponding to late adolescence), however, it did not influence EDP mechanisms in these animals. To sum up, these results proved a direct role of DISC1 in neuronal plasticity processes. They also suggested that there may exist the early “critical period” for DISC1 function in adult plasticity.

### 5.1.4 Where the plasticity defect originated in the DISC1-cc mice

In search for explanation of the observed EDP disruption we performed detailed anatomical and morphological analysis of pyramidal neurons from L2/3 in DISC1-cc mice injected with tamoxifen at P7 and their WT littermates. DISC1 is involved in a large number of protein-protein interactions and many of these proteins are important for neurite outgrowth (Camargo et al., 2007). Thus, we looked closer on dendritic elongation and elaboration of dendritic branching. We analysed two types of dendrites based on specific characteristics of pyramidal cells that have basal dendrites descending from the base of the soma and apical dendrites descending from the apex of the soma (Spruston, 2008). Our analysis showed that expression of DISC1-cc protein delays dendritic elongation and elaboration during the early phase of development in both types of dendrites. Nonetheless this change did not persist into adulthood since neurons represented similar morphology already at P21 (Fig. 7A, B). Similarly to our studies, Pletnikov et al. (2008) revealed attenuation of neurite outgrowth that led to a decreased complexity of neurite arbours in the primary neurons of their DISC1 mouse model. In contrast to our data, these changes appeared to be permanent. Nevertheless, their results do not have to be contradictory to ours. Pletnikov et al. (2008) used a different mouse model where modified DISC1 protein was expressed throughout entire postnatal life of a mouse. Hence, morphological changes from the early development to the adulthood might be explained by this permanently altered DISC1 function. Yet another parallel to our

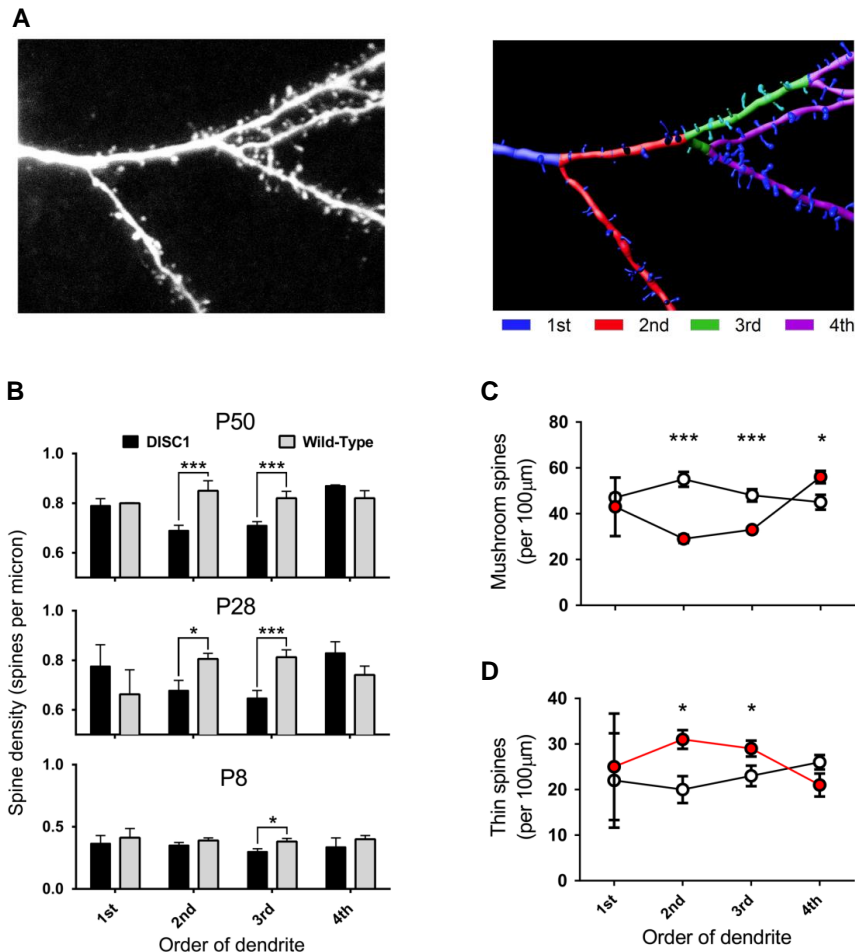


**Fig. 7** Changes in dendrite outgrowth evoked by DISC1-cc protein activation at P7. The basal (A) and the apical (B) dendrite development of L2/3 cells was retarded at P11 and P14 in DISC1-cc mice injected with tamoxifen (DISC1, in red). The difference from the WT animals reached statistical significance at P11 for the basal dendrite growth (\* $p < 0.005$ ) and at P14 for the apical dendrite growth. In both graphs blue lines show difference in means.

results may be drawn between our studies and results presented by Ozeki et al. (2003). Their cell culture studies showed that expression of the mutant DISC1 protein or suppression of the endogenous full-length DISC1 protein reduces neurite extension and decreases percentage of neurons bearing neurites.

Retardation in neurite outgrowth revealed in our model suggested an important role of DISC1 in the early development but it did not lead us to any good explanation of a long-lasting loss of the adult plasticity. Thus, we decided to check DISC1-cc neurons for more subtle morphological differences at the level of individual dendritic spines, structures that play an important role in the neuronal responsiveness and plasticity. Neurons were tested during the early development (P8), the late adolescence (P28) and during the adulthood (P50). We found that spine density was lower at P28 and P50 but only on basal dendrites of second- and third-order but no change in spine density was observed on the apical dendrites (Fig. 8A, B, C). Furthermore, spines on higher order dendrites revealed normal density at all time points. This result was not surprising if we take into account the fact that their development takes place after the period of DISC1-cc activation (after P9). Next, we analysed spine heads' morphology in the adulthood (P50). On second- and third-order dendrites we found fewer mushroom spines, an exaggerated population of thin spines and a decreased size of spine heads (Fig. 8D). These results may suggest crucial and time-dependent role of DISC1 protein during spine formation and spine maintenance. Indeed, further studies by the Fox's research group (de Haan et al., 2016) showed that when DISC1-cc activation was delayed to P9, second- and third-order spines presented density and morphology similar to WT control animals. Instead, at this time fourth- and fifth-order spines were affected. Interestingly, preliminary results regarding this later developmental period (P9-11) were much more variable than the ones obtained from P7-injected mice.

Decreased spine density in cortical pyramidal neurons had been shown previously in studies with autopsied brains from SZ patients which is in line with our findings (Garey et al., 1998; Glantz and Lewis, 2000). Because the density of dendritic spines reflects the number of glutamatergic excitatory inputs to pyramidal cells (DeFelipe and Farinas, 1992; Lewis et al., 2003), we analysed the content of glutamatergic receptors in spines. In electrophysiological recordings from brain slices we focused on AMPA and N-methyl-D-aspartate (NMDA) receptors. First EPSPs evoked by electric stimulation were pharmacologically modulated to obtain AMPA to NMDA ratios. We found that in DISC1-cc mice this ratio followed a standard development until P14, it shifted during the later postnatal development towards

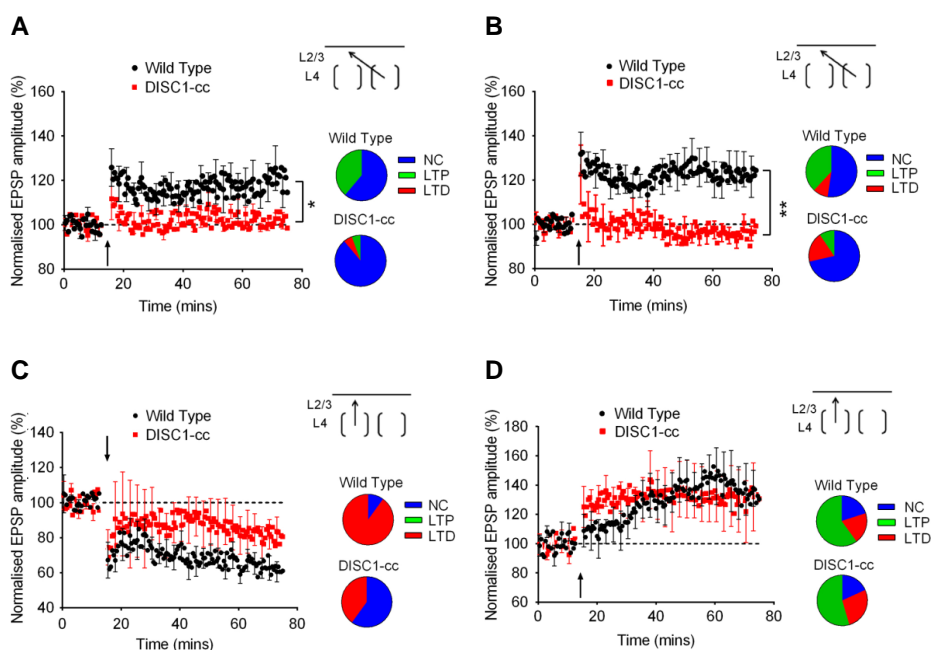


**Fig. 8 Changes evoked by DISC1-cc protein activation at P7 in basal dendritic spines.** (A) An example of L2/3 dendrites with spines on the left (scale bar 10  $\mu\text{m}$ ) and the same image on the right with colours used to show dendritic order. (B) A significant decrease in spine density on the second- and third-order dendrites was visible in DISC1-cc mice (DISC1, black bars) at P28 and P50, and at P8 only in third-order dendrites (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). (C, D) DISC1-cc mice (DISC1, red circles) had less mushroom spines and more thin spines at the second- and third-order dendrites (tested with two-way ANOVA for dendritic order and genotype).

lower values and it did not recover even in the adulthood. Furthermore, the proportion of NMDA receptor subunits in GluN2B to GluN2A increased, pointing to the reduction in the number of GluN2A. Altogether, those results suggest problems with a process of glutamate receptor insertion into synapses that leads to observed immature electrophysiological responses. Additionally, it is known that glutamate receptor insertion plays a major role in stabilizing structural development of spines (Bellone and Nicoll, 2007; Kopec et al., 2007; Nusser et al., 1998). It permits their increase in size during synaptogenesis. In fact, AMPA

receptor content is directly correlated with the size of spine heads (Kopec et al., 2007; Nusser et al., 1998). Therefore, our data showing smaller spine heads can be partially explained by problems with AMPA insertion. This explanation is even more convincing if we take into account that the most rapid synaptogenesis takes place during the early postnatal development (P7 to P13). This time corresponds directly to the “critical window” when appropriate DSIC1 signalling is necessary for adult plasticity.

Aforementioned changes may also lead to deficits in synaptic plasticity (Bellone and Nicoll, 2007; Kopec et al., 2007; Nusser et al., 1998). Therefore, in the next experiments, we tested long-term plasticity using classical stimulation protocols in the brain slices (see Methods). We revealed that in L2/3 a capability of inter-columnar LTP was entirely abolished at P28 as well as at P50 in DISC1-cc mice (Fig. 9A, B). At the same time, induction of LTD was possible but this LTD was altered: time course was slower, induction probability was lower,



**Fig. 9 Synaptic plasticity changes in DISC1-cc mice after P7 tamoxifen injection.** The capability for intercolumnar LTP was abolished by transient expression of DISC1-cc (P7-9) in L2/3 at P28 (A) and at P50 (B) (tested with two-way ANOVA for genotype and age). A significant drop in the proportion of cells expressing LTP from 33% in WT control mice to 5% in DISC1-cc mice at P28 (A) and from 43% to 9% at P50 is visible on the pie charts. (C) LTD expression was not statistically different between DISC1-cc mice and their WT littermates. However, the proportion of cells expressing LTD dropped from 90% in the WT mice to 40% in the DISC1-cc mice. (D) Reversal of LTD expression evoked by a complete whisker deprivation was unaffected in the DISC1-cc mice. This type of LTD unmasks PKA-dependent loss of depression.

and percentage of cells showing LTD in adult mice was much smaller (Fig. 9C). Next, we tested whether LTD reversal (deprivation-unmasked potentiation) was also impaired in DISC1-cc mice. Previous studies from the Fox's laboratory (Hardingham et al., 2008) showed that bilateral whisker deprivation for 7 days results in LTD occlusion in the barrel cortex, a state in which synapses favour LTD reversal. Interestingly, in DISC1-cc mice LTD reversal was possible in whisker-deprived DISC1-cc mice and similar to WT littermates (Fig. 9D), even though LTP was not a plasticity mechanism available in DISC1-cc mice. Finally, we checked time course for LTD showing that availability of the LTD mechanisms slowly decreases throughout life until it ends in the adulthood between P50 and P100. Knowing that often enough the SZ symptoms appear later in life (for instance during puberty), plasticity dynamics presented in our paper I may be helpful in the understanding of this phenomenon. Our data suggests that an early developmental disruption of DISC1 signalling affects mainly LTP expression but influences LTD parameters only slightly. Hence it is possible that the SZ symptoms stemming from disruptions of plasticity mechanisms can fully develop only when both forms of developmental plasticity, LTP and LTD, no longer function.

## 5.2 Fragile X Project (paper II)

It had been shown that patients with FXS often suffer from extreme sensitivity to sensory stimuli (Miller et al., 1999), including adversity to touch (Baranek et al., 1997; Baranek et al., 2008; Reiss and Freund, 1990). By the time that we began our Fragile X Project, several attempts had been made to reveal potential changes in the somatosensory system of the *Fmr1* KO mice in molecular and *in vitro* experimental settings. Furthermore, various behavioural phenotypes related to social withdrawal and depressive-like traits had been described in this strain (e.g. Spencer et al. (2011)). However, any query about sensory processing was largely missing in this landscape. Therefore, we decided to test *Fmr1* KO mice in the gap-crossing task, a whisker-dependent behavioural paradigm, generally used to study tactile-based learning. Preliminary results did not show any major difference in the task performance but we observed interesting changes in whisking behaviour. It looked as if *Fmr1* KO mice tried to avoid whisker-touching. Encouraged by those initial findings, we continued our detailed analysis of behavioural experiments in both aspects – task learning and whisking behaviour. In the meantime, we designed *in vivo* electrophysiological experiments to test whether the difference in whisking is accompanied by changes in the processing of the somatosensory information at the level of neural circuits. Our aim was to match methodology of our electrophysiological experiments with the behavioural ones for a better overview of potential changes. We moved individual whiskers mechanically, which corresponded to movements of

single whiskers analysed in the gap-crossing task. We used a juxtacellular recording technique that guarantees sampling of activity of single cells. It also provides the opportunity to label recorded cells at the end of the recording for better identification. Several studies revealed changes in neural network excitability suggesting hyperexcitability as the potential pathology underlying some of the symptoms of FXS pathology (Eichler and Meier, 2008; Markram and Markram, 2010). Therefore, in our electrophysiological experiments we carefully analysed not only neuronal responses but also receptive fields of the recorded cells. We expected that possible hyperexcitation in *Fmr1* KO mice may result in a spread of excitation over a larger cortical area, a consequence of changed somatotopic maps.

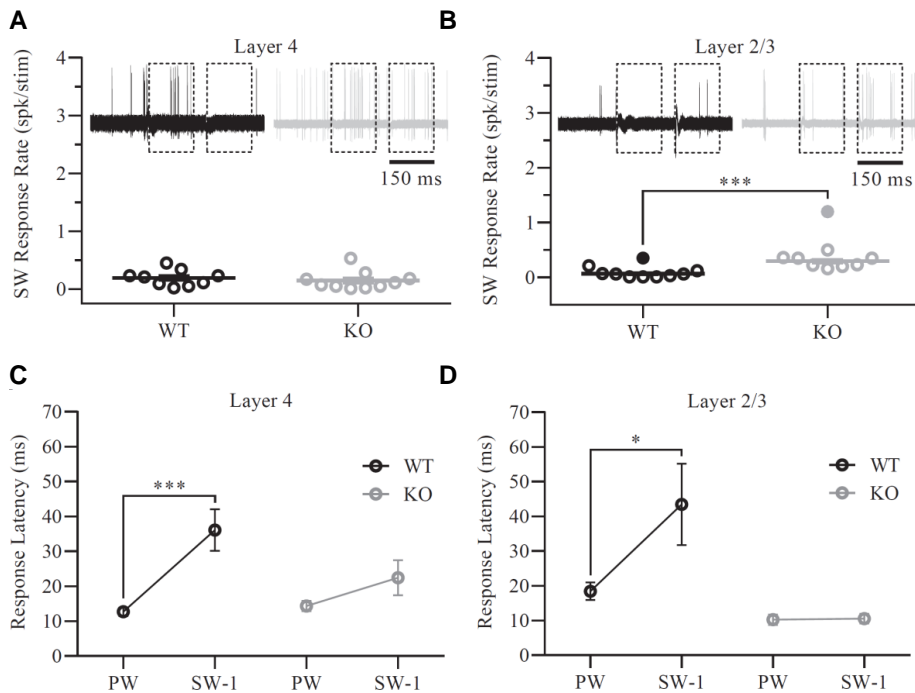
### **5.2.1 Receptive field changes in the *Fmr1* KO mouse**

In the first part of our project, we stimulated PW and SW with a piezo-electric stimulator, comparing basic properties of stimulation-evoked activity, response rate and response latency. Similarly to the DISC1 Project, we consecutively stimulated PW and all of the adjacent first order SWs using stimulation trains of 50 stimuli at 1-Hz stimulation frequency. Because it was not certain what kind of changes we would see in the response pattern, we used longer pulse duration (200 ms) that allowed us to analyse separately the ON- and OFF-components of the response, that is action potentials evoked by upward and downward movement of the stimulator. In both groups it rarely happened that a neuron responded to whisker-movements in both directions. Moreover, our analysis revealed no differences in temporal or spatial pattern of the ON- and OFF-responses. Thereupon, we consequently calculated a number of action potentials occurring during a 150-ms period either immediately after a stimulation onset or offset. To keep a consistent calculation window, for a few cells that responded to the whisker movements in both directions, we averaged the ON- and OFF-responses.

Several studies suggested hyperexcitability (for review, see Contractor et al. (2015)) and disruption in inhibition/excitation balance (for review, see Nelson and Valakh (2015)) as a primary cause of major FXS- and ASD-related symptoms. Localized activation of a given cortical area in response to the stimulation of a certain whisker is a characteristic feature of a distinctive somatotopic map organization in the barrel cortex (Feldman and Brecht, 2005). In a healthy brain of WT mice, the SW response is smaller than the PW response (fewer spikes) and it occurs later in time (longer response latency) (Armstrong-James and Fox, 1987). We used those characteristics of whisker-stimulation-evoked cortical activity to study potential changes in the flow of excitation in *Fmr1* KO mice. We looked at the response rate and the



response latency because differences in the neuronal receptive field may be represented by changes in these basic response parameters. Our recordings from excitatory cells of L4, the main input layer of the cortex, revealed no changes in the PW response rate (PW response) nor in the PW response latency (PW latency). The same was true for the SW response (Fig. 10A), however, the SW response occurred almost immediately after the PW response (Fig. 10C). Similarly, recordings from L2/3 revealed that the PW and the SW responses occurred roughly in the same time (Fig. 10D). Moreover, although the PW response rate did not change, the SW response was significantly higher in L2/3 (Fig. 10B). We also compared directly the PW and the SW responses in the WSI, a parameter designed by us to measure whisker selectivity (the higher the value, the better the selectivity). This analysis revealed that the WSI ratio was similar in L4 but it was significantly reduced in L2/3, suggesting that



**Fig. 10 Specific changes in the response rate and the response latency in *Fmr1* KO mice.** (A, B) A significant increase in the SW response rate was observed in L2/3 (\*\* $p = 0.0004$ ) but not in L4 of *Fmr1* KO mice. Insets represent example recordings from one stimulus train (50 stimulations) while dotted rectangles represent periods used for the calculation of the response rate (150 ms each). A grey filled circle was an outlier removed from the statistical comparison. (C, D) The SW response latency to the first spike was shorter in both layers of *Fmr1* KO mice. In contrast regular difference between the PW and SW response latency was visible in the control WT group (\*\* $p < 0.05$ ; \* $p < 0.001$ ).

whisker-stimulation-evoked response was not localized to the PW column. Instead, a single whisker movement activated a larger cortical area in L2/3, which translates into increased size of a neuronal receptive field. Larger receptive fields together with changes in the SW latency were signs of impaired information tuning that may lead to problems in accurate discrimination between deflections of different whiskers.

### **5.2.2 Shift in the frequency-encoding in the *Fmr1* KO mouse**

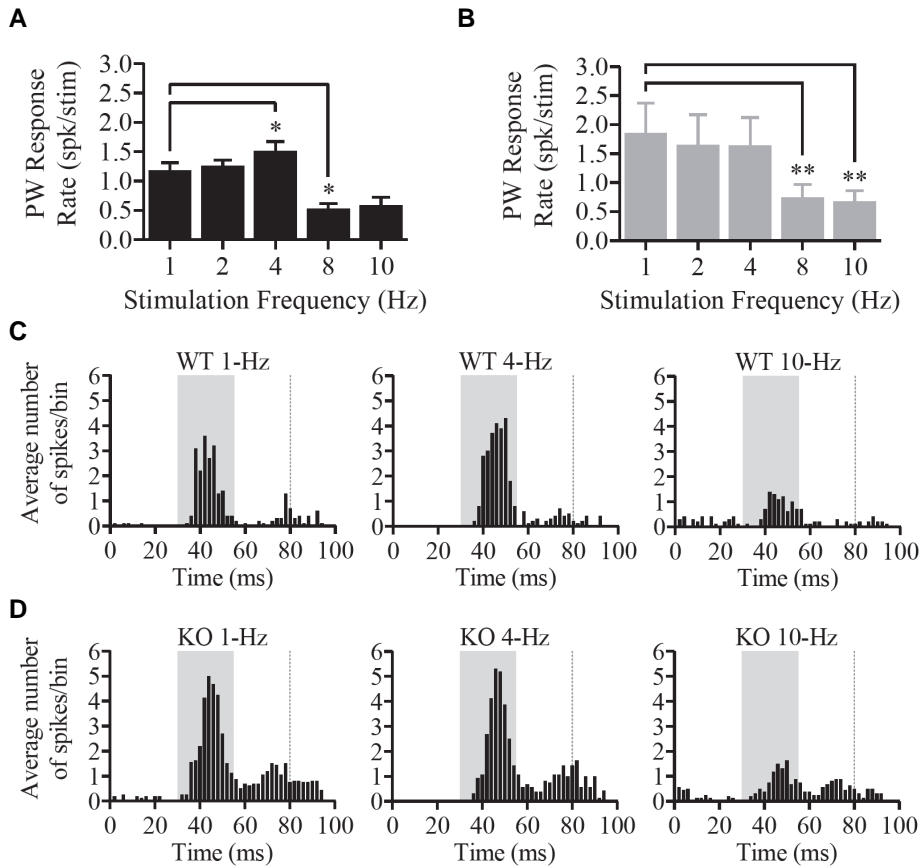
We learnt from the first part of the Fragile X Project that the somatosensory information that reaches the barrel cortex is partially impaired already at the level of L4. Because this is the main input layer of the barrel cortex (Diamond et al., 2008), the changes recorded can be interpreted also as a proximity of subcortical disruptions. Somatosensory processing impairments observed in L4 were augmented and even more complex at the level of L2/3 because not only a change in the response latency could be observed but also in the response rate. Although L2/3 receives projections from other brain structures as well, it is mainly involved in the cortico-cortical projections (Lubke et al., 2003). Therefore, we thought that L2/3 would be a good starting point to observe whether changes in the cortical processing are accompanied by disruptions in the encoding of the haptic information. Since whiskers can be moved with different frequencies along the objects that they touch, changes in the whisker-movement frequency may convey specific information about these objects (Berg and Kleinfeld, 2003; Carvell and Simons, 1990; Grant et al., 2009). Hence, in the second part of our study we focused on a detailed analysis of frequency encoding. We recorded cortical activity from L2/3 expecting more prominent changes in this layer based on our findings from the first part of the project. Furthermore, Ahissar et al. (2001) revealed in their studies on temporal frequency of whisker movement that frequency is differently encoded in different cortical layers. They showed that neurons from L2/3, but not from L4, tend to integrate various coding schemes. Both types of coding were observed in L2/3, by latency and by spike-count changes, which additionally supports our choice of this layer. In the frequency encoding part of our studies we had to shorten stimulation duration from 200 ms to 25 ms to be able to count evoked action potentials with the same time window (50 ms) for lower and for higher stimulation-frequencies. Because a protocol with various frequency stimulations was considerably longer than the 1-Hz stimulation protocol, we also reduced the number of repetitions during the stimulation train from 50 to 25. It allowed us to collect recordings from the same cell stimulating PW and all SWs with five chosen frequencies. Our frequency choice (1, 2, 4, 8, and 10 Hz) corresponded to frequencies chosen by Ahissar et al. (2001) (1 to 11 Hz) and stimulation duration likewise (25-ms in our studies, 20-ms in theirs).

In addition, when they reduced their initial stimulation duration from 50 ms to 20 ms, they noticed that the spike-counts were reduced in L4 but stayed at the same level in L2/3. Therefore, any potential change in the number of stimulation-evoked action potentials should be easier to notice in this layer.

In our studies of spatio-temporal frequency-encoding mechanisms we found that some basic properties of the frequency encoding were preserved in excitatory neurons recorded from L2/3 of both WT and *Fmr1* KO mice. Latency was increasing with the stimulation frequency similarly to results shown by Ahissar et al. (2001). Also, a sharpening of the receptive field described in the review by Moore (2004) was visible in our results. Sharpening of the receptive field reflects the spatial extent of cortical activation that is frequency-dependent. Response at the lower stimulation frequencies should have a significantly broader point spread than at the higher ones. In our data, a sharper receptive field was represented by more time-locked response noticeable as a sharper response peak in the post stimulus time histogram graphs. On the other hand, there were several changes revealing deficiencies in encoding various whisker movement frequencies. Although there was a similar overall difference between the cortical response rate for lower (1-, 2-, 4-Hz) and higher (8-, 10-Hz) frequencies of whisker movements in both *Fmr1* KO and WT littermates (Fig. 11), firing rates at lower frequencies were increased in the *Fmr1* KO mice. These responses were elevated to the level at which all low-frequency-stimulation-evoked firing rates were similar to the neuronal response to the 4-Hz stimulation. Moreover, just like in the first part of our studies, we also compared whisker selectivity using WSI calculation and the PW and the SW latency. In both cases, *Fmr1* KO mice presented changes that were independent of the stimulation-frequency. There was an overall decrease of the WSI values indicating larger and less specific neuronal receptive fields. Also, the difference between the PW and the SW latency was reduced at all stimulation-frequencies. To sum up, we thought that increased receptive field size (Feldman and Brecht, 2005; Fox et al., 2000) may be the underlying mechanism not only for the impaired information tuning but also for the frequency encoding problems present in the recorded excitatory cells. In addition, affected SW latency may have added to these effects.

### **5.2.3 Temporal spiking pattern and adaptation in the cortical response**

Temporal information contributes to tactile discrimination processes and it has been extensively studied (Foffani et al., 2009; Montemurro et al., 2007; Panzeri et al., 2001). The



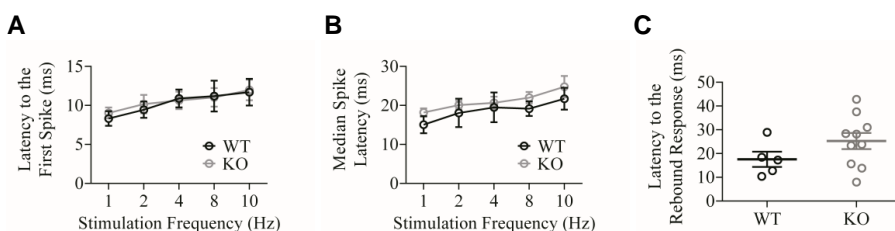
**Fig. 11 Frequency encoding by the response rate is altered in L2/3 of *Fmr1* KO mice.**

(A, B) Noticeable increase in the PW response rate for lower stimulation frequencies (1-4 Hz) and a drop in response for higher stimulation frequencies (8-10 Hz) in WT mice. In *Fmr1* KO mice response tuning for the lower stimulation frequencies was impaired (\* $p < 0.05$ ; \*\* $p < 0.005$ ). (C, D) Example recordings from one stimulus train (25 stimulations) shown as a PSTH (peristimulus time histogram) with 2 ms bins (the grey area marks the duration of the whisker deflection; dotted lines at 30 and 80 ms mark a time window used for the response calculation).

role of temporal precision may become even more important when another aspect of neural encoding, the response rate, is altered such as in the case of *Fmr1* KO mice. In the second part of the Fragile X Project related to stimulation at various frequencies, we found that neurons' response rates were similar despite changes in the stimulation frequency. Therefore, we performed a detailed analysis of the response latency and the response adaptation to see which components of the spatio-temporal coding are preserved in these mice. Similarly to the first part of the Fragile X Project, we analysed the onset latency to the first spike, but we

supplemented this analysis with the mean and the median latency measurements. All those types of analysis provide different information, so they can be complementary. While the onset latency to the first spike is more informative when it comes to discriminating sensory inputs, the mean and median latencies are thought to be more characteristic for each tested cell. The onset latency was the first spike evoked by stimulation in a stimulation train. The mean and the median latencies were calculated on the basis of timing of all spikes evoked by a stimulation train. All three measurements gave us statistically similar results, i.e. no difference in the PW response latency. Nonetheless, it seems that the median latency, but not the onset latency, revealed an interesting trend, in which it is longer at all stimulation frequencies in *Fmr1* KO mice (Fig. 12A, B). Because Fassihi et al. (2014) showed some evidence that the integration of sensory information takes place during the entire time of stimulation, a difference in the median latency could be a sign of alterations in sensory integration.

Response adaptation is another interesting measurement of the temporal precision of the response. It can be measured by means of an analysis of the response changes during the stimulation but also along consecutive stimulations in the stimulation train. If we pay attention to the Fig. 11C and 11D (the grand average of responses), it is possible to detect that after the initial responses (right next to the grey box) there is a silent period of WT neurons and a posterior rebound activity. However, in KO neurons the “silent period” does not exist and the posterior rebound seems to be higher. The “silent period” could be related to a local



**Fig. 12 Comparison of temporal precision of the whisker-stimulation-evoked response in L2/3.** (A, B) Latency to the first spike (the onset latency) as well as the median spike latency increased with the stimulation frequency in both WT and *Fmr1* KO mice. (C) Latency to the rebound response, a measurement of response adaptation, was not different for the WT and *Fmr1* KO mice. The rebound response latency was measured as a period between median latency of the response and the first spike occurring after the “silent period” which follows the first peak of the main response.

inhibition in the cortex, as it had been proposed in some works (Moore et al., 1999; Simons and Carvell, 1989), or inherited from the earlier stages of somatosensory processing, such as the thalamus or brain stem, as it had been suggested by others (Higley and Contreras, 2003; Higley and Contreras, 2005). The analysis of this “silent period” could help to understand whether in *Fmr1* KO mice their altered integration of sensory inputs was dominated by a lower local/subcortical inhibition. Therefore, we divided cells into three categories: cells that did not have any rebound activity (they were discarded), cells that had a response rebound within the first 50 ms (they were analysed), and cells that had a response rebound later than 50 ms (we checked what their proportion was). Although the data suggested a difference in the rebound activity, the proportion of cells showing a rebound with short or long latencies was not statistically significant. Also, the rebound response latency measured from the main response median to the first action potential in the rebound response did not vary (Fig. 12C). In addition to the “silent period” analysis in the frequency part of the Fragile X project, we measured OFF/ON response ratio for the first part of our project (recordings from L4 and L2/3). Longer stimulation in this part of our study (200-ms stimulation duration) allowed us to compare separately an OFF and an ON response between genotypes (stimulation onset response = ON response, stimulation offset response = OFF response). Although the OFF response rate was relatively higher, the response ratio did not reach statistical significance in any of the layers. Finally, for the frequency part of our studies, we analysed the temporal pattern between L2/3 cells in KO and WT, but we did not find any statistically significant differences either. We looked at the difference in the frequency adaptation during the stimulation course. We compared the time of the first whisker-stimulation-evoked spike between all consecutive stimulations using linear regression. In both genotypes a similar proportion of cells (about 50%) revealed statistical correspondence in those comparisons. Hence indicating that the ratio of “adaptive” to “non-adaptive” cells did not change in *Fmr1* KO mice.

To sum up, although our adaptation/inhibition analysis of the stimulation-evoked response did not show any adaptation effects, our experiments were not optimal to study adaptation mechanisms. Further experiments designed specifically for that purpose may be very informative for the understanding of not only sensory integration but also sensory perception in *Fmr1* KO mice. Assuming that sensory perception is a process requiring higher order cortices, one could argue that our electrophysiological data describes mostly a change in sensory integration, not perception. However, sensory integration is necessary for perception and we believe that changes in sensory integration that we report would also affect perception. Distinguishing between those two processes may be especially important in the

context of translation from an animal model to human disease. What is known in human FXS patients is a change in perception, but what our electrophysiological data mostly showed was a change in sensory integration.

The future experiments to specifically study adaptation/inhibition in relation to perception/integration in the FXS mouse model can be designed basing on the work performed in the Contreras' laboratory (Higley and Contreras, 2007a; Higley and Contreras, 2007b). In their studies, they focused on the mechanisms underlying cross-whisker suppression evoked by deflection of a single neighbouring SW preceding the deflection of PW. They changed different parameters of this preceding stimulation, from the number of pulses to the stimulation amplitude. Moreover, they recorded not only from the cortex but also from the thalamus. If applied to our project, recordings from both loci may be helpful in finding whether impairments observed in *Fmr1* KO mice are related to the cortical circuits or constitute a reflection of subcortical changes. Along this line, series of studies on temporal frequency of whisker movement (papers by Ahissar et al. (2001) and by Sosnik et al. (2001)) may also be a good guideline for experiments with simultaneous recordings along the somatosensory pathway since they recorded somatosensory responses in the cortex, thalamus and brain stem. In their studies they mostly focused on frequency encoding mechanisms. Adaptation can be also considered in the context of whisker vibrations that are thought to enhance coding efficiency in the barrel cortex (Adibi et al., 2013b). In the Arabzadeh's laboratory, they showed that exposure to sustained sensory stimuli evokes changes in the neuronal response (Adibi et al., 2013a; Adibi et al., 2013b). They focused on single- and multi-unit recordings of whisker-stimulation-evoked cortical activity where whisker stimulation was preceded by whisker vibrations at a high frequency (around 80 Hz) corresponding to the vibrations occurring in the natural setting. All the three aforementioned types of experiments may be easily adapted to our system and may shed new light on somatosensory problems in the FXS mouse model and, hopefully, translate into understanding of human medical conditions.

#### **5.2.4 Potential explanations of changes observed in the *Fmr1* KO mouse**

In the discussion part of the paper II, we presented some explanations of our results, mostly in the context of *in vitro* electrophysiology and anatomical data because there had not been many *in vivo* electrophysiological studies on this mouse model. We pointed out to the work of Bureau et al. (2008) where they showed that the development of excitatory connections between L4 and L2/3 was affected by the lack of FMRP. Bureau et al. (2008) revealed that

the probability of those connections was lower, their strength was reduced, and that they did not undergo EDP protocols. Moreover, the L4 to L2/3 projections were also spatially diffused. Interestingly, this change was specific to axonal descending projections within the barrel columns but not in the outer area in the septum-related columns, as discussed in detail also in their review (Bureau, 2009). Septal circuits are thought to play a differential part to barrel circuits. The former are involved in the whisker movements' information stream while the latter ones – in the temporal and spatial interactions between whiskers and external objects (Alloway, 2008). This observation opens yet another question about the tactile information processing in the septal circuits of *Fmr1* KO mice. It may be even more informative in the context of whisking behaviour and a central pattern generator that supposedly controls whisker movements in rodents (Gao et al., 2001).

Another interesting observation made in the paper by Bureau et al. (2008) were the changes in the axonal morphology. In short, axons of L4 neurons in *Fmr1* KO mice were at larger lateral distance from the cell somata during the first postnatal weeks. This change appeared to be a developmental delay rather than a permanent disruption because this difference was not observed at the age of 4 weeks, similarly to the spatial diffusion of L4 projections. Aforementioned timeline was similar to the one presented in the studies on spine development in *Fmr1* KO mice. Nimchinsky et al. (2001) showed that during the early development spine density and proportion of thin-headed spines was greater in this mouse model. However, by the week 4, spine density reached levels similar to WT mice and proportion of thin-headed spines was much closer to the control levels, which may suggest the transient nature of those changes. Other FXS-related mouse (Comery et al., 1997; Irwin et al., 2002) and human studies (Hinton et al., 1991; Irwin et al., 2001; Rudelli et al., 1985) revealed that thin-headed spines are overrepresented also in the adulthood. Therefore, it suggests that FMRP may play a crucial role in the neural circuit formation. Furthermore, changes evoked by the lack of this protein, especially during the early development, may result in permanent consequences, similarly to DISC1 protein function. On the other hand, it may be interesting to design a system blocking temporarily *Fmr1* gene or FMRP expression directly to find a specific developmental time-window for the action of this molecule that may be helpful in the search of future treatment.

Histological studies of brains obtained from FXS patients did not show any major anatomical abnormalities (Rudelli et al., 1985). Nonetheless, it was reported that abnormally long and thin spines were overrepresented in their brains and general synapse length was reduced. Obviously, morphological changes as such may influence functional balance between the



excitatory and inhibitory synapses. This kind of mechanism is thought to underlie mental retardation disorders including FXS and other diseases from ASD and SZ to Down, Patau or Rett syndrome (Eichler and Meier, 2008; Wondolowski and Dickman, 2013). Therefore, in the paper II discussion, we elaborated on the imbalance in the excitation-inhibition dynamics as a basis of the impairments observed in the neuronal receptive fields and problems in encoding of somatosensory information. It was shown that cortical neurons in *Fmr1* KO mice have stronger excitatory inputs (Olmos-Serrano et al., 2010) and less GABAergic interneurons that are less active (D'Hulst et al., 2006; Gibson et al., 2008; Paluszkiewicz et al., 2011). Nonetheless, we argued that our results were mostly related to changes in the excitatory circuits. We did not undermine the role of inhibitory impairment but we recalled inhibition studies on WT animals that were in line with our hypothesis. It was shown that, when blocking inhibition, a similar and proportional increase in responses to the PW- and the SW-stimulation was observed (Foeller et al., 2005; Kyriazi et al., 1998), whereas in our studies the changes were specific for the PW or the SW responses.

Extending the discussion from our paper 2, another interesting point that refers to the role of excitatory transmission in the FXS pathophysiology is the so-called “mGluR-theory” (mGluR – metabotropic GluR) developed in the Bear’s laboratory (reviewed in Bear et al. (2004)). This theory is based on the initial finding that the lack of FMRP potentially acting as a “translation brake” resulted in augmentation of mGluR-LTD (Huber et al., 2002). Bear et al. (2004) pointed out to two separate mechanisms of LTD: the one triggered by activation of postsynaptic NMDA receptors NMDA-LTD, and the one triggered by group 1 mGluRs activation mGluR-LTD. Both types of LTD could be evoked by the same synaptic stimulation protocol. Nonetheless, unlike NMDA-LTD, mGluR-LTD depended on the immediate translation of mRNA in the postsynaptic dendrites, hence they were down-regulated by FMRP expression. In fact, group 1 mGluRs, comprising mGluR1 and mGluR5, is required to activate synaptic protein synthesis following translation that is regulated by FMRP. Moreover, in contrast to NMDA-LTD, mGluR-LTD is irreversible and results in the loss of glutamate receptors that can be a prelude to synapse elimination. Bear et al. (2004) suggested that the lack of FMRP might be a major factor underlying excessive mGluR-signalling leading to development of FXS phenotypes. Hence relationship between mGluRs and FMRP was tested in further studies in the Bear’s laboratory by Dolen et al. (2007). In that paper, they attempted to rescue variety of phenotypes characteristic for *Fmr1* KO mice. To achieve this ambitious goal, they genetically diminished expression of mGluR5 by crossing *Fmr1* KO with mGluR5 KO mice. This breeding led to the selective reduction of mGluR5 signalling and reduction of protein synthesis in obtained *Fmr1* KO/mGluR5 KO mice. Most of the

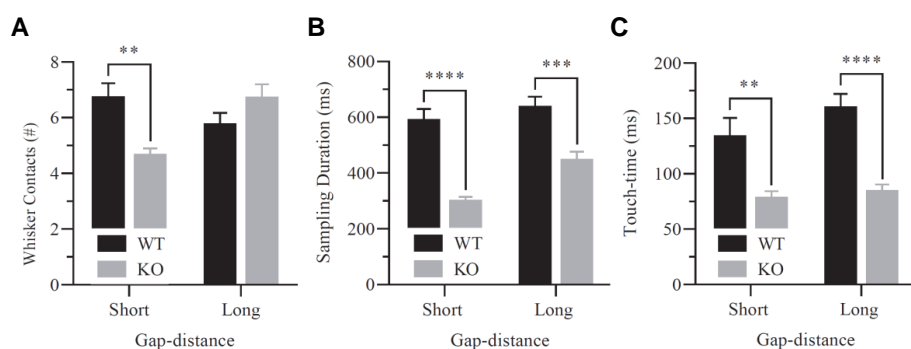
phenotypes ranging from the overrepresentation of immature thin-headed spines to behaviour were fully restored. Dolen et al. (2007) also tested ocular dominance plasticity protocol in the visual cortex during the early development. In normal conditions, it should lead to initial depression of responses to the deprived eye after 3 days of deprivation followed by later potentiation of responses to the non-deprived eye after 7 days. In *Fmr1* KO mice these changes were altered leading to substantial potentiation of the non-deprived eye already after 3 days. The lack of depression and faster potentiation resembled results of LTD reversal protocol where 7-day bilateral whisker trimming led to the deprivation-unmasked potentiation as we presented in the DISC1 Project. Therefore, it is tempting to hypothesize that from the beginning the cortex in *Fmr1* KO mice was in the state similar to the one occurring after the sensory deprivation. Thus, any further sensory deprivation would block or promote reversal of normally expected depression. In line with this idea is the data presented by Bureau et al. (2008) where they observed lack of whisker-trimming-induced weakening of L4 to L3 connections normally observed during the early development in the barrel cortex. On the other hand, Pilpel et al. (2009) showed interim increase in the hippocampal LTP evoked by low frequency pairing protocol applied during the early postnatal development. However, this change faded away with time. Furthermore, they also revealed significantly lower AMPA to NMDA ratio and, again, this alteration disappeared later during development (around 6-7 weeks of age). Changes in AMPA/NMDA ratio were caused by down-regulation of the AMPA and up-regulation of the NMDA receptor components. They suggested that the increase in NMDA receptors may be responsible for LTP increase observed in their studies. In contrast, decrease in AMPA receptors may play an important role in exaggerated LTD mechanisms observed in other FXS-related studies. In fact, increased internalization of AMPA receptors leading to decrease in available AMPA levels has been demonstrated in culture neurons (Nakamoto et al., 2007). To sum up, in *Fmr1* KO mice plasticity mechanisms seem to be altered during early postnatal development, similarly to changes shown by us in DISC1-cc mice. Furthermore, studies on both mouse models revealed mechanisms leading to alterations in the AMPA receptor function but those changes possibly had different underlying mechanisms of action. In DISC1-cc mice it was the AMPA receptor insertion, in *Fmr1* KO, the internalization. Time courses of FXS and SZ pathophysiology are similar referring to an early developmental disruption but they differ in terms of timing of the main symptoms onset. Because cognitive symptoms are core symptoms to both FXS and SZ, comparing their disease mechanisms may provide insight not only into their pathophysiology but also the cognition in general.

### 5.2.5 Altered whisking behaviour

Although FXS is characterized in humans by increased sensitivity to sensory stimuli, very few studies have examined *in vivo* sensory responses and behavioural consequences of sensory deficits in the mouse model of FXS, the *Fmr1* KO mouse. Therefore, in the third part of the Fragile X Project, we attempted to characterize behavioural deficits in a tactile-dependent learning task extending our *in vivo* studies of this FXS model. We wanted to test both aspects of the somatosensory processing: tactile integration, necessary for the task performance, and tactile perception with whisking behaviour used as its proximity measurement. In the gap-crossing task the animals made a simple go/no-go decision based on sensory information they collected when the whiskers were touching the platforms. Whisker touching provide information based on the activity of mechano-gated receptors that transfer this mechanical movement into electrical potential of neurons. Next this potential is processed to the somatosensory cortex and other brain structures. Our electrophysiological experiments were designed to mimic this behaviourally relevant situation: mechanical movements of the whiskers evoked by a piezo-electric stimulator were conveyed to the somatosensory barrel cortex where recording took place. Obviously, movements evoked by a piezo-electric stimulator in an anaesthetized mouse are not exactly the same as the natural whisker movements either in the movement frequency or in the movement dynamics. However, as mentioned above, the aim of our studies was to induce mechanical activation of the somatosensory system (similar to that occurring when touching), using its natural sensory input organ (whisker). We did not try to mimic the natural whisking frequency that is around 10-20 Hz, see e.g. Sofroniew and Svoboda (2015). Therefore, the absolute whisking frequency observed in behaving mice cannot be compared one-to-one to piezo-stimulation-evoked whisker movements in an anaesthetised animal. Furthermore, at whisker deflection frequencies lower than those occurring during natural exploratory whisking (10-15 Hz) sensory adaptation takes place, as shown in previous publications, see e.g. Ahissar et al. (2001). We also noticed this adaptation in our frequency experiments not only at 10-Hz but also at 8-Hz whisker deflection frequency. Nevertheless, despite these unavoidable shortcomings, our electrophysiological and behavioural methods correspond to one another in the closest possible manner. In fact, cortical activity recorded in the anaesthetized mouse was evoked by single whisker deflections and behavioural results were obtained from the single whisker gap-crossing task performed by an awoken mouse.

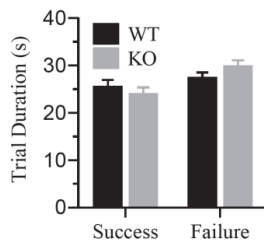
*Fmr1* KO mice appeared to have no problem learning the task. All major scores in performance parameters compared to the WT littermates revealed no differences. They spent

similar time on the gap area exploration, neither the number of attempts to cross the gap nor the number of successful crossing trials differed between the groups. However, we also analysed whisking behaviour to gain some insight into the process of acquisition of haptic information in *Fmr1* KO mice. In both genotypes, we divided our data into two major groups short and long gap distances. Mice during their tactile exploration rely not only on information collected by the whiskers but also by their nose (Celikel and Sakmann, 2007). Therefore, a “short distance” relates to distances reached by both (the nose and the whiskers) and a “long distance” is reached by the whiskers only. Our data showed that *Fmr1* KO mice had less whisker contacts with the platforms at shorter distances but there was no difference or even a trend towards the opposite at longer distances (Fig. 13A). These mice were also whisking less in the gap area making their whiskers’ “sampling duration” shorter (Fig. 13B) at both distances. Finally, a time of an individual whisker contact with a platform (a “touch-time”) was decreased at both distances as well (Fig. 13C). Altogether this data suggests that *Fmr1* KO mice avoided intensive touch since they made significantly less whisker contacts of shortened duration. Knowing human FXS phenotype with deficits in sensory processing as well as electrophysiological data showing changes in excitation/inhibition balance, we argued in the paper II that the changes observed in whisking kinematics may be a direct result of hypersensitivity in the somatosensory system. In the discussion of this paper we presented arguments against other potential explanations, especially important in the context of similar results in the task performance.



**Fig. 13 Alterations in whisking behaviour of *Fmr1* KO mice.** (A) *Fmr1* KO mice had less whisker contacts than their WT littermates at shorter gap distances (\*\*p = 0.001) but a trend towards the opposite was visible at longer gap distances. (B) Sampling duration was shorter at both gap distances in *Fmr1* KO mice (\*\*\*\*p < 0.0001; \*\*\*p = 0.0005). (C) Also the time of an individual whisker contact with a platform was decreased at both gap distances in *Fmr1* KO mice (\*\*p = 0.0051; \*\*\*\*p < 0.0001).

Differences in whisking behaviour that do not affect the overall task performance may be surprising at first sight considering the fact that mice collect haptic information necessary for task performance through deflections of their whiskers (Diamond and Arabzadeh, 2013; Diamond et al., 2008; Feldmeyer et al., 2013). It may even suggest that *Fmr1* KO mice are better learners with more effective sensory processing. However, we argued that this is not the case. Firstly, because they perform equally well – not better – than their WT littermates. Secondly, because they spend the same time around the gap deciding about the gap-crossing. Finally, based on the paper by Celikel and Sakmann (2007), we know that the gap-crossing task is rather a simple task for a mouse. Indeed, in this task it is sufficient that mice detect a platform without any additional complications, e.g. texture discrimination, so they possibly oversample during the task-performance. Thus, it seems logical that *Fmr1* KO mice simply avoided oversampling in our case, especially that they showed cognition related deficits in more complicated behavioural tasks (Kramvis et al., 2013; Padmashri et al., 2013; Santos et al., 2014; Spencer et al., 2011; van der Molen et al., 2014; Zhang et al., 2008). Another point that we explained is the fact that altered whisking is not likely to be caused by changes related to pain perception. Although FXS patients have alterations in the pain-processing pathways (Symons et al., 2010) and display self-injurious behaviours (Tranfaglia, 2011), *Fmr1* KO mice have normal acute nociceptive responses (Price et al., 2007; Zhao et al., 2005) and do not hurt themselves (Busquets-Garcia et al., 2013). We also argued against the idea that differences observed are a consequence of motor impairment or anxiety since *Fmr1* KO did not show any signs of these problems in their performance reaching the same gap distance over the same training time. Finally, an interesting observation not discussed in the paper II was the appearance of some signs of performance worsening with increasing difficulty of the task (when the gap was becoming wider). *Fmr1* KO mice made more not less whisker contacts in trials with longer gap distance (Fig. 13A). Furthermore, even though there were no statistically significant differences in the overall task performance, failed trials in *Fmr1* KO seemed to last longer than the successful ones (Fig. 14). This fact comes along with a reduced time of individual whisker touches. If a mutant mouse collects less information during a single whisker contact, it needs more touching attempts to collect the amount of information a normal mouse obtains in fewer touch attempts. As a result, a mutant may need more time to make a decision. In addition, similar behavioural experiments with the gap crossing task conducted in the McGee's lab (Arnett et al., 2014) showed that *Fmr1* KO mice displayed normal learning at shorter distances and impaired learning at longer ones.



**Fig. 14 Time spent at the gap during the successful and during the failed crossing trials.** No statistically significant effect was observed in the trial duration of the successful and failed gap-crossing trials.

To sum up, although we believe that our cortical hyperexcitation and resulting hypersensitivity in the somatosensory system may be the best explanation of the changes observed, it is not the only possible one. There are many steps between the activation of Merkel cells in the whisker follicle and the activation of the barrel cortex cells. Thus, we do not claim that physiological differences in the barrel cortex function observed in our experiments are the only factors contributing to a change in whisking behaviour in the gap-crossing task. Instead, we suggest that electrophysiological changes in the barrel cortex can be an important contribution to the changes observed in the altered whisking pattern. A remaining question is whether the somatosensory cortex is the most affected part of the somatosensory system in *Fmr1* KO mice or if there are changes in other structures along the somatosensory pathway.

### 5.3 The BC1 Project (paper III)

The main aim of the BC1 Project was extensive characterization of BC1 KO mice to determine a role of BC1 RNA in barrel cortex function. As reviewed in Iacoangeli and Tiedge (2013), BC1 forms ribonucleoprotein particles with different protein partners including FMRP. Hence we thought that elucidation of BC1 RNA function may be important, especially in the context of its relevance to neuropsychiatric phenotypes. In the initial part of the project, performed in Claudia Bagni's laboratory and collaborative laboratories, the focus was on: characterization of dendritic complexity; dendritic spine morphology; postsynaptic spine density size and makeup; and neuronal activity in BC1 KO mice. In short, it was shown that BC1 KO mice had decreased dendritic complexity but also had increased spine density in excitatory neurons of L2/3 and L4. Further experiments revealed a specific effect on the PSD – it was longer and thicker. These results aligned well with slice electrophysiology experiments that suggested higher spontaneous glutamatergic synaptic activity (sEPSCs). On the molecular side, involvement of the BC1 RNA in the activity-dependent translation was tested. Crucial role in repressing translation was confirmed in the analysis of: postsynaptic

expression of different subunits of glutamate receptors (GluR); expression and phosphorylation of the alpha-isoform of  $\alpha$ -CaMKII; relative amounts of F- and G-actin; translation of PSD-95; and global protein translation.

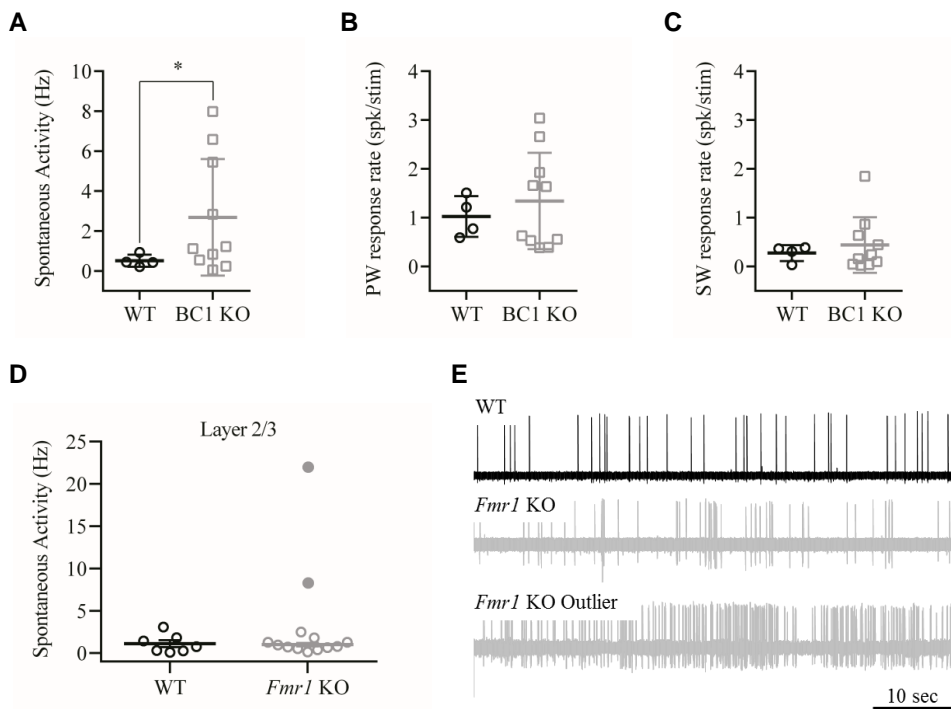
Just as in the DISC1 Project, the BC1 Project mice were tested for plasticity differences in the whisker deprivation experiments. This time, however, instead of whisker-plucking, a whisker-trimming protocol was used with 7-day-long deprivation protocol conducted on the six weeks old mice. Temporally-specific unilateral whisker-trimming induces changes in neuronal connectivity that are also reflected by concomitant changes in the spine number of corresponding neurons in the barrel cortex (Vees et al., 1998). In the WT littermate controls, opposite changes were shown in the brain hemispheres, an increase in spine density of the ipsilateral side and a decrease in the contralateral side. In contrast, increased overall spine density was observed in both hemispheres of BC1 KO mice. Spine density is a reliable measure associated with sensory deprivation used to assess structural plasticity changes that can be complemented by an analysis of synaptic levels of GluRs and PSD-95 (Butko et al., 2013). As expected, whisker trimming did not induce changes in BC1 KO mice in which the levels of these receptors were not statistically different between the whisker-deprived and whisker-undeprived hemispheres. Finally, behavioural tests revealed differences in novel object recognition, sociability, and grooming behaviour of BC1 KO mice. These results were in line with previously characterized phenotypes observed in rodent models of FXS and ASD (Santos et al., 2014).

### **5.3.1 Neuronal firing variability and spontaneous activity increase in the BC1 KO mice**

In Patrik Krieger's laboratory, we wanted to complement molecular and behavioural findings summarized above. We were looking for potential physiological changes at the level of neural circuit. We performed a set of *in vivo* experiments in anesthetized mice using a simplified version of the experimental protocol developed and optimized in the Fragile X Project. We investigated whether somatosensory maps were changed in the BC1 KO mice. We compared the PW and the SW responses but, also, the spontaneous activity of the excitatory cells. We recorded these neurons juxtacellularly focusing specifically on L2/3 of the barrel cortex. We expected that this layer may be a primary locus of changes in the neuronal receptive fields, similarly to the *Fmr1* KO mice. Contrary to our expectations, we did not find any difference in the response rate of neurons recorded in BC1 KO mice (Fig. 15B, C). However, we observed significant increases in the spontaneous firing activity (Fig.

15A). We averaged spiking rate based on 1-minute recordings collected before the beginning of the whisker stimulation train. Here again, the results collected from the BC1 KO mice were different from the ones from *Fmr1* KO mice in which the spontaneous activity was similar between genetically-modified and WT control mice (Fig. 15D, E).

Furthermore, we have also examined variability in the neuronal firing frequency by calculating coefficient of variation. Interestingly, neurons of BC1 KO mice had much higher variability in their firing frequency than WT control mice and this was true in both cases – whisker-stimulation-evoked and spontaneous activity. In the Fragile X Project we also tested variability of the spontaneous and stimulation-evoked neuronal activity. However, it did not reveal any statistically significant differences between *Fmr1* KO mice and WT control



**Fig. 15 Difference in the spontaneous activity firing of L2/3 excitatory cells recorded from BC1 KO and *Fmr1* KO mice.** (A, B) The PW and SW response rates were not statistically different in the BC1 KO mice when compared to their WT littermates. Note a high variability in the results in the BC1 KO group. (C) Spontaneous firing activity was significantly increased in BC1 KO mice (\*p < 0.05) and the neurons recorded represented much higher variability in firing rate than in the WT control group. (D) In contrast, spontaneous firing activity was neither more statistically different nor more variable in the *Fmr1* KO mice than in their WT littermates. Filled grey circles represent outliers that were removed from the statistical comparison. (E) Example recordings of the spontaneous activity recorded from the *Fmr1* KO mice and their WT littermates.



littermates. Interestingly though, in the *Fmr1* KO group we found a few outliers that were spontaneously firing at much higher rates than the rest of the neurons. They were excluded from the final analysis as “outliers” after testing with a detailed outliers test (ROUT method with  $Q = 1\%$ ). It is possible that in both animal models only a certain percentage of the excitatory cells is affected by the mutation, resulting in “outliers” identified in one case or increased variability noticed in another. However, to test this hypothesis, further experiments should be conducted to increase statistical power of the results discussed. Also, reconstruction of recorded cells followed by morphological analysis might be helpful in distinguishing whether we are seeing two different groups of cells. Finally, as mentioned in the introduction, the exact mechanisms of interactions between the FMR1 protein and BC1 RNA and their role are not clear on the molecular level. No doubt both of them play an important role in the repression of translation in similar pathways, but the question is whether their operational mechanisms are independent of one another. Thus, we should be especially careful when analysing any subtle differences in physiology between *Fmr1* KO and BC1 KO mice because studying them in detail may help us to better understand their specific roles.

### **5.3.2 Physiological differences between two FXS-related mouse models**

Because BC1 and FMRP are both translation repressors and operate along the same pathway (FMRP downstream of BC1 as reviewed by Iacoangeli and Tiedge (2013)), they may compensate for one another’s repressing function in quite an independent manner. Zhong et al. (2009) found that FMRP levels in the BC1 KO mice were similar to WT control mice, while in the next article some differences in phenotypes between single (FMRP or BC1) and double (FMRP and BC1) knock out animals were revealed (Zhong et al., 2010). Those results suggest that these two molecules might share some functional mechanisms in certain circumstances but be independent in others. We observed the differences between *Fmr1* KO and BC1 KO mice in our *in vivo* preparation. In the Fragile X Project, electrophysiological changes in the *Fmr1* KO mice were mainly related to the processing of somatosensory information in the brain after stimulation. In contrast, in the BC1 Project, the main noticeable difference concerned spontaneous activity. Moreover, in the BC1 project, we observed an increase in the firing variability, which was not the case in the Fragile X project. However, it is important to note that in *Fmr1* KO mice we observed a few outliers with higher spontaneous firing frequency that were not enough to create statistical differences between the groups (Fig. 15D, E). Thus, the proportion of cells susceptible to BC1 absence might be much higher than of those affected by the lack of FMRP.

In the Fragile X Project, apart from the regular spontaneous activity, we separately measured the inter-stimulus activity. The inter-stimulus activity is a measure reflecting the activity in the system during sensory processing. It characterizes the activity in-between stimulations during the stimulation train. The inter-stimulus activity was increased in *Fmr1* KO mice meaning that once the somatosensory system was activated, the neurons were more excitable. Zhong et al. (2010) found differences in propensity for prolonged synchronized bicuculline-induced neuronal discharges. Namely, they showed that synchronized discharges depend directly on de novo protein synthesis. Because protein synthesis depends on sensory experience, it could vary during processing of sensory information and during spontaneous activity. If this is true, the importance of FMRP/BC1 function may differ depending on more active/passive states of the brain.

### **5.3.3 Spontaneous activity and basic properties of excitatory neurons in the BC1 KO mice**

In the BC1 KO mice, although we showed an increase in the amplitude of sEPSCs, we did not observe any changes in their frequency. Neither were there differences in the basic cell membrane properties, like miniature EPSC amplitude and frequency, resting membrane potential, input resistance, and action potential threshold and halfwidth (data not shown in the manuscript). These data may be surprising considering the increased spontaneous activity observed by us *in vivo*. Nevertheless, it is in line with the data presented by Zhong et al. (2010), where, similarly to our studies, no differences were found between the passive properties of neurons recorded in slice of BC1 KO mice (only the specific change in propensity to synchronized discharges mentioned earlier). Furthermore, the lack of difference between genotypes in *in vitro* sEPSC frequency and the increase in spontaneous firing in BC1 KO mice *in vivo* need not be contradictory. Larger sEPSCs amplitude alone may lead to more spikes *in vivo* since in this setting neurons may achieve a spiking threshold more easily. In addition, potentially expected higher frequency of sEPSCs in BC1 KO mice need not necessarily translate directly into higher rate of action potentials *in vivo*. Even with the higher frequency of sEPSCs, the timing of these events may be the most important variable. Therefore, synchronization of oscillations or UP/DOWN states may be a crucial factor influencing the frequency of sEPSCs.

Zhong et al. (2010) showed *in vivo* several changes in oscillatory properties in BC1 KO mice, e.g. higher dependence of theta and beta oscillations on mGluR activity and excessive gamma frequency oscillations. Gamma-oscillations are generated by interneurons and entrain

principal neurons (Bartos et al., 2007). Thus, their synchronization may be crucial for principal neuron activity. On the other hand, UP and DOWN state dynamics are not clearly translatable into cell activity. For instance, the duration and the frequency of firing during the active UP states change independently. We discussed this concern using examples from the experiments conducted on *Fmr1* KO mice in the Fragile X Project paper.

Finally, the *in vivo* spontaneous spiking we recorded from L2/3 pyramidal neurons reflects a combination of excitatory inputs from both cortical and thalamic neurons. In the slice preparation, the active source of sensory excitatory drive from the thalamus is missing, which may account for the observed differences. While we did not measure thalamic activity *in vitro* or *in vivo*, we did observe comparable increases in spine density in BC1 KO neurons from L2/3 and L4 suggesting enhanced excitability throughout the thalamo-cortical input pathway. The aforementioned increase in the cortical spine density in BC1 KO mice may be also expected to result in higher frequency of sEPSCs that was not observed. This discrepancy may be attributed to a different fraction of silent/active synapses between the two genotypes. BC1 KO mice may have more spines with silent synapses but similar numbers of active synapses (and hence similar sEPSC frequency) in comparison to WT mice. Interestingly, Harlow et al. (2010) observed an increased proportion of silent synapses in the barrel cortex of the developing *Fmr1* KO mice that normally should be eliminated during postnatal development. As pointed out in this paper, the proportion of AMPA to NMDA receptors and the number of NMDA-only silent synapses contribute to the EPSC and may influence its amplitude.



## 6 CONCLUDING REMARKS

In the DISC1 Project we found several characteristics of DISC1 protein function in the context of whisker-deprivation-induced cortical plasticity. We revealed that DISC1 is involved in experience-dependent plasticity mechanisms and its role depends on time, playing a major part in the early critical period. We showed that this critical period for DISC1 function in plasticity occurs in the early postnatal days around P7-P13, which overlaps with the time of maximum synaptogenesis. Furthermore, we showed that the spine formation process was affected, shifting spine morphology towards more thin-headed, immature spines. This effect persisted into adulthood and had its physiological consequences. Further *in vitro* electrophysiological recordings revealed that the changes evoked by short-term activation of mutated DISC1-cc protein resulted in reduced AMPA receptor proportion that appeared around adolescence and remained throughout adulthood. This finding was in line with changes in spine morphology since spine head size varies with AMPA receptor content. Moreover, we showed that DISC1-cc mice presented a total absence of LTP expression and altered dynamics of LTD mechanisms, namely a slower time course of LTD expression and a lower probability of LTD induction. We also checked the time course for LTD expression throughout life and our data suggested that the LTD mode of plasticity is available until P50 and disappears before P100. To sum up, in the DISC1 Project, we observed time course of changes in plasticity mechanisms suggests that plasticity dynamics correspond directly to the progressive development of SZ pathophysiology. Furthermore, a recent study from the Fox's laboratory (Hardingham and Fox, 2016) related to the plasticity mechanisms in the prefrontal cortex of DISC1-cc mice discovered plasticity changes similar to the ones observed in the somatosensory cortex. Therefore, future in-depth understanding of changes in the somatosensory barrel cortex might shed some new light on the mechanisms of plasticity changes in SZ pathophysiology also in other cortical areas.

In the FXS-related projects we focused on the somatosensory processing mechanisms in the barrel cortex. First, in the *Fmr1* KO mice, we showed that whisker-stimulation-evoked neural responses were increased specifically in the barrel columns surrounding the PW column. Furthermore, the latency to the SW response was shortened, suggesting potential problems with the encoding of tactile information. Hence in the second part of our studies, we analysed responses to stimulation at various frequencies to look closer at changes in the somatosensory encoding mechanisms. We tested stimulation frequencies between 1 and 10 Hz. Differences in the response rate between the low and high stimulation frequencies were present in both genotypes, *Fmr1* KO mice and their WT littermates. Although the *Fmr1* KO mice exhibited

alterations in the fine-tuning in the response at lower frequencies, the response rate did not differ between 1 and 4 Hz, in contrast to WT mice. Further analysis revealed that the increase in the SW response rate in *Fmr1* KO mice was noticeable also at various stimulus frequencies as well as in the shortened response latency. Both effects were independent of the stimulation frequency. Altogether, all these changes indicate problems with whisker selectivity and alterations in the tactile processing mechanisms in *Fmr1* KO mice. Next we checked whether the impairments observed in our electrophysiological experiments resulted in behavioural changes. We chose a gap-crossing task, a whisker-dependent decision-making paradigm used in testing of sensory-motor learning capabilities. *Fmr1* KO mice did not display any problems with performing the gap-crossing task. However, we decided to look closer at the whisking behaviour of those mice and this original idea led us to very interesting observations. It seemed that *Fmr1* KO mice not only touched with their whiskers for a shorter time than their WT littermates but also tended to avoid whisker-touching. This behavioural change corresponded directly with the known human FXS phenotype, a hypersensitivity to sensory stimuli. Because hypersensitivity problems manifesting as “tactile defensiveness” or “tactile sensitivity” are common symptoms in FXS, ASD and related diseases, we believe that the understanding of the basic physiological mechanisms underlying these problems in *Fmr1* KO mice may be applicable to other diseases involving sensory processing deficits. Furthermore, because hypersensitivity was observed not only in the somatosensory system but also in other sensory modalities, mechanisms of the observed disruptions may be of a more universal nature.

Finally, in the second FXS-related project, we performed electrophysiological experiments on the somatosensory whisker system of BC1 KO mice using a stimulation protocol similar to the one used in the *Fmr1* KO mice. We did not find any differences in the response rate or the response latency in BC1 KO mice. Nevertheless, we revealed that response variability was significantly higher in these mice. Moreover, BC1 KO mice had increased spontaneous activity, which is a measure of activity when the system is not activated. This contrasted with the findings in *Fmr1* KO mice that presented changes in the somatosensory system during sensory processing. Because BC1 RNA has been found to repress translation at the initial stage of this process, while the FMR1 protein is thought to participate in this process at the later stages, there are many questions about the specific role and type of interactions between these two molecules. So far, there is no consensus on their interactions. While some groups claim direct interaction between the FMR1 protein and BC1 RNA, others suggest separate mechanisms of actions. Therefore, the differences found between *Fmr1* KO and BC1 KO

mice may help to address the questions which are difficult to answer on the molecular level only.

The work presented in this thesis led us to reinterpret experiments performed on the somatosensory whisker system in the past in the context of clinically relevant questions. Experimental protocols that we optimized in our projects can be applied to further studies in various mouse models of disease. They can be useful not only in the models corresponding directly to the somatosensory impairments but also in the ones revealing cortical plasticity deficits. Moreover, when analysing our data from SZ and FXS mouse models jointly in a wider context of the literature available, we identified intriguing similarity between those pathologies. In both the case of SZ and FXS, cognitive impairments appear to be a problem central to the disease, but what makes them different is the onset time of their symptoms. What underlies this difference? While in SZ the onset is usually delayed until late adolescence, in FXS the progression of symptoms begins already in early postnatal life. Because both pathologies are thought to be neurodevelopmental and reveal changes in plasticity mechanisms, we could gain a new insight into their dynamics by analysing those plasticity changes in the context of similarities and differences. May the critical period for DISC1 protein function in plasticity be proved to be a more universal period that applies to other molecules involved in plasticity processes including the FMR1 protein? Can the cognitive problems potentially related to sensory deficits be stopped by medical treatment at the early developmental stages? Should the altered cortical activity be challenged with some additional external stimulations to improve the formation of connections in the early sensory circuits? All these questions remain unresolved as of today. However, I believe that we can improve our efficiency in searching for effective treatments by focusing on the basic mechanisms of those disorders rather than their symptomatology.





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could result in a higher cell-specific energy demand in the deeper layers at Site CO020 and may explain why microbial abundance was only a small fraction of the size predicted by the global regression line (Fig. 1A and figs. S7 and S14).

Our findings provide a comprehensive view of the deep seafloor biosphere associated with coal beds. Despite energetic challenges, this environment appears to have maintained some of the taxonomic groups that populated the original shallow depositional setting and have contributed to carbon cycling ever since.

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## SUPPLEMENTARY MATERIALS

[www.sciencemag.org/content/349/6246/420/suppl/DC1](http://www.sciencemag.org/content/349/6246/420/suppl/DC1)  
Materials and Methods  
Figs. S1 to S14  
Tables S1 to S7  
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## NEURODEVELOPMENT

# Adult cortical plasticity depends on an early postnatal critical period

Stuart D. Greenhill,<sup>1,\*</sup> Konrad Juczewski,<sup>2,\*</sup> Annelies M. de Haan,<sup>1</sup> Gillian Seaton,<sup>1</sup> Kevin Fox,<sup>1</sup> Neil R. Hardingham<sup>1,†</sup>

Development of the cerebral cortex is influenced by sensory experience during distinct phases of postnatal development known as critical periods. Disruption of experience during a critical period produces neurons that lack specificity for particular stimulus features, such as location in the somatosensory system. Synaptic plasticity is the agent by which sensory experience affects cortical development. Here, we describe, in mice, a developmental critical period that affects plasticity itself. Transient neonatal disruption of signaling via the C-terminal domain of “disrupted in schizophrenia 1” (DISC1)—a molecule implicated in psychiatric disorders—resulted in a lack of long-term potentiation (LTP) (persistent strengthening of synapses) and experience-dependent potentiation in adulthood. Long-term depression (LTD) (selective weakening of specific sets of synapses) and reversal of LTD were present, although impaired, in adolescence and absent in adulthood. These changes may form the basis for the cognitive deficits associated with mutations in DISC1 and the delayed onset of a range of psychiatric symptoms in late adolescence.

**D**isrupted in schizophrenia 1 (DISC1) is a protein that, when mutated, predisposes the human carrier for a number of mental disorders including schizophrenia, bipolar disorder, recurrent major depression, and autism (1, 2). DISC1 interacts with a surprisingly large number of signaling molecules, including phosphodiesterase 4, glycogen synthase kinase 3, kalirin-7, fasciculation and elongation protein  $\zeta$  1, kendirin, lissencephaly 1 (Lis1), and nudE neurodevelopment protein 1-like 1 (Nudel) (3–8). DISC1 affects diverse aspects of neuronal development, such as proliferation, migration, and neurite extension. In addition, DISC1 is known to be expressed in cortical neurons during both development and adulthood (9) and to reside at the postsynaptic density (6, 10–12), although very little is understood of the role it plays there. In this study, working with mice, we asked whether DISC1 protein-protein interactions early in development are critical for synaptic

plasticity in adulthood. We disrupted transiently DISC1's interaction with Lis1 and Nudel during early development, at a time after cortical neurogenesis and cell migration [which are complete by about postnatal day 7 (P7) in the mouse] but before synaptogenesis and dendrite formation dominate.

We studied adult plasticity in the mouse barrel cortex, a primary sensory cortical area that receives tactile information from a normal array of 40 large whiskers. We removed all but one whisker on one side of the face of adult mice (13) to invoke cortical plasticity. The single-whisker experience normally leads to expansion of the cortical territory responding to the spared whisker (Fig. 1A). To manipulate DISC1 interactions with Lis1 and Nudel, we used a conditional transgenic mouse expressing the DISC1 C-terminal domain (DISC1<sub>CT</sub>; residues 671 to 852), which interacts with Lis1 and Nudel (14–16) in a tamoxifen-sensitive construct. Within this system, a single tamoxifen injection affects DISC1 signaling for 6 to 48 hours (P7 to P9) (15). Spatial expression of DISC1<sub>CT</sub> is restricted to excitatory neurons in the forebrain by the calcium/calmodulin-dependent protein kinase II subunit  $\alpha$  (CaMKII) promoter, and its activity is controlled by tamoxifen. We studied

<sup>1</sup>School of Biosciences, Cardiff University, Cardiff, CF23 3AX, UK. <sup>2</sup>National Institute on Alcohol Abuse and Alcoholism, NIH, Rockville, MD 20852, USA.

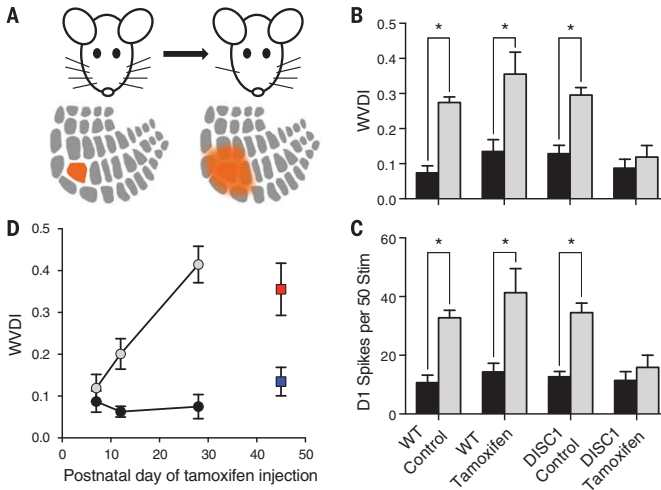
\*These authors contributed equally to this work. †Corresponding author. E-mail: [sbmrh@cardiff.ac.uk](mailto:sbmrh@cardiff.ac.uk)

the effect of a single subcutaneous injection of tamoxifen at P7 on single-whisker plasticity in adulthood (age range P70 to P130).

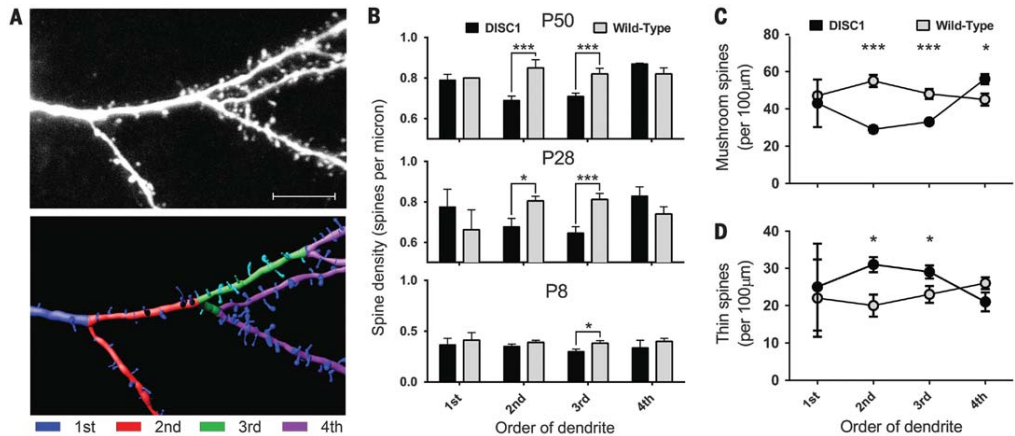
We found that adult DISC1cc mice injected with tamoxifen at P7 and with whiskers intact

developed a normal barrel pattern, as well as normal cortical layers, cell density, and receptive fields (fig. S1). However, experience-dependent potentiation invoked by whisker loss was absent in DISC1cc mice injected with tamoxifen [ $F_{7,51} =$

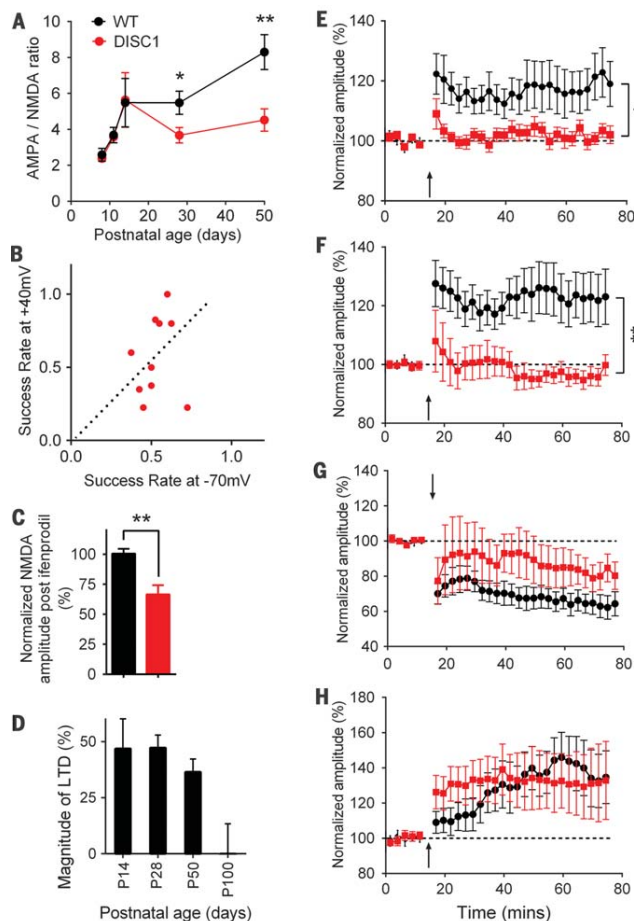
6.9,  $P < 0.001$ , three-way analysis of variance (ANOVA)] (see Fig. 1). Plasticity in cortical layers 2 and 3 (L2/3) was normal in wild-type mice receiving tamoxifen, which indicated that tamoxifen only acted in conjunction with the mutant



**Fig. 1. Plasticity is impaired in adults by transient impairment of DISC1 C-terminal interactions at P7.** (A) Whisker deprivation and expansion of spared whisker domain (orange area) (13). (B) Weighted vibrissae dominance index (WVDI) for spared versus principal whiskers across experimental groups (total  $n = 52$  mice, 496 cells; naïve mice, black bars; deprived mice, gray bars). WVDI increases with deprivation except for in DISC1cc mice injected with tamoxifen at P7 [ $F_{7,51} = 10.6$ ,  $P < 0.001$ , three-way analysis of variance (ANOVA)]. Tamoxifen only affected plasticity in DISC1cc mice and not wild types [interaction between genotype and tamoxifen ( $P < 0.0005$ )]. (C) Spared (D1) whisker response increased with deprivation (gray bars), directly correlated with WVDI [correlation coefficient ( $R$ ) = 0.93;  $P < 0.0001$ ]. (D) The WVDI increases in DISC1cc mice injected with tamoxifen on P11 to P13 [ $t_{12} = 4.97$ ,  $P < 0.05$ ; black, naïve; gray, deprived] but only attains levels seen in wild types (red square) when injected at P28; WT mice injected on P7 were not different from DISC1cc mice injected on P28 ( $t_{12} = 0.61$ ,  $P = 0.45$ ) (interaction between age and deprivation  $F_{2,2} = 10.46$ ,  $P < 0.0003$ , ANOVA). The WT control data are plotted at P45 for clarity (red, deprived; blue, naïve), but all mice were injected with tamoxifen on P7. All plasticity values were measured in adulthood.



**Fig. 2. Enduring effects of transient impairment of DISC1 C-terminal interactions at P7 on dendritic spines.** (A) Example of L2/3 dendrites showing spines and dendritic order. Scale bar, 10  $\mu$ m. (B) DISC1cc mice had lower spine density on second- and third-order dendrites at P28 and P50. Spine density was lower at P8 on third-order dendrites in DISC1cc mice. (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .) (C and D) DISC1cc mice had a lower density of mushroom spines and a higher density of thin spines on second- and third-order dendrites. (ANOVA: interaction between dendrite order and genotype for mushroom spines:  $F_{1,124} = 58.64$ ,  $P < 0.0001$  and for thin spines  $F_{1,124} = 7.40$ ,  $P < 0.01$ ).



**Fig. 3. Persistent functional consequences of transient impairment of DISC1 C-terminal interactions at P7.** (A) The AMPA/NMDA ratio of WT and DISC1cc mice diverge after P14, and this ratio in DISC1cc mice remains at a low level into adulthood (P50 WT ratio is  $8.29 \pm 0.97$ ; DISC1 ratio is  $4.52 \pm 0.61$ ; and  $t_{15} = 3.29$ ,  $P < 0.01$ ). (B) Of P50 DISC1cc recordings, 50% had minimal-stimulus excitatory postsynaptic current success rates at +40mV higher than rates at -70mV, indicative of the presence of silent synapses. (C) NMDA currents in adult DISC1cc mice (red bar) showed enhanced sensitivity to ifenprodil application when compared with WT mice (black bar; DISC1cc  $0.67 \pm 0.08$ , WT  $1.01 \pm 0.04$ ,  $t_{10} = 3.9$ ,  $P < 0.005$ ). (D) LTD in WT mice was consistent at P14, P28, and P50 but was absent at P100 [average depression of  $47 \pm 13\%$  at P14 ( $P < 0.01$ ),  $47 \pm 6\%$  at P28 ( $P < 0.001$ ),  $36 \pm 6\%$  at P50 ( $P < 0.001$ ), and  $-1 \pm 14\%$  at P100 ( $P > 0.05$ )]. (E) Normalized peak excitatory postsynaptic potential amplitude is plotted versus time. Transient expression of mutant DISC1 at P7 abolishes the capability for intercolumnar LTP in L2/3 at P28 and (F) at P50 (effect of genotype  $F_{1,74} = 14.27$ ,  $P < 0.0003$  and not age  $F_{1,74} = 0.13$ ,  $P < 0.71$ , ANOVA). The percentage of cells showing statistically significant LTP drops from 33% in WT mice to 5% in DISC1cc mice (P28) and 43% in WT mice to 9% in DISC1cc mice at P50. (G) Average LTD values are similar in WT and DISC1cc mice ( $F_{1,18} = 3.44$ ,  $P < 0.08$ , ANOVA), although the percentage of cells showing LTD drops from 90% in wild types to 40% in DISC1cc mice. (H) Complete whisker deprivation unmasks the reversal of LTD that depends on adenosine 3',5'-monophosphate-dependent protein kinase (26), and this is unaffected in the adult mouse by P7 DISC1cc activation ( $F_{1,19} = 0.16$ ,  $P < 0.87$ , ANOVA).

protein and not by perturbing estrogen signaling ( $t_{11} = 2.9$ ,  $P < 0.02$ ). Plasticity was also normal in DISC1cc mice given just vehicle at P7, which indicated that background levels of DISC1cc availability are effectively zero (Fig. 1) ( $t_{11} = 2.4$ ,  $P < 0.05$ ). (Note that the mutated ligand-binding domain fused to DISC1cc does not bind natural estrogen, only tamoxifen.) The weighted vibrissae dominance index was unchanged in whisker-deprived DISC1cc animals receiving tamoxifen, because the spared whisker responses did not potentiate (Fig. 1 and fig. S2), and consequently, the spared whisker domain did not expand into the deprived barrels surrounding the D1 barrel (fig. S3). The lack of plasticity in the DISC1cc mice was robust across two background strains (Fig. 1 and fig. S4). These results show that normal DISC1 interaction with Lis1 and Nudel is vital during a brief period in neonatal development for the adult animal to exhibit experience-dependent plasticity.

Transient disruption of DISC1/Lis1/Nudel interactions later in development had a smaller effect on L2/3 plasticity. Disrupting DISC1 C-terminal interactions at P11 to P13 reduced plasticity less than it did at P7 and had no effect at P28 (Fig. 1D). This indicates that a critical period exists in early development with long-lasting consequences for plasticity expressed much later in adulthood.

We studied the early development of the DISC1cc mice to see where the defect originated. We found that disrupting DISC1 C-terminal signaling at P7 retarded dendritic elongation and elaboration of dendritic branching (figs. S5 and S6), but both had recovered by P21. The paired-pulse ratio, which is a measure of presynaptic maturation in the L4 to 2/3 pathway (17) was also delayed (fig. S7). Retardation of neuronal development demonstrates the immediate effect of disrupting DISC1 C-terminal interactions at P7 but does not explain the long-lasting loss of adult plasticity.

The long-lasting effects of transient disruption of DISC1/Lis1/Nudel interactions were to be found at the level of the spines rather than the dendrites. At the start of the critical period for adult plasticity, the neurons highest-order basal dendrites are mainly second- and third-order branches and are destined to become 50% of adult basal dendrites (Fig. 2 and figs. S5 and S6). We found lower spine density on second- and third-order dendritic spines in DISC1cc mice at P28 ( $t_{31} = 2.36$ ,  $P < 0.03$ ; and  $t_{41} = 3.82$ ,  $P < 0.0005$ , respectively) and P50 ( $t_{30} = 4.78$ ,  $P < 0.0001$ ; and  $t_{43} = 4.66$ ,  $P < 0.0001$ , respectively). The fourth- and higher-order dendrites, which mainly develop after the period during which we impaired DISC1 C-terminal interactions, showed normal spine density at P28 ( $t_{20} = 0.96$ ,  $P = 0.35$ ; interaction between dendrite order and genotype  $F_{4,104} = 4.48$ ,  $P < 0.005$ ) and at P50 ( $t_{30} = -1.318$ ,  $P = 0.20$ ; interaction between dendrite order and genotype  $F_{4,117} = 7.29$ ,  $P < 0.0001$ ). The spine density deficit was only found on basal dendrites, not on apical dendrites ( $F_{4,131} = 0.86$ ,  $P = 0.49$ ).

The period when DISC1 C-terminal signaling is critical for adult plasticity (P7 to P13)

corresponds to a period of rapid synaptogenesis across the brain, as well as in L2/3 of barrel cortex (18), when experience is necessary for AMPA insertion within synapses (19). Altered neonatal experience during this period leads to defocused receptive fields in adulthood (20). As the size of spine heads are correlated with their AMPA receptor content (21, 22), we investigated spine head size and classification. At P50, there were fewer mushroom spines (both as a percentage of the whole and in absolute terms) on the second- and third-order dendrites of DISC1cc mice than on their first-, fourth-, and fifth-order dendrites ( $t_{35.8} = 8.76$ ,  $P < 0.0001$ ), and fewer than on the second- and third-order dendrites of wild-type mice ( $t_{40.9} = 8.72$ ,  $P < 0.0001$ ). Furthermore, there were more thin spines on the second- and third-order dendrites in the DISC1cc mice ( $t_{75} = 3.07$ ,  $P < 0.005$  compared with wild types, and  $t_{68} = 4.10$ ,  $P < 0.0005$  compared with other dendrite orders within the DISC1cc mice). Finally, the spine heads were smaller on the thin spines in the DISC1cc mouse second- and third-order dendrites than in the wild types ( $t_{76} = 3.31$ ,  $P < 0.01$ ) (Fig. 2). These findings imply a lower level of AMPA receptor insertion in DISC1 mutants.

We investigated synaptic function further in DISC1 mutants and found that, whereas the AMPA/N-methyl-D-aspartate (NMDA) ratio followed a normal developmental trajectory up to P14, it diverged at P28 ( $t_{18} = 2.33$ ,  $P < 0.05$ ) and did not recover by P50 ( $t_{18} = 3.29$ ,  $P < 0.01$ ) (Fig. 3A). Consistent with this finding, silent synapses were present in DISC1 L2/3 cells at P50 (Fig. 3B), whereas in wild types they had converted to functional synapses by this age (23). The NMDA component of the synaptic response was also immature and contained a higher proportion of GluN2B versus GluN2A subunits than normal ( $t_{10} = 3.9$ ,  $P < 0.005$ ) (Fig. 3C) (24, 25). In contrast, inhibition appeared to be unaffected in DISC1cc mice (fig. S8). Low levels of GluN2A and AMPA receptors are consistent with the spine size defects, which implies that glutamate receptor insertion is affected by transient disruption of DISC1 C-terminal interactions in early development. These factors predict that synaptic plasticity should also be deficient in DISC1cc mice (21, 22, 25).

On investigation, we found that long-term potentiation (LTP) was absent in the P7 tamoxifen-treated DISC1cc mice at P28 and P50 (Fig. 3, E and F), which indicated that development of LTP was abolished rather than delayed. Long-term depression (LTD) was affected although not abolished: The time-course of LTD was slower and the probability of LTD induction was lower in DISC1cc mice (fig. S9), although it was possible to induce LTD in the mutants as in the wild types (Fig. 3G). This suggested that it might be possible to reverse LTD in these synapses despite their lack of LTP. Previous studies had shown that 7 days of complete bilateral whisker deprivation can occlude LTD in the barrel cortex and reset the synapses to a state that favors LTD reversal (26). We found that it was possible to reverse LTD in the completely whisker-deprived

DISC1cc mouse (Fig. 3H). These results show that developmental disruption of DISC1 signaling blocks or impairs selective aspects of synaptic plasticity.

Adult plasticity is different from many forms of developmental plasticity (27, 28). In the somatosensory and visual cortex, adult plasticity is dependent on CaMKII and closely related to LTP (29, 30). Developmental forms of synaptic plasticity, such as tumor necrosis factor- $\alpha$ -dependent synaptic scaling, experience-dependent depression, and LTD are gradually reduced with age (30, 31). We found that the normal period of LTD expression in the barrel cortex ends between P50 and P100 (Fig. 3D), after which LTD and reversal of LTD are not available modes of plasticity. Therefore, the loss of adult LTP only becomes critical at an age when developmental forms of plasticity have decreased to low levels. The latent effect of ablating prospective adult plasticity during an early critical period may therefore help explain the late onset of some schizophrenia symptoms.

How might a loss of LTP affect psychiatric conditions? Working memory is defective in schizophrenia and relies on persistent modes of network firing (32). Persistent neuronal activity requires formation of attractor states in neuronal networks, as has recently been shown in monkey prefrontal cortex during context-dependent integration of visual information (33). Therefore, a loss of plasticity such as we describe here is likely to disrupt working memory function by preventing formation of stable attractor states.

The C-terminal domain of DISC1 expressed in the DISC1cc mouse is known to reduce wild-type DISC1-Nudel and DISC1-Lis interactions (15). DISC1-Nudel interactions are thought to depend on the C-terminal domain's ability to form dimeric and tetrameric states (16). DISC1 and Nudel interact strongly at P7, less so by P16, and negligibly by 6 months (14). The DISC1-Nudel complex is therefore available to be disrupted only when spines form rapidly on cortical neurons during the critical period we describe here for adult plasticity. Nudel and DISC1 also both bind to Lis1 (14), and Lis1 haploinsufficiency has been shown to decrease spine density, specifically on second- and third-order dendrites (34), in striking similarity to the present results. Human induced pluripotent stem cells from schizophrenia and depression sufferers carrying a DISC1 C-terminal mutation also exhibit deficits in synapse formation (35). By restricting DISC1 C-terminal dysfunction to a short period of development, we have been able to show that adult plasticity (i) depends on synapse formation during this early critical period, (ii) cannot be recovered despite continued expression of normal DISC1, and (iii) is independent of DISC1-Nudel interactions in adulthood.

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## SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/349/6246/424/suppl/DC1  
Materials and Methods  
Figs. S1 to S9  
References (36–46)

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## Adult cortical plasticity depends on an early postnatal critical period

Stuart D. Greenhill, Konrad Juczewski, Annelies M. de Haan, Gillian Seaton, Kevin Fox and Neil R. Hardingham (July 23, 2015)  
*Science* **349** (6246), 424-427. [doi: 10.1126/science.aaa8481]

### Editor's Summary

#### Keeping synaptic plasticity plastic

Neuronal synapses in the brain adjust according to shifting demands as we experience the world. This synaptic plasticity forms the basis for critical periods in the visual and somatosensory systems. Greenhill *et al.* have now found, in mice, a critical period for the development of plasticity itself. At the core is a protein that in its mutant form is associated with schizophrenia. Disrupting this protein's function temporarily during early development caused a failure in brain plasticity in adult mice.

*Science*, this issue p. 424

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## Supplementary Materials for

### **Adult cortical plasticity depends on an early postnatal critical period**

Stuart D. Greenhill, Konrad Juczewski, Annelies M. de Haan, Gillian Seaton, Kevin Fox,  
Neil R. Hardingham\*

\*Corresponding author. E-mail: [sbinrh@cardiff.ac.uk](mailto:sbinrh@cardiff.ac.uk)

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#### **This PDF file includes**

Materials and Methods  
Figs. S1 to S9  
References

**Correction (24 August 2015):** The originally posted file was in error, the current version includes Fig. S9, and the volume number is 349. The originally posted version can be seen [here](#).

## **Materials and Methods**

### **Subjects**

Tamoxifen-inducible DISC1cc mice (15) were obtained from Alcino Silva at UCLA and maintained by inbreeding or crossbreeding with C57Bl/6J mice (Harlan Labs, UK) or C57Bl/6N mice (Taconic, Ry, Denmark). Animals were social-group housed with *ad libitum* food and water in a 12:12 hour normal light/dark cycle. Both male and female mice were used throughout the study. All animal care and use was performed in compliance with the UK Animals (Scientific Procedures) Act 1986.

### **Transgene induction**

Mice received a single subcutaneous (s.c.) injection of tamoxifen (20 mg/kg, Sigma, UK) in corn oil at postnatal day 7, or 28 or for the P11-13 group, one injection on P11 and one on P13. Control animals received s.c. injections of corn oil on the same postnatal days. Both transgenic animals and their wild-type littermates were used to control for any possible effects of tamoxifen on plasticity and neurophysiology.

### **Whisker Deprivation**

Mice (aged P50-75 at start of deprivation) were briefly anaesthetised with isoflurane for 1-2 minutes. The vibrissal pad was visualised under a dissecting microscope and the whiskers removed by a gentle pulling motion to leave the follicle intact. This process does not damage the follicle innervation and allows the whisker to regrow(36). The process was repeated every 2-3 days as necessary to remove any regrown whiskers. For *in vivo* plasticity studies the whiskers on the right-hand side were deprived with only the D1 whisker spared to provide a period of single whisker experience for 18 days (with 5-7 days regrowth after). For *in vitro* studies all whiskers were removed bilaterally for 7 days.

### ***In vivo* electrophysiology**

*Subjects:* Anaesthesia was induced with isoflurane and maintained by urethane (1.5g/kg body weight i.p. with a trace amount of acepromazine) in adult mice (age range P50-113, n=89). Hindlimb and corneal reflexes, breathing rate and cortical activity were used to monitor anaesthesia levels and maintain animals in a state similar to stage 3-4 sleep(37). Supplemental doses of urethane (10% initial dose)

were administered as required. Topical analgesic (lidocaine) was applied to the ears and scalp.

*Surgery:* Anaesthetised mice were placed in a stereotaxic frame (Narashige, Japan) and body temperature maintained at 37°C throughout surgery and recording by a thermostatically controlled heating blanket (Harvard Apparatus, Kent, UK). A 2x2mm section of the left parietal cranium was thinned with an electric dental drill over the barrel cortex (0-2mm caudal from bregma, 2-4mm lateral from midline). A small fleck of thinned skull was removed from the area with a 30G hypodermic needle to create a hole just large enough to allow the carbon fibre electrode access for each penetration. The dura was left intact as the electrode was strong enough to break through it without resection.

*Recordings:* Carbon-fibre electrodes(38) were used to make recordings from barrels corresponding to those whiskers immediately surrounding the spared whisker. Recordings were made at even intervals of 50µm from the surface to the bottom of layer 4. Action potentials were amplified with a Neurolog system isolated using a window discriminator to provide single-unit recordings (Digitimer, Welwyn Garden City, UK) and digitised with a CED 1401 and Spike2 software (CED, Cambridge, UK) running on a Windows desktop PC. Whiskers were stimulated one at a time using a glass rod attached to a piezo wafer driven by a Digitimer DS-2 isolated stimulator. Stimuli were applied as single 10ms 200µm upward deflections at 1Hz, repeated 50 times.

*Histology:* The locations of the extracellular recording penetrations were confirmed by micro-lesions made at the end of each recording penetration. Small electrical lesions (1 µA DC, tip negative, 10 seconds) were made at an estimated depth of 350µm. At the end of the recording session the mouse was deeply anaesthetised and transcardially perfused with 0.1M phosphate-buffered saline, followed by 4% formaldehyde in PBS. The brain was removed after fixation and the recorded hemisphere's cortex dissected and flattened between two glass slides as previously described (39). The flattened cortex was then postfixed for 24h in 4% formaldehyde/20% sucrose in PBS and then transferred to 20% sucrose PBS until sectioning. Tangential sections (35µm) were cut on a freezing microtome and stained for cytochrome oxidase activity by reaction with diaminobenzidine and cytochrome C

(40). The lesions from recording were then correlated with the histology to confirm in which barrel each cell was recorded.

### ***In vitro* methods**

A total of 287 mice aged between 8 and 70 days old were used (WT and DISC1cc mice both injected with tamoxifen at P7). Recordings were analysed from 960 cells. For experiments carried out following whisker deprivation, we used a deprivation period of 7 days as this has been shown to have the greatest effect on layer 2/3 cortical plasticity (41) (see above for deprivation methods).

### ***In vitro* recording conditions and stimulation protocols**

Coronal slices (400µm thick) containing barrel cortex were taken from mice using a Micron MM650V microtome (Thermo-Scientific, UK). Slices were maintained in a submersion chamber continually perfused (2-3ml/min) with artificial cerebro-spinal fluid (ACSF) containing (in mM): 119NaCl, 3.5KCl, 1NaH<sub>2</sub>PO<sub>4</sub>, 2CaCl<sub>2</sub>, 1MgSO<sub>4</sub>, 26NaHCO<sub>3</sub>, 10 glucose and 10µM picrotoxin to block IPSPs. The solution was bubbled with 5% CO<sub>2</sub>-95% O<sub>2</sub> and slices were kept at room temperature (21-24°C). Intracellular electrodes contained (in mM): 110 K-gluconate, 10KCl, 2MgCl<sub>2</sub>, 0.3 Na<sub>2</sub>ATP and 0.03 NaGTP. Biocytin (Sigma, UK) was routinely added to the electrode filling solution at a concentration of 5mg / ml. Electrode resistance was 10-15MΩ.

Slices were placed in the recording chamber under an upright microscope (BX 50 WI, Olympus, UK). Pyramidal neurons were chosen in layer 2/3 of the somatosensory cortex directly above the layer IV barrels under visual guidance, using a 40x water immersion objective, differential interference contrast (DIC) optics and infrared illumination. Whole-cell recordings were made from pyramidal cells in the current clamp configuration. Signals were amplified using an Axoclamp 2B amplifier (Axon Instruments, USA), low pass filtered at below 1-3kHz (Digitimer, UK) and sampled at 5kHz for analysis off-line on a PC computer (RM, UK).

After recordings were obtained, the neurons were electrophysiologically characterised. Firstly, the input resistance of the neuron was recorded by injection of long pulses of current. I/V relationships of cells were obtained by injecting varying negative and positive sub-threshold currents into the neurons. Postsynaptic action potentials were measured in response to long pulses of depolarising current injection.

Responses to short hyperpolarising pulses of current were also measured in order to calculate the final time constant of the membrane. The series resistance was continually monitored during the recording and recordings were rejected if it changed by over 20% during the experiment.

The identity of neurons as pyramidal was subsequently confirmed by histological processing. After neurons had been electrophysiologically identified as pyramidal, monosynaptic EPSPs were evoked via a monopolar stimulating electrode placed accurately in layer 4 of either the home barrel or the adjacent barrel column. The stimulus intensity was adjusted to produce an EPSP amplitude of roughly 5mV in the postsynaptic neuron. Monosynaptic components of EPSPs were recorded and had reversal potentials close to 0 mV ( $-2 \pm 3$  mV). Trains of EPSPs were evoked in responses to 10 stimuli at 10Hz or 20 stimuli at 20Hz and amplitudes measured post-hoc in order to quantify short-term plasticity.

### **Plasticity Experiments**

For LTP experiments, after a control period of recording (stimulating at a frequency of 0.1Hz), the post-synaptic neuron was subjected to a paired pre- and post-synaptic spiking protocol, where the presynaptic stimulus was timed to occur 10ms before a postsynaptic action potential evoked by somatic current injection. This pre-post interval has been shown to be effective in inducing LTP in layer 2/3 barrel cortex of a similar age to the current study (41). Trains of paired activity consisted of 50 paired action potentials at a frequency of 2Hz. The paired pulse ratio was defined as being the peak amplitude of the second EPSP divided by the peak amplitude of the first EPSP. The LTP protocol consisted of four sets of trains of paired activity with a minute between the trains. After the pairing protocol, the evoked EPSP was again recorded for a further hour. For LTD experiments, the post-synaptic action potential was timed to occur 50ms before the presynaptic stimulation. As LTD is difficult to induce in mature brain slices, the protocol consisted of 800 reverse-paired stimulations at 2Hz. When investigating the age dependence of LTD, the same LTD experiment was performed on P14, P28, P50 and P100 cortex.

### **EPSP Measurement**

EPSPs were measured using an automated routine that compared a window in the baseline membrane potential shortly before the EPSP with the peak EPSP amplitude. Amplitudes were binned into 30 second epochs for data analysis.

### **AMPA to NMDA ratios**

AMPA to NMDA ratios of evoked EPSPs were obtained pharmacologically (42). At resting membrane potentials, EPSPs were mediated by AMPA currents and entirely blocked by addition of CNQX to the ACSF. Stable periods of AMPA mediated potentials were recorded under control conditions and then AMPA mediated EPSPs were blocked and NMDA mediated EPSPs simultaneously unmasked using a modified ACSF solution containing 0mM magnesium and 20 $\mu$ M CNQX or 10 $\mu$ M NBQX. Rise times and half widths of PSPs were recorded and confirmed the existence of AMPA and NMDA potentials. Potentials recorded in zero magnesium and CNQX were entirely blocked by 50  $\mu$ M APV. Ratios were calculated of the amplitudes of the AMPA mediated and NMDA mediated potentials (42). In order to calculate the NR2B component of the NMDA mediated PSPs, PSPs were recorded before and after application of 3 $\mu$ M ifenprodil (43) and stable periods of recording in control conditions and after perfusion of ifenprodil were averaged and the reduction in NMDA EPSPs calculated.

### **IPSC recordings**

During recordings of layer 2/3 neurons, slices were perfused with oxygenated ACSF containing 2mM kynurenic acid and 1 $\mu$ M tetrodotoxin to block ionotropic glutamate receptors and voltage-gated sodium channels, respectively. We recorded a population of spontaneously occurring inward currents at the normal resting potentials (-70mV) using a high chloride containing intracellular solution (140mM CsCl, 4mM NaCl, 10mM HEPES, 1mM MgCl<sub>2</sub>, 2mM Mg-ATP, 0.05mM EGTA) from 4 week old mice (44). Currents were completely blocked by the GABA<sub>A</sub> receptor antagonist picrotoxin (50 $\mu$ M). mIPSCs amplitudes and frequencies were measured for 100 second epochs of recording and averaged between genotypes and animal. The threshold for event detection was set at two to three times the signal to noise ratio at 5pA.

### **Silent Synapses**

Recordings measuring the incidence of silent synapses were made as previously described (23). Layer II/III pyramidal cells ( $n = 10$ ) from tamoxifen-treated (P7) DISC1-cc animals were patched in voltage clamp mode, with the addition of 5mM QX-314 in the intracellular solution. A unipolar stimulating electrode was placed in layer IV directly below the patched cell and EPSCs evoked at 0.1 Hz. Stimulus intensity was reduced until a failure rate of approximately 50% was observed at a holding potential of -70 mV. Forty EPSCs were then evoked at -70 mV holding and the success rate noted. Holding potential was changed to +40 mV and the cell allowed to equilibrate for 2-3 minutes (until holding current stabilised). Forty more EPSCs were evoked with the same stimulus parameters and the success rate at +40 mV noted. EPSC success rates were then compared for the two holding potentials; if there was a higher probability of evoking an EPSC at depolarised potential then the cell was considered to contain silent synapses.

### **Anatomical reconstructions**

Following recordings, slices were fixed overnight at 4°C in 100 mM phosphate buffered saline (PBS, pH 7.3) containing 4% paraformaldehyde (BDH, USA). Slices were then transferred to PBS and histologically reconstructed by conventional methods described previously (41). Slices were incubated for 30 minutes in PBS with 0.3% hydrogen peroxide containing 0.4% Triton X-100 (Sigma, UK). They were then washed in PBS with Triton and then incubated for 2 hours in PBS-avidin-biotinylated horseradish peroxidase (ABC, Vector Labs, USA). Slices were then reacted using 3,3'-diaminobenzidine (DAB, Sigma, UK) using nickel as the chromogen until the soma and dendritic arborisations were clearly visible (viewed under the light microscope, Olympus, UK). After several further rinses in PBS the sections containing the neurons were mounted on slides.

### **Morphological reconstructions of biocytin-filled neurons**

The 2D representation of the cells was achieved using a camera lucida (Olympus, UK) drawing of the filled neurons (Figs. 2a & 2b). The neurons' dendritic fields were then analysed using Sholl analysis(45). We measured the number of occasions that dendrites crossed the Sholl shells at increasing distances from the soma (dendritic counts(41)).

### **Measurements of spine density and classification**

Neurons were filled with biocytin and fixed with paramformaldehyde in an identical manner to that used for dendritic quantifications. Thereafter, slices were incubated for 18 hours in PBS supplemented with 1% Triton X-100 and 0.2% streptavidin Alexa Fluor 488 conjugate (Invitrogen) at 4-6°C (46). After washing with PBS, cells were located in slices under a fluorescent microscope and subsequently imaged under a 2-photon microscope (Prairie Systems) and spine density measured from z stacks (ImageJ). Spines were classified into four groups (mushroom, thin, and stubby spines, and filopodia) based on head-to-neck ratio and neck length (Mushroom spines – head:neck ratio  $>1.15$ , neck length  $<0.09\mu\text{m}$ ; thin spines - head:neck ratio  $>1.15$ , neck length  $>0.09\mu\text{m}$ , stubby spines: head:neck ratio  $<1.15$  and length  $<1.1\mu\text{m}$ ). Filopodia were easily distinguished by having no detectable spine head, were infrequent and not included in this report. Further spine analysis was conducted using Imaris software (Bitplane, Andor Technology, Belfast).

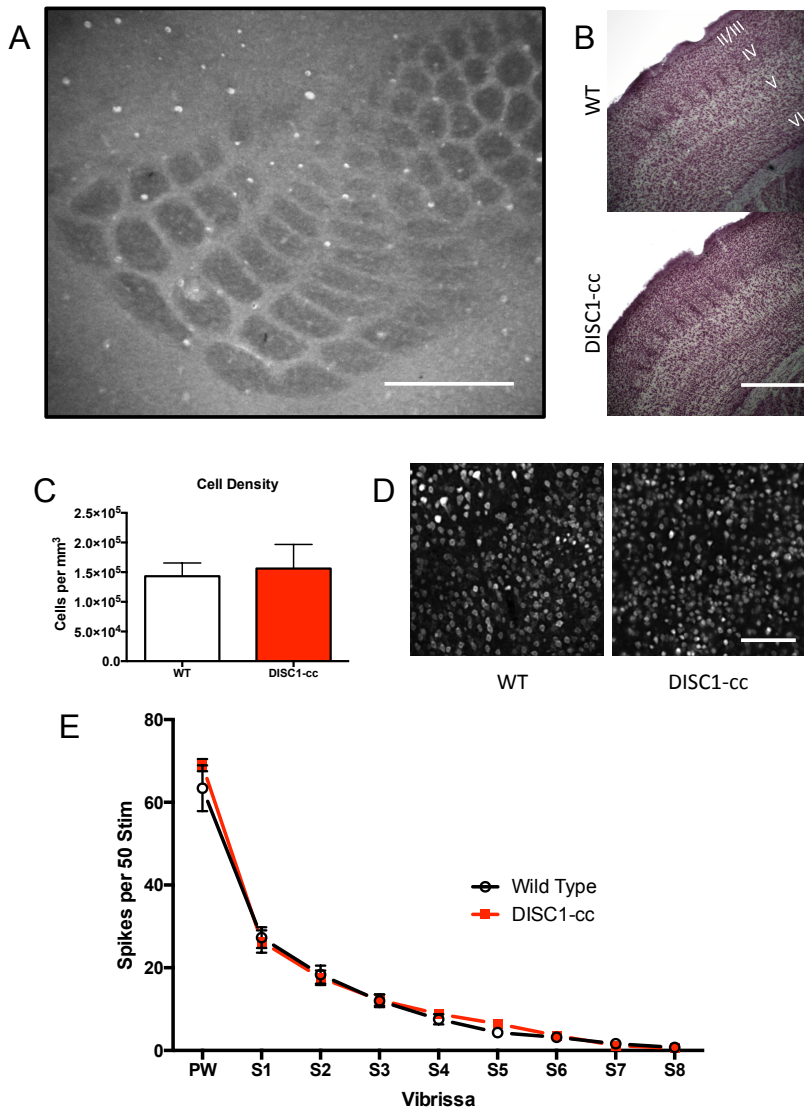
### **Layer depth and cell density measurement**

WT and DISC1cc mice previously subjected to tamoxifen injection at P7 were transcardially perfused at P50-P70 (n=4 per group) and fixed as described above. Coronal sections were cut at  $40\mu\text{m}$  on a freezing microtome and transferred to PBS. Sections were mounted on subbed slides, defatted with acetone and processed for Nissl staining (Thionin, 1%).

*NeuN immunostaining for cell density:* Sections were blocked in 5% Normal Goat Serum in 0.1M PBS and 0.1% TritonX-100 for 1 hour. After blocking sections were incubated in a primary antibody mix (in 0.1M PBS, 0.1% TritonX-100 and 3% Normal Goat Serum) of mouse monoclonal anti-NeuN (Millipore MAB377, 1:100 dilution) for 2 hours at room temperature, 18 hours overnight at 4°C and a further 2 hours at room temperature. After 3x 30 minute washes in 0.1M PBS and 0.1% TritonX-100 the secondary antibody Alexa594 goat anti-mouse (Life Technologies A11032, 1:200 dilution) was applied (in 0.1M PBS, 0.1% TritonX-100 and 3% Normal Goat Serum). Slices were incubated in the secondary mix for 3.5 hours at room temperature, and then after a further 3x 20 minute washes in 0.1M PBS and 0.1% TritonX-100 were mounted in Vectashield DAPI hardset (Vector H1500). The same protocol was applied to tangential slices alternating with slices stained for cytochrome oxidase to localise viral spread across barrels.



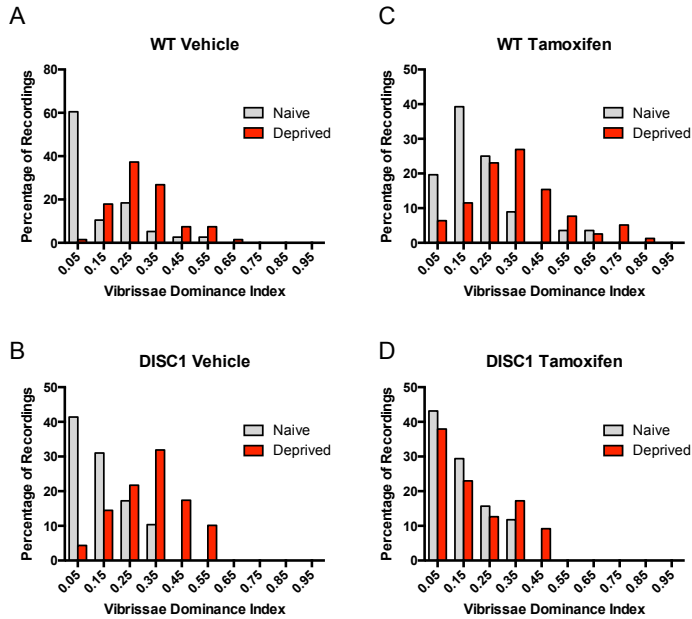
Sections were visualized using a Leica TCS SP2 confocal microscope. Cell density in confocal images was automatically quantified with Imaris F1 7.7.2 (Bitplane, Andor Technology, Belfast).



**Figure S1.** Development of the barrel cortex is normal at the macroscopic scale in animals with transient expression of mutant DISC1 at P7.

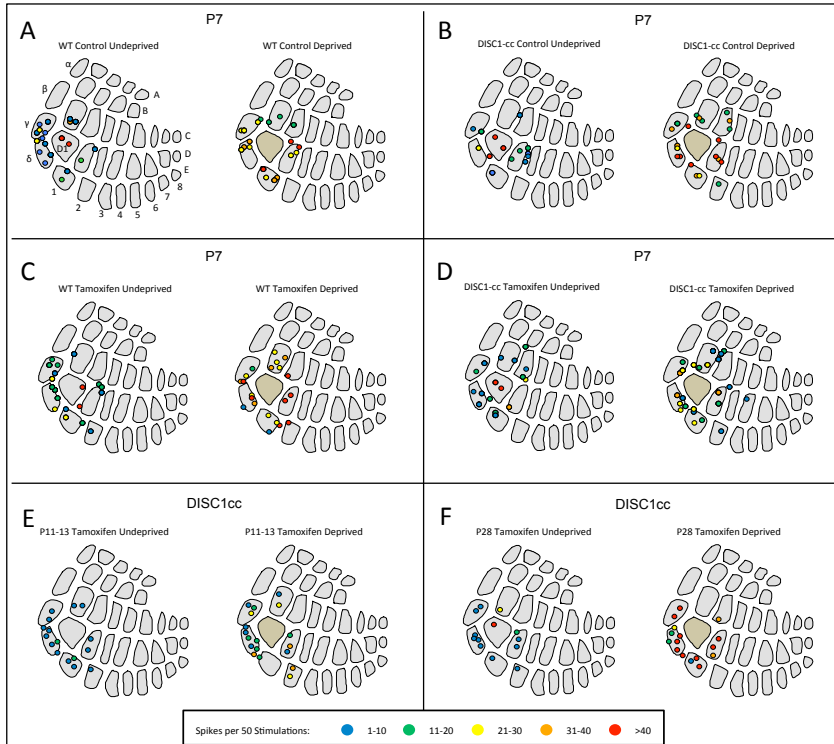
**A)** The normal barrel cortex pattern develops in Layer 4 in DISC1cc mice as shown with this cytochrome oxidase stained section. The area of the D1-D5 barrels were measured in wild-types and DISC1cc mice that had either been treated with

tamoxifen or vehicle; values were compared across tamoxifen treatment and genotype and were found not to be different (no effect of tamoxifen  $F_{(1,18)}=0.05$ ,  $p=0.81$ ; no effect of genotype  $F_{(1,18)}=0.63$ ,  $p=0.44$ , 2-way ANOVA). Note the micro-lesion made in the C1 barrel to mark the location of the recording penetration (scale bar =  $500\mu\text{m}$ ). **B)** Cortical layers have the same thickness in DISC1cc mice as in WT mice as shown in these Nissl stained coronal sections of barrel cortex. The depth of the junctions between layers was measured for DISC1cc and WT mice and found to be similar in absolute thickness (microns) and in thickness relative to the total cortical depth (L2/3 thickness: WT  $313\pm 11\mu\text{m}$ , DISC1cc  $334\pm 6\mu\text{m}$ ; L2/3/4 thickness: WT  $458\pm 6\mu\text{m}$ , DISC1cc  $453\pm 8\mu\text{m}$ ; total cortical depth: WT  $987\mu\text{m}$ , DISC1  $979\mu\text{m}$ ) (L2/3,  $F_{(1,6)}=1.7$ ,  $p=0.22$ ; L2/3/4,  $F_{(1,6)}=0.005$ ,  $p=0.94$ ) (scale bar =  $500\mu\text{m}$ ). NB: this is in contrast to studies where DISC1 mutations are active during cell migration, which results in thinner L2/3 (1) **C)** Layer 2/3 cell densities are similar between DISC1cc mice as in WT mice ( $F_{(1,14)}=0.62$ ,  $p=0.44$ ). **D)** Examples of barrel cortex in DISC1cc and WT mice showing the similarity of cell density between genotypes (neurons labelled with Neu-N; scale bar =  $100\mu\text{m}$ ). **E)** Receptive field profiles for undeprived WT and DISC1cc mice. The responses are plotted in order from the anatomically defined principal whisker (PW) and then by the greatest to least responding surround whisker (S1, S2, ... S8). There were no differences in adulthood between the undeprived receptive fields in WT mice and DISC1cc mice treated with tamoxifen at P7.



**Figure S2.** Vibrissae dominance histograms show P7 activation of DISC1cc impairs adult plasticity.

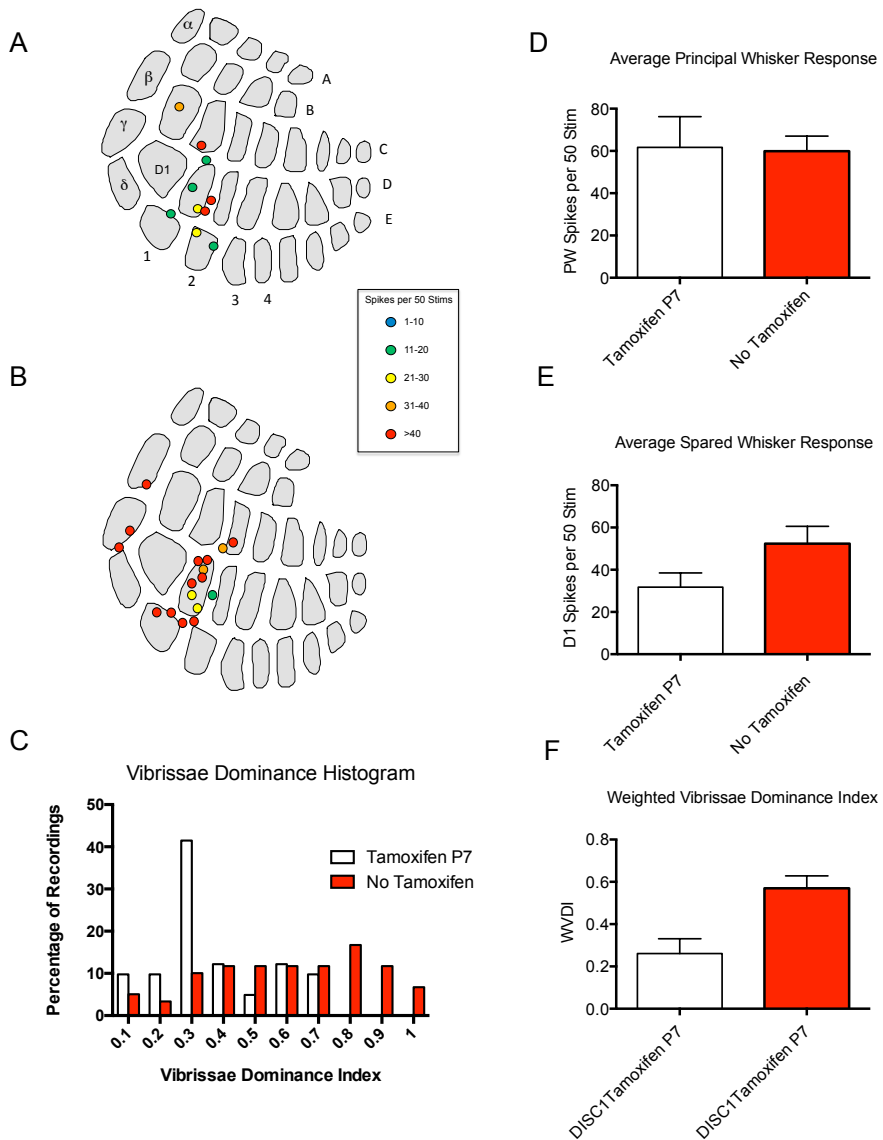
**A, B)** Vibrissae dominance histograms for adult WT and DISC1cc mice treated with corn oil vehicle at P7. Naïve mice display a left-shifted dominance histogram as the average PW response far outweighs the D1 response (see Figure 1). Modal VDI was 0.05 for both WT and DISC1cc naïve vehicle-treated mice. After 18 days D1-spurred deprivation the modal VDI was 0.25 for WT and 0.35 for DISC1cc vehicle-treated mice. **C)** WT mice treated with tamoxifen at P7 still displayed a large shift in VDI after 18 days single whisker experience (modal VDI = 0.15 naïve, 0.35 deprived) **D)** In contrast to the other cohorts, DISC1cc mice treated with tamoxifen at P7 did not exhibit a VDI shift in deprived animals (modal VDI = 0.05 for both naïve and deprived groups) suggesting that adult experience-dependent plasticity is abolished in these animals.



**Figure S3.** Penetration maps showing the average spike rate of recorded cells to D1 whisker stimulation.

The heat map indicates the strength of responses within a penetration (key below figure). Deprived animals exhibiting experience-dependent plasticity would be expected to show a greater spike rate in response to spared whisker stimulation in the barrels surrounding D1. **A)** In vehicle treated wild-type mice, the proportion of penetrations with a mean spike rate of 31 or above (per 50 stimulations) is 1/18 in naïve mice and 6/19 in deprived animals (5.5% vs 31.6%,  $\chi^2(1,37) = 4.08$ ,  $p < 0.05$ , Pearson's chi-square test). **B)** Similarly, in vehicle treated DISC1cc mice, 1/12 penetrations were high-spiking in control mice and 13/21 responded strongly to D1 stimulation in deprived animals (8.3% vs 61.9%,  $\chi^2(1,34) = 9.74$ ,  $P < 0.01$ , Pearson's chi-square test). **C)** Treatment of WT mice with tamoxifen at P7 did not ameliorate the shift in favour of D1 in deprived animals, with 1/18 cells responding strongly to D1 stimulation in naïve mice and 11/21 in deprived mice (5.5% vs 52.4%,  $\chi^2(1,39) = 9.98$ ,  $P < 0.01$ , Pearson's chi-square test). **D)** In contrast to the control conditions shown in A-C, treatment of DISC1cc animals with tamoxifen at P7 resulted in no

difference in the proportion of strong D1-responding penetrations when comparing naïve and deprived mice, suggesting a deficit in experience dependent plasticity (Naïve = 1/15, 6.6% vs deprived 2/22, 9.1%,  $\chi^2(1,37) = 0.07$ ,  $P > 0.05$ , Pearson's chi-square test). **E)** DISC1-cc mice treated with tamoxifen at P11-13 did not show a significant difference in the proportion of highly responsive cells between naïve and deprived animals (Naïve = 0/16, 0% vs deprived 3/18, 16.7%,  $\chi^2(1,34) = 2.92$ ,  $P = 0.087$ , Pearson's chi-square test), although other measurements of plasticity (e.g. weighted vibrissae dominance) suggest that some experience-dependent plasticity does occurs in this group (Figure 2, main text). **F)** Treatment with tamoxifen at P28 did not hinder experience-dependent plasticity, with deprived mice showing a significantly higher proportion of D1-biased penetrations (Naïve = 0/12, 0% vs deprived 12/16, 75%,  $\chi^2(1,28) = 15.75$ ,  $P > 0.0001$ , Pearson's chi-square test).

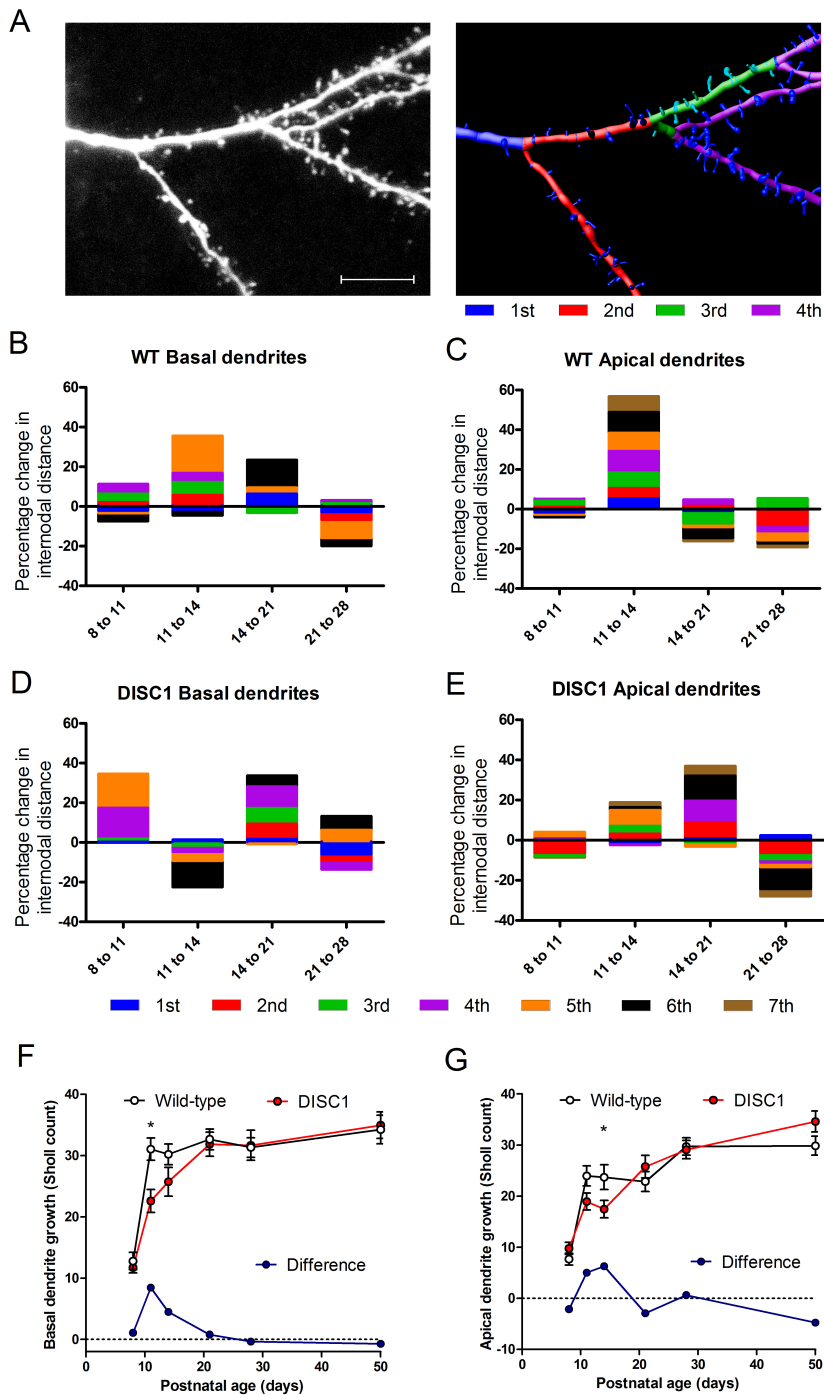


**Figure S4.** Transient DISC1cc expression at P7 blocks experience-dependent plasticity in mice with a different background strain from those shown in Figure 1.

DISC1cc mice were bred to a C57BL/6N Taconic background. Mice were either injected with tamoxifen **(A)** or vehicle control on P7 **(B)**. **A**) The aggregate penetration map is shown for recordings from 6 animals. The colour code shows the average response of L2/3 cells in a particular penetration (cells recorded at 50 micron intervals from 50 microns to 250 microns depth in each penetration). Many

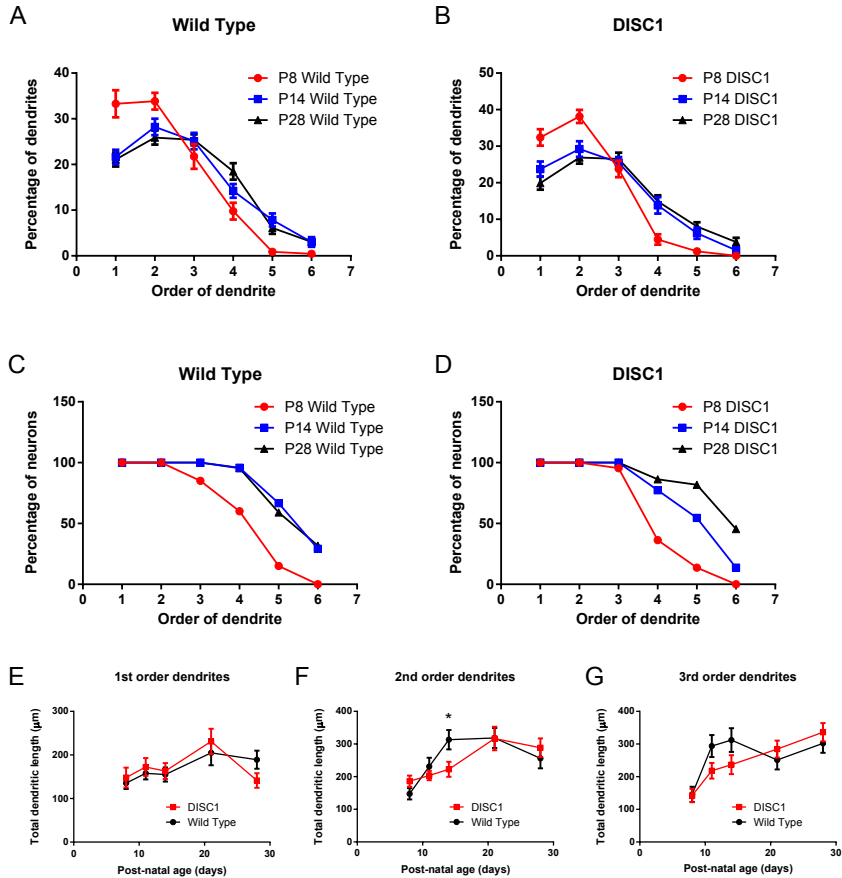
fewer penetrations show potentiated responses to D1 stimulation (red and orange coded penetrations) in columns surrounding the D1 barrel-column (4/10) in Tx treated mice compared with controls (8/11) shown in **B. C)** Vibrissae dominance histograms for the same animals showing the relative response of cells to the spared D1 vibrissa versus their anatomically defined principal vibrissa. The proportion of cells in each bin is plotted where the Vibrissae Dominance Index (VDI) for a given cell is given by  $VDI = D1/(D1+PW)$ , where D1 is the average response to D1 whisker stimulation and PW the average response to principal whisker stimulation. **D)** Principal whisker responses were not different across Tx treated and vehicle cases ( $F_{(1,10)}=0.12$ ,  $p=0.91$ ). **E)** The D1 whisker response was 165% greater in mice that had not received Tx at P7 than in DISC1 mice that had ( $F_{(1,10)}=5.68$ ,  $p<0.05$ ). **F)** The average weighted vibrissae dominance index (see Methods) was also significantly greater for P7 Tx treated animals than controls ( $F_{(1,10)}=16.2$ ,  $p<0.005$ ).





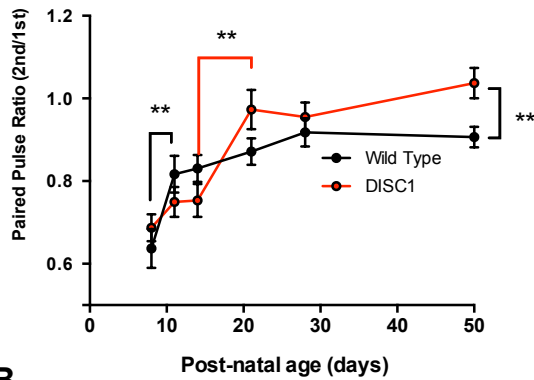
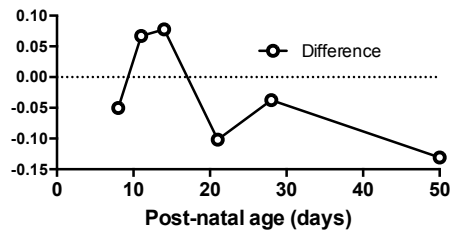
**Figure S5.** Dendritic development in animals transiently expressing DISC1cc.

**A)** Example of L2/3 cell dendrites showing spines and dendritic order. Scale bar=10 microns. **B-E)** Growth in dendrites charted as an increase in internodal distances. The change in median internodal distance is given as percentage of the internodal distance at the start of the period. **B)** Wild-type basal dendrites: the main periods of elongation are between 11 and 21 days. Negative values are due to increased branching in that order of dendrite (P21-28). **C)** Wild-type apical dendrites: Almost all the elongation occurs between P11 and 14. **D)** DISC1 basal dendrites: Development of the basal dendrites show a different timecourse to the wild-types. Growth of the 2<sup>nd</sup> and 3<sup>rd</sup> order dendrites (red and green) is delayed until P14-21. **E)** DISC1 apical dendrites: Note that 2<sup>nd</sup> and 3<sup>rd</sup> order dendrites mainly elongate between P14 and P21 whereas in wild-types the dendrites elongate earlier (P11-14, C). **F)** A plot of basal dendritic growth versus age group shows rapid development between P8 and P11 in wild types (black line white circle). In comparison, the DISC1 dendritic growth (red line) is retarded at P11 and P14 (blue line shows difference in means) and significantly different at P11 ( $t_{(40)}=3.23$ ,  $p<0.005$ ). **G)** The apical dendritic development of layer 2/3 cells is again retarded at P11 and P14 and is significantly different from the wild-types at P14 ( $t_{(40)}=2.12$ ,  $p<0.05$ ).



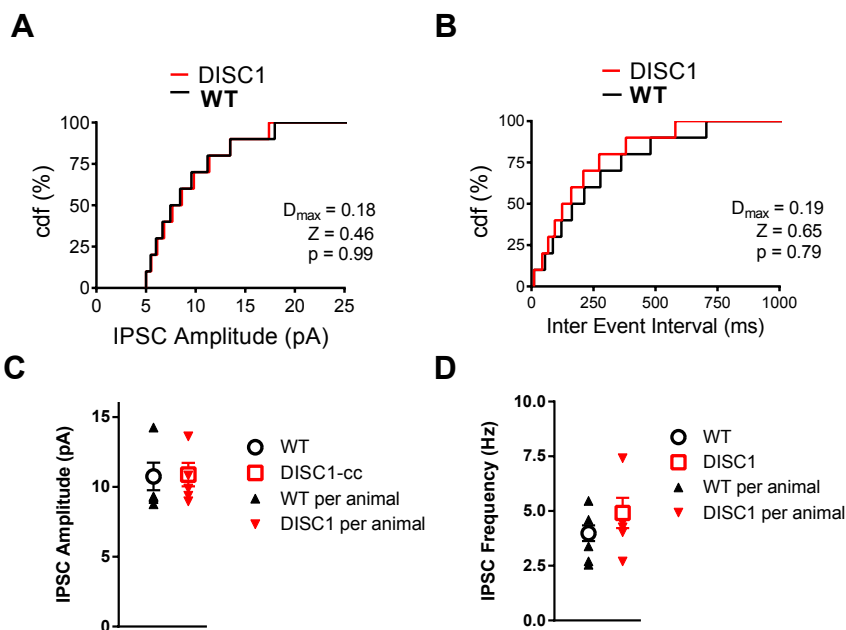
**Figure S6.** Development of basal dendrites in animals transiently expressing DISC1cc.

**A)** Wild-type L2/3 neurons: The number of each order of dendrite present at P8 to P28 is expressed as a percentage of the total number of dendritic branches at that age. **B)** The same data as in (A) for DISC1 neurons, showing very similar proportions of each dendrite order between DISC1 and WT animals. **C)** The percentage of neurons possessing each order of dendrite in wild-types **D)** The same form as in (C) plotted for DISC1 mice. **E-G)** The development of dendritic length for each order of dendrite in DISC1 (red) and Wild-type (black) animals. Note that the growth rate in DISC1cc cells is delayed in the 2<sup>nd</sup> and 3<sup>rd</sup> order dendrites and significantly smaller at P14 for the 2nd order dendrites (F) ( $t_{(42)}=2.39$ ,  $p<0.05$ ).

**A****B**

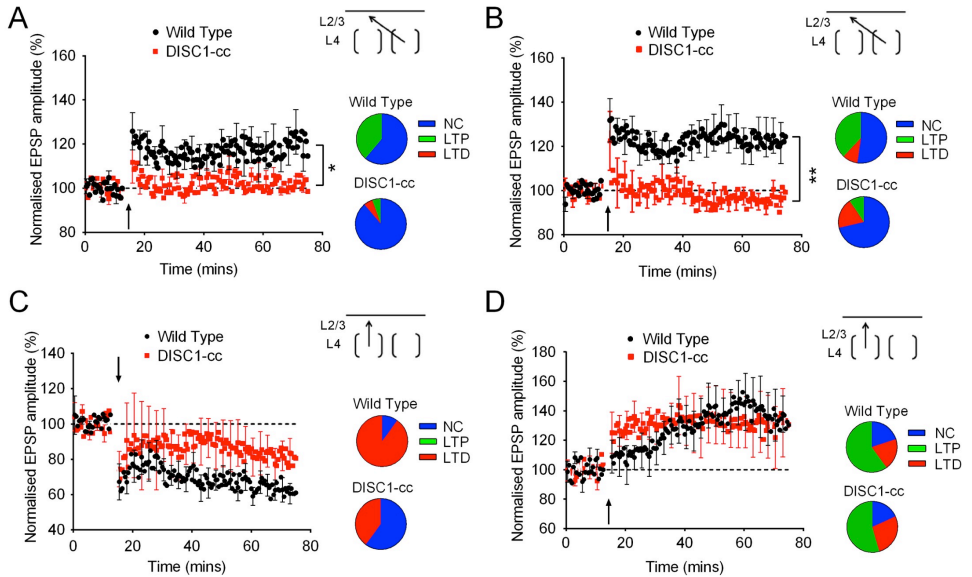
**Figure S7.** Paired pulse ratio development is delayed by transient release of DISC1cc.

**A)** The developmental increase in paired pulse ratio is delayed in DISC1cc mice (interaction between age and genotype  $F_{(5,5)}=2.59$ ,  $p<0.03$ ). In WT mice, paired pulse ratio increases close to adult values between P8 and P11 ( $t_{(41)}=2.76$ ,  $p<0.01$ ), whereas in DISC1 mice the increase is delayed and occurs between P14 and 21 ( $t_{(41)}=3.46$ ,  $p<0.005$ ). Adult values of paired pulse ratio are higher in DISC1 mice at P50 ( $t_{(46)}=2.78$ ,  $p<0.01$ ). **B)** The delay in development and subsequent overshoot can be clearly seen when the difference in paired pulse ratio between WT and DISC1cc animals is plotted.



**Figure S8.** Transient DISC1cc release does not significantly affect inhibitory activity in the barrel cortex.

**A)** IPSC amplitudes from WT and DISC1 mice were not different to one another (KS test,  $D_{\max}=0.18$ ,  $Z=0.46$ ,  $p>0.05$ ) **B)** IPSC inter-event intervals from WT and DISC1 mice were not different to one another (KS test,  $D_{\max}=0.19$ ,  $Z=0.65$ ,  $p>0.05$ ). **C)** Mean IPSC amplitudes for WT and DISC1 mice grouped by animal (triangles) and overall mean values (open symbols; WT =  $10.76 \pm 0.99$ , DISC1 =  $10.89 \pm 0.85$ ,  $t_{(23)}=0.11$ ,  $p>0.05$ , grouped by animal  $t_{(8)}=0.29$ ,  $p>0.05$ ). **D)** Mean IPSC frequencies for WT and DISC1 mice grouped by animal (triangles) and overall mean values (open symbols; WT =  $3.99 \pm 0.36$ , DISC1 =  $4.91 \pm 0.69$ ,  $t_{(23)}=1.18$ ,  $p>0.05$ , grouped by animal  $t_{(8)}=0.87$ ,  $p>0.05$  ).



**Figure S9.** LTP and LTD but not deprivation-unmasked potentiation is impaired in adults by transient DISC1cc release at P7.

**A)** Transient release of DISC1cc at P7 abolishes the capability for inter-columnar LTP in layer 2/3 at P28 and **B)** at P50 (effect of genotype  $F_{(1,74)}=14.27$ ,  $p<0.0003$ , not age  $F_{(1,74)}=0.13$ ,  $p<0.71$ , ANOVA). The percentage of cells showing statistically significant LTP drops from 33% in wild-types to 5% in DISC1 (P28) and 43% in wild-types to 9% in DISC1cc at P50 (see pie charts, NC = no change). **C)** Average LTD values are not statistically different in WT and DISC1cc mice ( $F_{(1,18)}=3.44$ ,  $p<0.08$ , ANOVA), though the percentage of cells showing LTD drops from 90% in wild-types to 40% in DISC1. **D)** Complete whisker deprivation unmasks PKA dependent loss of depression(20) and this is unaffected in the adult mouse by P7 DISC1cc ( $F_{(1,18)}=0.16$ ,  $p<0.87$ , ANOVA).

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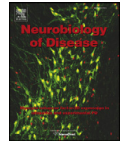


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# Somatosensory map expansion and altered processing of tactile inputs in a mouse model of fragile X syndrome



Konrad Juczewski<sup>a,\*</sup>, Helen von Richthofen<sup>a</sup>, Claudia Bagni<sup>b,c,f</sup>, Tansu Celikel<sup>d</sup>, Gilberto Fisone<sup>a</sup>, Patrik Krieger<sup>e,\*\*</sup>

<sup>a</sup> Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

<sup>b</sup> VIB Center for the Biology of Disease, KU Leuven, Leuven, Belgium

<sup>c</sup> Department of Biomedicine and Prevention, University of Rome Tor Vergata, Rome, Italy

<sup>d</sup> Department of Neurophysiology, Donders Institute for Brain, Cognition and Behaviour, Radboud University, Nijmegen, The Netherlands

<sup>e</sup> Department of Systems Neuroscience, Medical Faculty, Ruhr University Bochum, Bochum, Germany

<sup>f</sup> Department of Fundamental Neuroscience, University of Lausanne, Lausanne, Switzerland

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## ABSTRACT

Fragile X syndrome (FXS) is a common inherited form of intellectual disability caused by the absence or reduction of the fragile X mental retardation protein (FMRP) encoded by the *FMR1* gene. In humans, one symptom of FXS is hypersensitivity to sensory stimuli, including touch. We used a mouse model of FXS (*Fmr1* KO) to study sensory processing of tactile information conveyed via the whisker system. *In vivo* electrophysiological recordings in somatosensory barrel cortex showed layer-specific broadening of the receptive fields at the level of layer 2/3 but not layer 4, in response to whisker stimulation. Furthermore, the encoding of tactile stimuli at different frequencies was severely affected in layer 2/3. The behavioral effect of this broadening of the receptive fields was tested in the gap-crossing task, a whisker-dependent behavioral paradigm. In this task the *Fmr1* KO mice showed differences in the number of whisker contacts with platforms, decrease in the whisker sampling duration and reduction in the whisker touch-time while performing the task. We propose that the increased excitability in the somatosensory barrel cortex upon whisker stimulation may contribute to changes in the whisking strategy as well as to other observed behavioral phenotypes related to tactile processing in *Fmr1* KO mice.

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## 1. Introduction

Fragile X Syndrome (FXS) is a neurodevelopmental disorder with multiple symptoms including cognitive problems. FXS is associated with mutation of the fragile X mental retardation 1 gene (*Fmr1*) that results in an abnormally numerous repetition of a non-coding CGG trinucleotide (Bagni and Oostra, 2013; McLennan et al., 2011; Tranfaglia, 2011). As a consequence, *Fmr1* gene function may be significantly reduced or entirely silenced and a product of its expression, the fragile X mental retardation protein (FMRP), is partially or fully absent in the affected organism (Hagerman et al., 2014). FMRP is an RNA-binding

protein that regulates translation of several pre- and postsynaptic transcripts (Darnell et al., 2011), a process especially important for synaptic plasticity (Bear and Malenka, 1994; Malenka and Bear, 2004). Lack of FMRP leads to altered synaptic development and impaired neural circuits formation that may underlie sensory deficits and cognitive symptoms observed in FXS patients (Bassell and Warren, 2008). FXS is the most common heritable form of intellectual disability (Farzin et al., 2006; Bhogal and Jongens, 2010) and the best characterized cause of autism spectrum disorders (Dolen and Bear, 2009). It evokes various disruptions in the central nervous system causing learning deficits, abnormal social behaviors, and extreme sensitivity to sensory stimuli (Miller et al., 1999). We focused our studies on the somatosensory system knowing that many FXS patients present hypersensitivity to touch (Cascio, 2010). Furthermore, also people suffering from depression, other autism spectrum disorders (ASD), or attention-deficit-hyperactivity disorder (ADHD) appear to have similar problems with their sense of touch (Weber and Newmark, 2007).

Touch is an important source of sensory information. Disturbances to the development of the somatosensory system have serious consequences for social behavior (Shishelova and Raevskii, 2010). The *Fmr1*

\* Correspondence to: K. Juczewski, NIH/NIAAA, 5625 Fishers Lane, Rockville, MD 20852, USA.

\*\* Correspondence to: P. Krieger, Dept. of Systems Neuroscience, Ruhr University Bochum, Universitätsstrasse 150, 44801 Bochum, Germany.

E-mail addresses: [konrad.juczewski@nih.gov](mailto:konrad.juczewski@nih.gov) (K. Juczewski), [patrik.krieger@rub.de](mailto:patrik.krieger@rub.de) (P. Krieger).

<sup>1</sup> Current affiliation: National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, USA.

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knock-out (KO) mouse model (The Dutch-Belgian Fragile X Consortium, 1994) used in our studies has phenotypes similar to those observed in human FXS patients (van den Ouweland et al., 1994). Additionally, the sense of touch is a well-studied system in mice and constitutes an important source of information necessary for their functioning and social interactions (Kazdoba et al., 2014; Santos et al., 2014). Mice also use whisker information to distinguish features of nearby objects and localize themselves in space (Brecht, 2007). The mouse whisker system consists of special facial hair (whiskers) and corresponding regions in the primary somatosensory cortex (barrel cortex) (Woolsey and Van der Loos, 1970). The whisker system is a useful model for research on the somatosensory system due to similarities between human and mouse tactile processing. Mechano-gated receptors in the skin serve as an input in both human touch and in the whisker system, and the flow of excitation goes through the same brain structures (brainstem and thalamus). Therefore, the whisker system represents a relevant model for understanding of tactile processing in humans (Diamond, 2010).

Sensory processing studies on the impaired somatosensory system are of vital importance for our understanding of the mechanisms underlying sensory deficits in FXS and neurodevelopmental disorders mentioned above. In our study, we performed *in vivo* single-cell electrophysiological experiments to gain new insight into systems-level hyperexcitability in FXS, adding to the known molecular mechanisms of the disorder (Chen et al., 2010; Coffee et al., 2012; Santoro et al., 2012; Zhang et al., 2014). We investigated the processing of touch in the somatosensory barrel cortex of *Fmr1* KO mice and their wild type (WT) littermates using juxtacellular recordings. Analyzing whisker-stimulation-evoked responses, we found that *Fmr1* KO mice show an abnormally large area of cortical activation in response to sensory stimuli, i.e. an expansion of the somatosensory map, and an impaired encoding of the stimulation frequency. In addition, we tested *Fmr1* KO and WT mice in a gap-crossing task, a simple whisker-dependent behavioral paradigm (Celikel and Sakmann, 2007; Harris et al., 1999; Hutson and Masterton, 1986; Papaioannou et al., 2013). The gap-crossing task can be used to study exploratory locomotor behavior and basic learning capabilities in a whisker-dependent task. In addition using high-speed imaging whisker kinematics can be studied which is useful for assaying how the animal has acquired sensory data; for example, in a tactile task parameters such as number of contacts and contact time are important factors to understand sensory information acquisition and its further processing. We did not find any difference in task performance but, interestingly, we observed altered whisker kinematics in *Fmr1* KO mice.

## 2. Materials and methods

### 2.1. Animal preparation

All procedures were performed in accordance with ethical permits approved by the local ethics committee. 30 Male *Fmr1* KO mice and 30 of their WT littermates of C57Bl/6J background strain were used (The Dutch-Belgian Fragile X Consortium, 1994). Animals were housed with *ad libitum* food and water at 21 °C and a 12-h light/dark cycle.

### 2.2. *In vivo* electrophysiology: experiment preparation and procedures

Experiments were conducted under general anesthesia introduced with isoflurane (Baxter, UK) inhalation and maintained by intra-peritoneal injection of a mix of urethane (dosage 1.0 g urethane/kg mouse weight; Sigma-Aldrich, USA) and acepromazine maleate (dosage 3 mg/kg; Pharmaxim AB, Sweden) dissolved in water. An initial injection constituted 70% of the maximum dose and it was repeated at lower doses (10% initial dose) when anesthesia depth was diminishing. Anesthesia depth was assessed continuously based on the animal's breathing rate, hind leg withdrawal and corneal (blinking) reflexes. Oxygen was provided constantly during preparation as well as during the

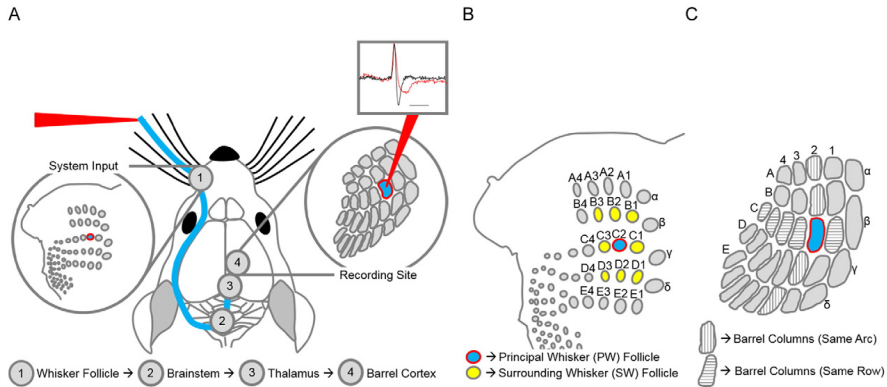
experiment in order to improve breathing and, thus, the survival rate of the animals. Animal temperature was maintained and kept stabilized at 37 °C.

The mouse was mounted in the stereotaxic apparatus with ear bars and a nose bar to stabilize its skull. Using a dental drill, a skull area above the barrel cortex (2–4 mm lateral from midline and 0–2 mm posterior from bregma) was thinned to create small, electrode-tip-sized holes with a fine needle. Each hole was made separately just before introduction of the electrode to a new position in the brain. After surgery, ear bars and the nose bar were removed and substituted with a metal plate attached to the top of a skull with glue and dental acrylic (Paladur, Germany). This allowed better access to mouse's whiskers. 18 WT and 18 *Fmr1* KO animals were used for the experiments. *In vivo* juxtacellular recordings were performed with electrodes (resistance 4 to 8 M $\Omega$ ) pulled from borosilicate filamented glass (Hilgenberg GmbH, Germany) on a Sutter P-97 puller (Sutter Instruments, CA, USA). Electrodes were filled with the following solution (in mM): 135 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub> and 5 HEPES; pH was adjusted to 7.2 with NaOH. 0.9% NaCl was used as a bath solution covering the skull surface. Recordings were made from layer 2/3 (L2/3) and layer 4 (L4) of the somatosensory barrel cortex. Cells classified as L2/3 were located at 100–350  $\mu$ m and those classified as L4 at 350–500  $\mu$ m below pia (Groh et al., 2010). An increase in electrode resistance while lowering the electrode was used to locate the brain surface. Later, it helped in identification of the recorded cell depth. Only excitatory cells from L2/3 and L4 were chosen for the final analysis. Other recorded cells, classified as interneurons or L5 pyramidal cells, were discarded due to a low number of recordings.

Cell sampling was based on a standard classification method (Armstrong-James and Fox, 1987; Bruno and Simons, 2002; Niell and Stryker, 2008). Fast-spiking units (presumably interneurons; black trace, inset Fig. 1A) were distinguished from regular-spiking units (presumably excitatory cells; red trace, inset Fig. 1A) based on their spike's peak-to-trough duration (the interneurons had a very short duration, <0.3 ms), a symmetrical up and down deflection (an integral value for the interneurons is close to zero), and high-frequency-bursts characteristic of these inhibitory cells. Cells were recorded with similar anesthesia level corresponding to stage 3–4 sleep (Armstrong-James and Fox, 1988) to avoid variability in brain activity. In both WT control and *Fmr1* KO animals, cells were sampled according to the same criteria to ensure equal cell sampling strategy. These criteria included: spike shape (to exclude interneurons); whisker response latency (to determine principal whisker response); spontaneous activity (cells with no spontaneous activity were not included). We also performed an outlier analysis and results were the same with or without outliers included in the analysis.

### 2.3. Whisker stimulation protocols

Whiskers were cut to a length of around 10 mm to ensure equal movements when stimulated with a glass capillary glued to a piezo-wafer (PL140.11, Physics Instruments, Germany). The glass capillary tip was placed in loose contact with the whisker, approximately 5 mm away from the whisker pad. Stimulation was controlled with an amplifier and a filter (Sigmann Elektronik, Germany) and consisted of square pulses. Displacements of the stimulated whisker were about 0.7 mm in the dorsal to ventral direction. The max ringing amplitude was approximately 80  $\mu$ m and the average ringing frequency was 30 Hz. Whiskers were subdivided in two categories, a principal whisker (PW) and surrounding adjacent whiskers (SWs). Neurons in a given barrel column preferentially responded to stimulation of one whisker, the PW, and weaker to SWs. A recording electrode was placed in a barrel column and a putative PW was initially identified using a hand held probe (a small wooden stick). We carried on with the piezo-stimulation protocol only when the cell was clearly responding to dorso-ventral movements of the hand-held probe. The Whisker Selectivity Index (WSI; see Results) is calculated on the basis of evidence that a cell has a similar



**Fig. 1.** Overview of the mouse somatosensory system. (A) A schema of the whisker to barrel somatosensory pathway from the whisker (system input) to the recording site in somatosensory barrel cortex. Single whiskers were mechanically moved with a piezoelectric stimulator while simultaneously recording, *in vivo*, cortical response from single cells with a glass electrode placed in a barrel column corresponding to the stimulated principal whisker (PW). Inset: Two different main types of juxtacellular units were recorded, interneurons (black trace) and excitatory neurons (red trace); see [Materials and methods](#) for details. Scale bar 1 ms. (B) The mouse snout with the different whisker follicles outlined. The principal whisker (PW) and the first-order surrounding whiskers (SW) are marked. In this example C2 is the PW. During an experiment the electrode was recording from one cell and a whisker-evoked response was recorded when alternatingly stimulating the PW or each of the SWs. (C) A general schema of the somatosensory barrel cortex. Input from the C2 whisker (in panel B), in normal conditions causes the largest response from cortical cells in the corresponding C2 barrel column.

directional tuning to both PW and SW whisker deflection (Kida et al., 2005). Manual identification of the PW was highly accurate, as confirmed by the off-line analysis, where the PW was defined off-line as the whisker eliciting the strongest response (the highest number of action potentials evoked in the recorded cell), with the shortest latency (Armstrong-James et al., 1992).

Two stimulation protocols were used in two different parts of this study. In the first part, focusing on the characterization of the response to 1-Hz stimulation, the following stimulation parameters were used: 1-Hz stimulation frequency; 200-ms square pulses; rise/fall time of 8 ms; piezo-deflection amplitude of about 0.7 mm; 50 stimulation repeats. Cells from both layers, L2/3 and L4, were recorded. "Inter-stimulus Activity" of the cell was calculated based on the average number of action potentials occurring in 150-ms periods preceding stimulation of the whisker. The whisker-stimulation-evoked response was calculated as the number of action potentials occurring during a 150-ms period, 150-ms from the start or the end of the whisker stimulation were used depending on the cell characteristics (some cells responded specifically to ON- or OFF-stimulation). In a few cases where cells responded equally well to both parts of the stimulation, we averaged ON- and OFF-responses to keep a consistent 150-ms window for later analysis. Inter-stimulus Activity was subtracted from the number of spikes evoked after whisker stimulation to calculate the whisker-evoked response rate. In the second part, focusing on the differences in frequency coding, the following stimulation parameters were used: 1-, 2-, 4-, 8-, and 10-Hz stimulation frequency; 25-ms square pulses; rise/fall time of 4 ms; piezo-deflection amplitude of about 0.7 mm; 25 stimulation repeats. The whisker-stimulation-evoked response was calculated as the number of action potentials occurring during the 50-ms period following whisker stimulation onset. Averaging over a shorter period (50-ms instead of 150-ms as in the previous experiments) was done to keep the period constant for various stimulation frequencies. Only cells from L2/3 were recorded in this part of the study. Changes undertaken in the second protocol were the adjustments necessary to achieve the most informative procedure to discover differences in frequency coding.

Whisker response latency was analyzed in two different ways. In the first part of the manuscript, the average latency to the response onset

(ON-latency) or offset (OFF-latency) was calculated (the first spike evoked by a stimulation in a stimulation train). ON-, OFF- or an average from ON- and OFF-latency was used depending on the cell characteristics (some cells responded specifically to ON- or OFF-stimulation, some responded to both; also ON- and OFF-latency were exactly within the same time-range). Latency to the PW response was compared with the latency to the SW-1 response (SW-1 is the surrounding whisker with the highest response rate). In the second part, focusing on the characterization of differences in frequency coding, latency analysis was extended to check different aspects of the latency encoding (see [Results](#)). In addition to the average latency to the first spike, the median latency of the response was calculated for all of the stimulation-frequencies.

#### 2.4. Gap-crossing task: the experimental set up

The gap-crossing task apparatus was built as previously described using two identical moveable platforms made of transparent Plexiglas (width = 0.5 cm) (Papaioannou et al., 2013). The platforms (75 × 220 mm) were elevated 25 cm off the surface, surrounded on three sides with 20-cm-high walls, and placed end-to-end facing each other. Each platform was equipped with two motion sensors to monitor animal movements on the platform and to calculate off-line variables of decision making during the gap-crossing task. Additionally, a high-resolution infrared video camera (PIKE 032B; Allied Vision Technologies, Germany) was placed above the gap to record whisker activity during gap-crossing attempts. The platform in the field of view of the camera was called the "target platform" and the platform on the other side of the gap was called the "home platform" ("target" and "home" are not used to denote a preferred direction of crossings). Data on whisker kinematics and nose position were collected only when animals were approaching the gap from the home platform because the camera was placed over the target platform. An IR-backlight (880 nm; Microscan, WA, USA) positioned below the gap provided necessary contrast for tracking animal and whisker motion. A liquid-cooling block was placed underneath the IR-backlight to ensure that a constant temperature was maintained. Extraneous noise was masked with white noise (~75 dB).

A schematic of the gap-crossing apparatus is presented in the Fig. 7A and B.

### 2.5. Testing protocol

Animals (12 WT and 12 *Fmr1* KO littermates; age 9–13 weeks) were habituated to the experimenter and to the gap-crossing apparatus 2 days prior to a behavioral test. Each day of a habituation procedure consisted of two 5-minute sessions of handling, during which the experimenter was interacting with the animals extensively by allowing them to explore the experimenters' hands and by picking them up. The habituation also included 20 min inside the apparatus with the platforms pushed together, so that the animals could cross between the platforms without a gap between them. On the first day, an animal was placed inside the apparatus with background white noise and lights on; on the second day, lights were turned off. After the second habituation session, all whiskers, except C2 on both sides of the snout, were removed to facilitate whisker tracking. It has been shown that mice are able to learn the gap-crossing task with a single whisker or multiple whiskers alike (Celikel and Sakmann, 2007). During the whisker removal procedure isoflurane anesthesia was used. The removed whiskers were trimmed with scissors to fur-level or plucked as needed throughout testing, following the daily test session to avoid stress during the task.

Testing consisted of one 20-minute session per day for 8 consecutive days. Animals were placed inside the apparatus with white noise in the background and in complete darkness. They were allowed to freely explore and cross the gap spontaneously. The gap distance was changed in increments of 0.5 cm after each successful cross according to a pseudo-random protocol that weighted larger distances towards the end of the session. The protocol was divided into 4 blocks. Within each block, distances were selected randomly from a predetermined range unique to that block, and the number of successful crossings that were needed before proceeding to the next block was 1 or 3. The exact protocol was as follows: Day 1: block 1 (3 crossings before switching to the next block) = 4–4.5 cm, block 2 (3 crossings) = 4.5–5.5 cm, block 3 (3 crossings) = 5–6.5 cm, block 4 = 5.5–7 cm; Day 2–4: same as Day 1 except that there was only 1 successful crossing necessary in block 1, before switching to block 2; Day 5–8: block 1 (1 crossing) = 4–4.5 cm, block 2 (3 crossings) = 5.5–6 cm, block 3 (3 crossings) = 6–7 cm, block 4 = 6–7.5 cm. This pseudo-random protocol allowed mice to work up to the greater distances while maintaining a degree of unpredictability. The exact distances within these ranges varied for each mouse and each session. After each session the animal was placed back in its home cage and the test apparatus was cleaned with 70% ethanol.

"Catch trials" were performed to ensure that gap crosses were based on sensory input from the whiskers. During these sessions 4 trials with distances generated by the pseudo-random protocol were followed by one trial at 8 cm, a distance unreachable by the whiskers. None of the tested animals attempted to cross at a distance of 8 cm.

### 2.6. Analysis of locomotor behavior and whisking

Movement of each mouse within the behavioral apparatus was monitored with infrared motion sensors (MS). The ON- and OFF-time of the beam breaks from each motion sensor were analyzed using custom-written MATLAB routines (MathWorks, MA, USA), to quantify animal behavior (Voigts et al., 2008). An "Attempt" was recorded when the animal activated (by breaking the beam) one of the motion sensors closest to the gap (MS2 or MS3 in Fig. 7A) and a "Successful Attempt/Trial" was recorded when the animal crossed over the gap to reach the opposite platform. The variable "Exploration Duration" represented the amount of time spent at the gap (onset of MS2-ON until MS2-OFF or MS3-ON until MS3-OFF). A "Trial" started with the activation of MS1 and ended with activation of MS4. "Trial Duration" was the time from the start of the trial at MS1 to its end at MS4. In addition to the motion

sensor data, we quantified more specifically how the animal used its whiskers to explore the gap. The quantification was based on the whisking behavior data from the successful attempts only (Fig. 8). "Tactile Sampling Duration" was defined as the time the whiskers were in contact with the platform during a single "Trial". "Whisker Touch-time" was calculated as the average sampling duration for a single whisker contact (single whisker touch). All contact events were grouped and averaged for each distance separately. Thus, the difference between "Exploration Duration" and "Tactile Sampling Duration" was that the former was the time spent exploring the gap (this duration includes the time when touching and when not touching the platform), whereas the latter measured specifically the time during which the whiskers were in physical contact with the platform. "Tactile Sampling Duration" is thus a subset of "Exploration Duration". Whisker contacts were counted by human observers, who used a custom-written interface to determine whether there was a contact with a target (a platform) on a given frame.

### 2.7. Data analysis and statistics

Data were acquired with Axoclamp 2B (Molecular Devices) and analyzed off-line using pClamp 9 (Molecular Devices). Statistical tests and identification of outliers were performed using GraphPad Prism6 (GraphPad Software, CA, USA). Outliers were identified in the same manner (ROUT method with  $Q = 1\%$ ) for all datasets presented in this article and their removal did not affect overall results. ANOVA analysis with post-hoc tests or unpaired *t*-tests was used to determine statistical significance. Data reported as mean  $\pm$  SEM.

## 3. Results

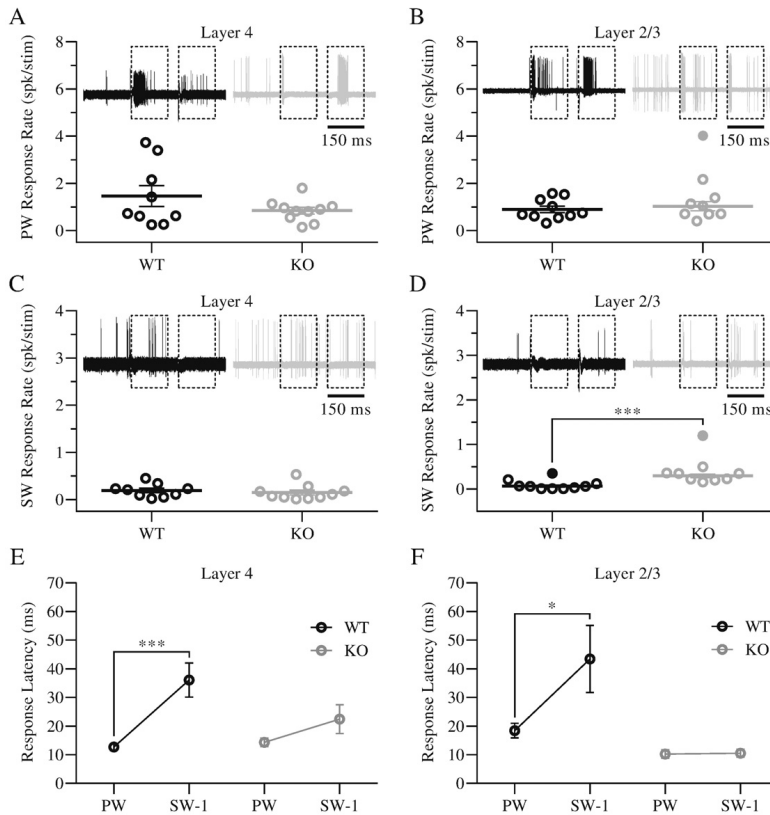
### 3.1. Excitation spread differences in cortico-cortical connections of *Fmr1* KO mice

A characteristic feature of a somatotopic map is the localized activation of a given cortical area in response to stimulation of a specific peripheral body area. In rodents, one cortical area with a distinctive somatotopic organization is the somatosensory barrel cortex (Feldman and Brecht, 2005). Tactile stimulation of each individual whisker evokes a localized activation of the barrel cortex in the area corresponding to this whisker. In the experimental paradigm described below deflection of a given whisker evokes the largest response in a particular barrel column. The whisker that elicits the strongest response in that particular barrel column is called the Principal Whisker (PW). A smaller, but still noticeable, activation can be observed in a PW column when other whiskers from the PW neighborhood are stimulated. The whiskers in the immediate neighborhood of PW are called first order Surrounding Whiskers (SW) (Fig. 1).

To investigate possible hyperexcitation, resulting in a spread of activation over a larger cortical area, we analyzed both the PW- and SW-stimulation-evoked cortical response in the PW column. In our studies we used an *in vivo* juxtacellular-recording technique in anesthetized mice. After placing the recording electrode in the barrel column and obtaining a juxtacellular recording, the whiskers on the mouse snout were mechanically deflected using a piezo-electric stimulator. Subsequently, we stimulated single whiskers connected to this column directly (PW) and indirectly (SW) while recording from the same cell. These were excitatory cells of layer 4 (L4) and layer 2/3 (L2/3) of the somatosensory barrel cortex (see Methods). A graphical representation of this experimental paradigm is presented in Fig. 1.

Whiskers were deflected 50 times at 1 Hz with the piezo-electric stimulator. The evoked-response rate was calculated as the average number of action potentials (spikes) evoked per single whisker deflection. PW was defined as the whisker that evoked the highest number of spikes when deflected. The average response rate for PW stimulation (Fig. 2A, B) was similar (unpaired *t*-test,  $p > 0.2$ ) for KO and WT mice in



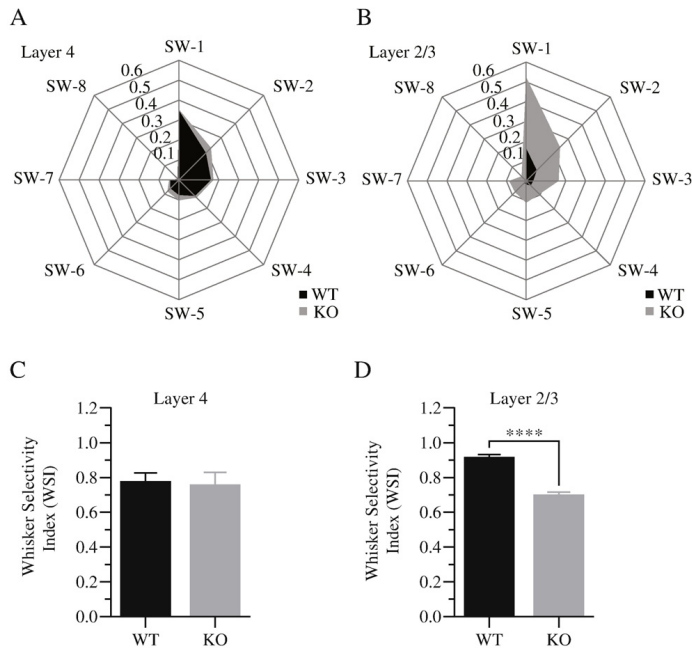


**Fig. 2.** *Fmr1* KO mice have specific changes in the flow of horizontal excitation at the level of somatosensory cortical connections. (A, B) No difference was found in the PW evoked response in cells recorded from L4 ( $n = 9$  for WT,  $n = 10$  for KO,  $p = 0.2143$ ) or L2/3 ( $n = 10$  for WT,  $n = 9$  for KO,  $p = 0.2688$ ). One outlier marked as a grey filled circle was removed from the statistical comparison. (C, D) The average response for SW stimulation was also similar in L4 ( $p = 0.5194$ ), but there was a significant increase in L2/3 for KO mice (\*\*\* $p = 0.0004$ ; two outliers marked as filled circles – one grey and black circle – were removed from the statistical comparison). Inserts in all graphs are *in vivo* electrophysiology recordings from 50 superimposed sweeps (one stimulus train). Each vertical line represents a single spike. The dotted rectangles start at the onset of whisker deflection and mark the 150-ms periods used to calculate the whisker-evoked response. Whisker deflection duration was 200 ms. Unpaired *t*-tests. (E, F) The average response latency to the first spike for PW and SW-1 response recorded in L4 and L2/3 (see [Materials and methods](#) for details). The latency was similar for PW but it was significantly shorter for SW-1 in KO mice in both layers. Two-way repeated measures ANOVA with post-hoc Sidak's multiple comparisons test: \* $p < 0.05$ , \*\*\* $p < 0.001$ .

L4 and L2/3 (L4 KO:  $0.84 \pm 0.15$  spike/stimulus (spk/stim),  $n = 10$  cells; L4 WT  $1.46 \pm 0.45$  spk/stim  $n = 9$ ; L2/3 KO:  $1.03 \pm 0.20$  spk/stim  $n = 8$  [outlier at 4.02 spk/stim excluded]; L2/3 WT  $0.90 \pm 0.14$  spk/stim,  $n = 10$ ). The PW response can be an indicator of a vertical transmission of excitation within barrel columns. In contrast, the SW response depends mostly on horizontal transmission across barrel columns (Armstrong-James et al., 1992; Feldmeyer et al., 2002; Laaris and Keller, 2002; Schubert et al., 2003). There was no difference in L4 in the SW response rate either (KO:  $0.15 \pm 0.05$  spk/stim,  $n = 10$ ; WT:  $0.19 \pm 0.05$  spk/stim,  $n = 9$ ; unpaired *t*-test,  $p > 0.5$ ) (Fig. 2C). On the other hand, in L2/3 the groups differed significantly (Fig. 2D). The average response rate for KO was five times higher than for WT control mice (KO:  $0.30 \pm 0.04$  spk/stim,  $n = 8$ ; WT:  $0.06 \pm 0.02$  spk/stim,  $n = 9$ ; unpaired *t*-test,  $p = 0.0004$  [outliers excluded: at 0.35 spk/stim in WT; at 1.20 spk/stim in KO]). To analyze if not only the response rate but, also, the timing of spikes was affected, we calculated the PW and SW

response latency – the average latency to the first spike (see [Materials and methods](#) for details). We found that the SW latency was two-three times longer than the PW latency in WT animals in both layers (L4: PW =  $12.68 \pm 1.19$  ms, SW =  $36.09 \pm 5.96$  ms,  $n = 9$ ; L2/3: PW =  $18.43 \pm 2.54$  ms, SW =  $43.42 \pm 11.72$  ms,  $n = 10$ ) (Fig. 2E, F). In contrast, in KO animals there was not a major difference between the PW and SW latency (L4: PW =  $15.30 \pm 1.66$  ms, SW =  $22.44 \pm 5.00$  ms,  $n = 8$ ; L2/3: PW =  $10.23 \pm 1.49$  ms, SW =  $10.56 \pm 1.40$  ms,  $n = 9$ ) (Fig. 2E, F). Statistical analysis confirmed this observation (two way repeated measures ANOVA, L4: genotype:  $p = 0.1860$ , whisker:  $p = 0.0008$ , interaction:  $p = 0.0550$ ; Sidak's multiple comparisons test WT: PW-SW,  $p < 0.0005$ ; KO: PW-SW,  $p > 0.05$ ; L2/3: genotype:  $p = 0.0065$ , whisker:  $p = 0.0565$ , interaction:  $p = 0.0626$ ; Sidak's multiple comparisons test WT: PW-SW,  $p < 0.05$ ; KO: PW-SW,  $p > 0.05$ ).

A graphical representation of the relatively larger SW response in KO L2/3, but not in KO L4 is shown in the Fig. 3A, B. Normalizing the SW



**Fig. 3.** *Fmr1* KO mice display impairments in information tuning in L2/3 of somatosensory cortex. (A, B) Responses recorded from the cells located in PW column when stimulating corresponding SW. In the spider web graphs responses to SW stimulation are in order from highest to lowest whisker-stimulation-evoked response (SW-1 to SW-8, respectively). Numbers show the ratio of SW response/PW response. No noticeable difference was observed in L4 ( $n = 10$  for KO,  $n = 9$  for WT) but the excitation spread was visibly larger in L2/3 ( $n = 9$  for KO,  $n = 10$  for WT) in KO mice. (C, D) The Whisker Selectivity Index ( $WSI = 1 - (SW / PW)$ ) is a measure of how selective the stimulation of one whisker is to one given barrel column. In L4 there was no difference between WT and KO ( $p = 0.8554$ ), whereas in L2/3 in KO mice the WSI was significantly lower ( $****p < 0.0001$ ). The lower WSI in KO mice means that, the PW stimulation activates more of the surround barrel columns compared to WT mice. Unpaired *t*-tests.

response to the PW response, shows that in L4 the WT and KO responses are similar (similar area in Fig. 3A). In contrast, in L2/3 the SW response in KO is relatively larger compared to WT (larger area in Fig. 3B). This increased SW response rate in L2/3 along with shorter SW latency in both layers of *Fmr1* KO mice is an indicator of increased excitatory spread in cortico-cortical connections or due to subcortical changes (Kwegyir-Afful et al., 2005).

### 3.2. Impaired information tuning in L2/3 of *Fmr1* KO mice

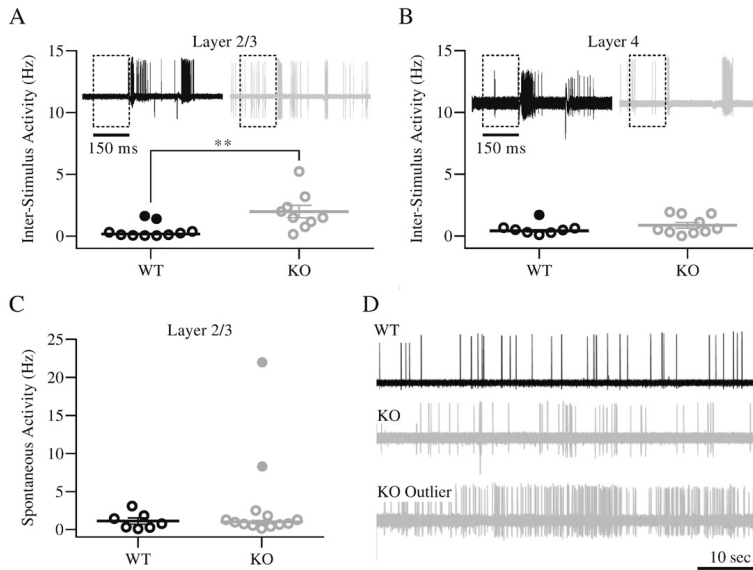
Under normal conditions cortical response to the PW stimulation is much higher compared to the response to the SW stimulation (Armstrong-James and Fox, 1987). However, in pathological conditions, the cortical localization of the excitation and its spread can be changed. Thus, the difference in the cortical response to the PW and the SW deflection may be smaller, making it more difficult to identify a PW based on the evoked-response measurements and, likely, more difficult for an animal to differentiate between deflections of different whiskers. To analyze how cortical activation is localized in response to whisker movement (i.e., PW column versus SW column) we defined a new parameter called Whisker Selectivity Index (WSI).

The WSI was calculated as  $WSI = 1 - (SW / PW)$ . The WSI varies between 1 and 0; "0" means that the cell responded equally well to stimulation of the PW and all of the SW (by definition  $PW \geq SW$ ), and "1" means a response localized to the PW column only. In line with the

findings on the whisker-stimulation-evoked response, in L4 the WSI ratio was similar between WT and KO suggesting that there is no difference in the response localization (KO:  $WSI = 0.75 \pm 0.08$ ,  $n = 10$ ; WT:  $WSI = 0.77 \pm 0.06$ ,  $n = 9$ ; unpaired *t*-test,  $p > 0.8$ ; Fig. 3C). However, a significantly reduced WSI was observed in L2/3 of KO mice (KO:  $WSI = 0.69 \pm 0.03$ ,  $n = 9$ ; WT  $WSI = 0.91 \pm 0.03$ ,  $n = 10$ ; unpaired *t*-test,  $p < 0.0001$ ) (Fig. 3D). These differences in the WSI, together with the differences in the evoked-response rates, may be a sign of impaired information tuning, in the sense that animals may be unable to discriminate accurately between deflections of different whiskers.

### 3.3. Increased inter-stimulus activity in L2/3 of *Fmr1* KO mice

Further indication that in *Fmr1* KO animals the L2/3 cells are more active can be inferred from recordings of spiking activity in the absence of whisker deflections. We analyzed this activity in two ways. Firstly, we measured cells' "Inter-stimulus Activity" as the average number of action potentials occurring during the 150-ms period immediately preceding each of the whisker deflections in a stimulus train (see Methods). Secondly, we recorded "Spontaneous Activity" from 1-min recordings immediately preceding a whisker stimulation train. The difference between these two measurements is that the Spontaneous Activity is a measure of activity when the system is not activated by whisker deflections, the Inter-stimulus Activity may more closely reflect



**Fig. 4.** Inter-stimulus Activity in L2/3 somatosensory cortex of *Fmr1* KO mice is increased when the whisker system is activated. (A, B) The Inter-stimulus Activity in KO mice is significantly increased in L2/3 (\*\* $p = 0.0067$ ) but not in L4 ( $p = 0.3279$ ) (WT L2/3:  $n = 10$ , KO L2/3:  $n = 9$ ; filled black circles are outliers not included in the statistical analysis; WT L4:  $n = 9$ ; KO L4:  $n = 10$ ). Inter-stimulus Activity is the average spiking frequency during a 150-ms period preceding each stimulus in a whisker stimulation train (the dotted rectangles). Inserts are 25 superimposed single sweeps from the electrophysiology recordings of spikes evoked by PW stimulation. (C) Spontaneous Activity did not differ significantly between WT and KO mice (WT:  $n = 7$ ; KO:  $n = 13$ ; two outliers marked as grey filled circles were removed from the statistical comparison;  $p = 0.8159$ ). Spontaneous Activity was calculated as the average from 1-minute recordings taken before the stimulation protocol. Spontaneous Activity was measured when the whisker system is not activated by whisker deflections, whereas Inter-stimulus Activity was recorded during a train of whisker stimulations. The two different types of measurements thus reflect different states of activation. (D) Raw traces from the L2/3 Spontaneous Activity recordings, the upper trace from a WT mouse, the middle from a KO mouse, and the bottom recording shows an outlier from a KO mouse. Unpaired  $t$ -test.

the activity in the system during sensory processing (Sachdev et al., 2004).

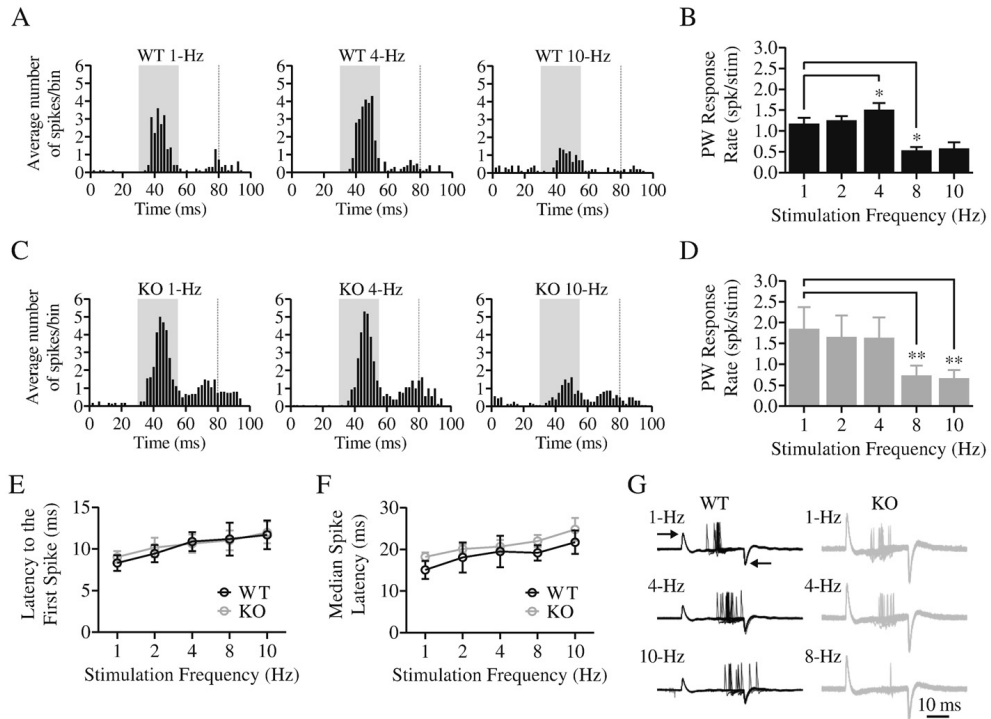
We show (Fig. 4A) that in the L2/3 excitatory cells of KO mice the Inter-stimulus Activity was significantly higher compared to WT control mice (KO:  $1.99 \pm 0.50$  Hz,  $n = 9$  and WT:  $0.16 \pm 0.05$  Hz,  $n = 8$ ; unpaired  $t$ -test,  $p = 0.0067$  [outliers at 1.41 and 1.65 Hz removed]). In line with our finding that the whisker-stimulation-evoked responses in L4 were similar in KO and WT animals, the Inter-stimulus Activity was also similar (KO:  $0.87 \pm 0.23$  Hz,  $n = 10$ ; WT:  $0.42 \pm 0.08$  Hz,  $n = 7$ ; unpaired  $t$ -test,  $p = 0.3279$  [outlier at 2.63 Hz removed]) (Fig. 4B). The Spontaneous Activity (the activity before the onset of the whisker stimulation train) in L2/3 (Fig. 4C, D) was also similar between KO and WT animals (KO:  $1.00 \pm 0.21$  Hz,  $n = 11$ ; WT:  $1.11 \pm 0.41$  Hz,  $n = 7$ ; unpaired  $t$ -test,  $p = 0.8159$  [outliers at 2.2 and 8.3 Hz removed]). The significantly increased Inter-stimulus Activity may be caused by an increase in sub-threshold activity occurring during whisker deflections (Moore and Nelson, 1998). They presumably sum up leading to more spiking when the whisker system is being activated. In contrast, in the silent state (preceding the whisker stimulation train) when Spontaneous Activity was recorded, a difference between WT and KO was less prominent (with the exception of two KO cells).

### 3.4. Altered frequency coding in *Fmr1* KO mice

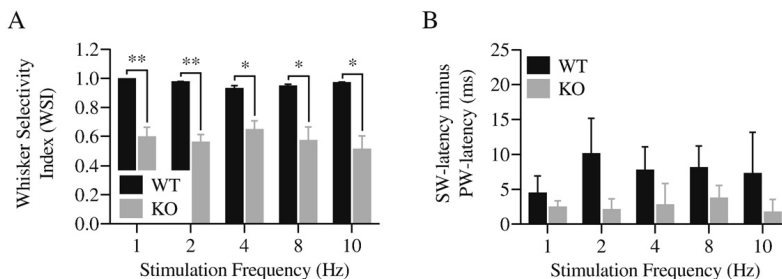
Whiskers can be actively moved with various frequencies, and vibrate when touching objects (Berg and Kleinfeld, 2003; Carvell and Simons, 1990; Grant et al., 2009). Different frequencies can convey specific information, such as the shape or the texture of the object being

touched. Therefore, next we tested whether *Fmr1* KO mice, which we had showed to have increased activity and spread of excitation at the level of L2/3 pyramidal cells, also show impairments in frequency coding of somatosensory information. The average PW response to 1-, 4- and 10-Hz whisker stimulation is shown as a PSTH (peristimulus time histograms) in Fig. 5A and C (this is the average of all recorded cells). In L2/3 pyramidal cells of WT mice (Fig. 5B), the stimulation-evoked response rate increased significantly between 1- and 4-Hz (1-Hz:  $1.12 \pm 0.20$  spk/stim; 2-Hz:  $1.19 \pm 0.16$  spk/stim; 4-Hz:  $1.45 \pm 0.23$  spk/stim; one-way RM ANOVA,  $p = 0.0118$ ) and then dropped when comparing 1- to 8- and 10-Hz stimulation (8-Hz:  $0.47 \pm 0.14$  spk/stim; 10 Hz:  $0.52 \pm 0.21$  spk/stim;  $n = 7$ ; one-way RM ANOVA,  $p = 0.0285$ ). In contrast, in KO animals (Fig. 5D), there was no significant difference in the stimulation-evoked response between 1- and 4-Hz (1-Hz:  $1.79 \pm 0.58$  spk/stim; 2-Hz:  $1.59 \pm 0.58$  spk/stim; 4-Hz:  $1.58 \pm 0.55$  spk/stim;  $n = 13$  cells; one-way RM ANOVA,  $p = 0.2177$ ). Nevertheless, the response to 8- and 10-Hz stimulation dropped significantly (8-Hz:  $0.68 \pm 0.29$  spk/stim; 10-Hz:  $0.61 \pm 0.25$  spk/stim;  $n = 13$ ; one-way RM ANOVA,  $p = 0.0056$ ), similarly to WT animals. These results imply that KO animals have deficiencies in coding various whisker movement frequencies. Possibly, the L2/3 pyramidal cells of these animals reach a response plateau sooner compared to WT control animals.

In addition to analyzing the number of spikes per stimulus in PW response, we calculated the average latency to the first spike (PW latency to the first spike, see Materials and methods for details) (Fig. 5E, G). The PW latency to the first spike was similar in WT and KO mice, and in both cases the latency increased with increasing stimulation frequency (two-way RM ANOVA, frequency:  $p = 0.0036$ ; genotype:  $p = 0.8621$ ,



**Fig. 5.** *Fmr1* KO mice show impaired frequency encoding mechanisms. (A, C) The average PW response for 1-, 4- and 10-Hz whisker stimulation is shown as a PSTH (peristimulus time histogram) with 2 ms bins. Data show the total number of spikes recorded on average from a cell in response to 25 whisker deflections (in A, 7 cells; in C, 13 cells). The grey area marks the duration of the whisker deflection. Whisker responses were calculated for 50 ms from the stimulation onset (from 30 ms until 80 ms, dotted grey line). In WT, but not in KO mice the PW response increased from 1- to 4-Hz whisker deflection. This data is summarized and quantified in B and D. (B) In WT mice ( $n = 7$ ) there is a gradual increase in the response up to 4-Hz, followed by a drop in the response rate at 8- and 10-Hz (Dunnett's multiple comparisons test;  $p < 0.05$ ). (D) In contrast to WT in KO mice ( $n = 13$ ), there was no increase in response rates up to 4-Hz-whisker-stimulation. Similar to WT, there was a drop in the response at 8-Hz- and 10-Hz-stimulation (Dunnett's multiple comparisons test;  $p < 0.005$ ). (E, F) In both WT and KO mice the latency to the first spike as well as the median spike latency evoked by the PW stimulation, increased with increasing stimulation frequency and this trend was visible in both WT and KO mice (two-way RM ANOVA, frequency;  $p = 0.0036$  and  $p = 0.0040$ , respectively). (G) Examples of raw recordings used for the latency analysis. Each of the six traces show 25 superimposed sweeps of 1-, 4-, 8- or 10-Hz-stimulation. Arrows mark whisker stimulation artefact.



**Fig. 6.** Whisker selectivity deficit in *Fmr1* KO mice is independent of the whisker-stimulation-frequency. (A) In L2/3 pyramidal cells there was a decreased Whisker Selectivity Index (WSI) in KO ( $n = 13$ ) compared to WT ( $n = 6$ ) at all tested stimulation frequencies (1- to 10-Hz). This indicated that SW deflections evoked a relatively larger response in KO L2/3 pyramidal cells, which could contribute to an overexcitation and thus likely a less specific encoding of the exact whisker that was stimulated. Two-way repeated measures ANOVA with post-hoc Sidak's multiple comparisons test;  $p < 0.05$ ,  $p < 0.005$ . (B) The lack of input specificity was also manifested in the response latency to the first spike. In WT it is longer for the SW compared to the PW stimulation, in KO mice it was the same for PW and SW (two-way ANOVA, genotype,  $p = 0.005$ ; frequency,  $p = 0.8547$ ; interaction,  $p = 0.8342$ ).

interaction:  $p = 0.9613$ ). In addition to the importance of single spikes for encoding (Foffani et al., 2009; Panzeri et al., 2001), there is evidence (Fasshi et al., 2014) that during a tactile perception task rodents integrate sensory information during the entire time of the presented stimuli. Thus, the overall spiking during the entire stimulation is important in the encoding process as well. For this reason, we calculated not only the average latency to the first spike but also the median latency of the PW response (PW median spike latency). The latencies increased in both WT and KO with increasing stimulation frequency (two-way RM ANOVA, frequency:  $p = 0.0040$ , genotype:  $p = 0.3187$ , interaction:  $p = 0.9712$ ) (Fig. 5 F, G). Combining the findings of a decreased WSI (Fig. 3D) and the deficiency in frequency coding based on the evoked-response rate (Fig. 5D), we next investigated whether the alteration in WSI, and the difference between response latency for the PW and SW, was dependent on the frequency of whisker deflection.

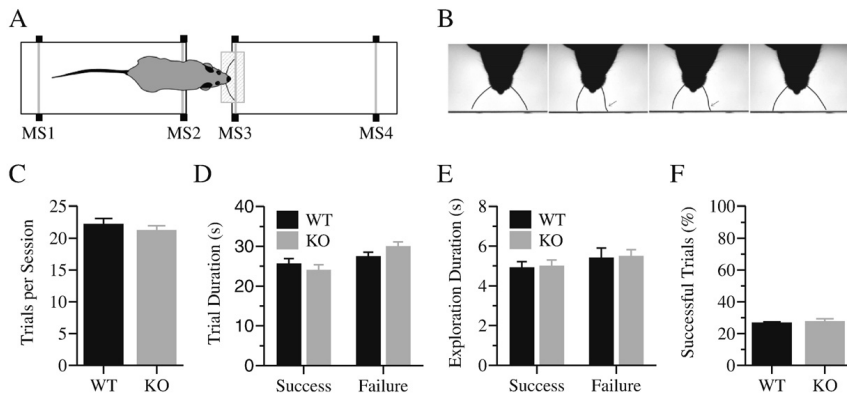
### 3.5. Increased receptive fields cause problem in encoding stimulation frequency

The whisker-stimulation-evoked response varied considerably more for 8- and 10-Hz stimulation as compared to 1- to 4-Hz-stimulation (coefficient of variation for 8- and 10-Hz was 2–4 times higher). Therefore, we analyzed data from 1- to 4-Hz-stimulation and 8- to 10-Hz-stimulation separately. We found that the mean WSI was decreased significantly in KO ( $n = 13$ ) in comparison to WT cells ( $n = 6$ ) in response to 1- to 4-Hz-stimulation (Fig. 6A; two-way RM ANOVA, genotype:  $p = 0.0019$ , frequency:  $p = 0.7239$ , interaction:  $p = 0.1546$ ). At 8- and 10-Hz-stimulation the mean WSI was also statistically smaller in KO (Fig. 6A; two-way RM ANOVA, genotype:  $p = 0.0017$ , frequency:  $p = 0.8681$ , interaction:  $p = 0.7168$ ). We observed that already at 8-Hz cells were less likely to spike. The same was true for 10-Hz-whisker-stimulation causing a larger variability in response rate. A further measure of the lack of input specificity, measured over different deflection frequencies, is that in WT, but not in KO, the response latency (measured as latency to the first

spike) was shorter for the PW than for the SW response (Fig. 6B). The difference in latencies was quantified by taking the SW response latency minus the PW response latency. In WT the SW response, averaged over all deflection frequencies, was  $9.8 \pm 0.12$  ms after the PW response, whereas in KO the response latency was not statistically different ( $2.3 \pm 0.05$  ms, two-way ANOVA; Fig. 6B). This part of our studies showed that SW stimulation caused comparatively large activation of recorded cells over a wide range of whisker deflection frequencies, and hence reduction in the WSI. This effect can be interpreted as an increased receptive field size (Feldman and Brecht, 2005; Fox et al., 2000) of these cells and it may underlie problems with encoding stimulation frequencies.

### 3.6. *Fmr1* KO mice show an altered whisker exploration strategy in the gap-crossing task

We tested the animals in a whisker-dependent behavioral task to investigate what consequences the map expansion could have on sensory processing of whisker mediated information. Rodents use their whiskers for tactile exploration of their environment; therefore analyzing mouse behavior in a whisker-dependent task can reveal changes in somatosensory processing. *Fmr1* KO and WT control animals were examined in the gap-crossing task, a tactile-dependent behavioral test designed to investigate sensory-motor learning capabilities (Celikel and Sakmann, 2007; Hutson and Masterton, 1986). In this task, animals were trained for 8 days to cross a gap between two plastic platforms (Fig. 7A, B). The gap distance was progressively increased once the animal learned how to cross the gap (see Methods). KO and WT control mice (KO:  $n = 12$ , WT:  $n = 12$  animals) performed this task equally well with no significant difference in any parameters of task performance (unpaired *t*-test): “Trials per Session” ( $p = 0.56$ ; Fig. 7C), “Trial Duration” ( $p = 0.29$ ; Fig. 7D), “Exploration Duration” ( $p > 0.8$ ; Fig. 7E), and “Successful Trials” ( $p = 0.55$ ; Fig. 7F). We used “Trials per Session” and “Trial Duration” to describe general locomotor activity but

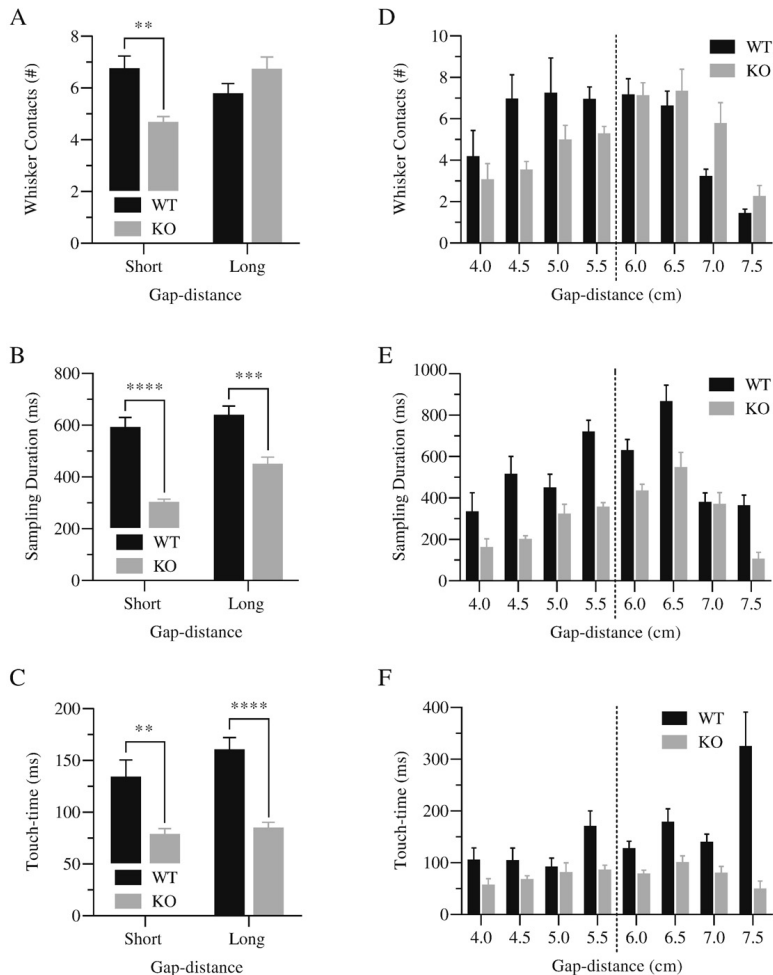


**Fig. 7.** *Fmr1* KO mice performance in a whisker-dependent behavioral task. (A) The gap-crossing test apparatus: the two rectangles are plastic platforms and the 8 black squares are infrared motion sensors (MS). The animal uses touch, including whisker touch, to localize the opposite platform and subsequently crosses the gap to reach the opposite platform. When the animal crosses the line between detectors (in grey) a movement is registered. Data from the motion sensors are quantified in panels C, D, E, and F. (B) A video camera was used to collect information on whisker touches of the opposite platform when the mouse is exploring the gap. The video frames show (from left to right) how the animal approaches the platform with its whisker (frame 1) and then (frame 2 and 3) touches the platform. The arrow points to the bending of the whisker, that occurs during its contact with the platform. In the last frame (frame 4) the whiskers have detached from the platform and will be retracted. The whiskers are marked in black to improve visualization. In the Fig. 2 the whisker contacts were quantified. (C, D, E, F) Motion sensor data collected during the gap-crossing task were compared between WT ( $n = 12$ ) and KO ( $n = 12$ ) mice. No significant differences were found in any measure: (C) number of Trials per Session ( $p = 0.56$ ); (D) Trial Duration when the mouse crosses the gap (success,  $p = 0.55$ ) or decides not to cross (failure,  $p = 0.29$ ); (E) Exploration Duration, the time that a mouse was active in the gap area ( $p = 0.89$  for success;  $p = 0.92$  for failure). (F) percentage of Successful Trials, i.e., trials when the mouse crosses the gap ( $p = 0.81$ ). Unpaired *t*-tests.

also, more specifically, as indicators of the animal's behavioral strategy to solve the gap-crossing task. "Exploration Duration" was used to depict the time that animal spent exploring the gap area and "Successful Trials" to report the percentage of attempts in which the mouse crossed the gap.

The gap-crossing task is not only a behavioral task used to study exploratory locomotor behavior but can also be used to analyze whisker kinematics which is important for understanding how tactile sensory

information is acquired. To learn more about whisking behavior, we analyzed the following parameters: "Whisker Contacts" made with the target platform; "Tactile Sampling Duration" defined as the average time that the whiskers made physical contact with a platform; and "Touch-time", average duration of a single whisker touch. Note that the measure of "Exploration Duration" refers to the time the mouse spends exploring the gap area so it is a measure of animal movement. Whereas, "Tactile Sampling Duration" refers specifically to how the



**Fig. 8.** *Fmr1* KO mice show decreased whisker touching during the gap-crossing task. (A, B, C) Whisker kinematics data were analyzed in two categories: short-gap distances (4- to 5.5-cm;  $n = 90$  for KO,  $n = 92$  for WT) and long-gap distances (6.0- to 7.5-cm;  $n = 114$  for KO,  $n = 112$  for WT). (A) The number of Whisker Contacts during tactile sampling varied significantly between WT and KO mice for shorter gap-distances ( $^{**}p = 0.001$ ) and was not significantly different for longer gap-distances ( $p = 0.1727$ ). (B) Tactile Sampling Duration, the average time of physical contact between a whisker and a platform during a single trial, was significantly reduced in KO mice in both categories ( $^{***}p < 0.0001$  for short-gap-distances;  $^{***}p = 0.0005$  for long-gap-distances). (C) Similarly, the average duration of a single whisker contact during the tactile sampling (Touch-time) was significantly shorter in KO mice in both categories ( $^{**}p = 0.0051$  for short gap-distances;  $^{****}p < 0.0001$  for long gap-distances). (D, E, F) All whisker kinematics data are presented as the averages for each gap-distance separately: number of Whisker Contacts (D), Tactile Sampling Duration (E), and whisker Touch-time (F). Vertical dashed lines divide gap-distances for the shorter and the longer ones as presented in panels A, B, and C. Unpaired *t*-tests.



mouse uses its whiskers to contact objects. The “Tactile Sampling Duration” is a subset of the “Exploration Duration” since the mouse makes whisker contact (“Tactile Sampling Duration”), during part of the time that it spends exploring the gap (“Exploration Duration”). Because mice in their tactile-exploration process use not only whiskers, but also their nose (Celikel and Sakmann, 2007), we sorted the whisker kinematics data into two categories: short gap-distances (4.0 to 5.5 cm; number of successful trials:  $n = 90$  for KO,  $n = 92$  for WT) that are reachable for both whisker and nose exploration, and long-gap distances (6.0 to 7.5 cm; number of successful trials:  $n = 114$  for KO,  $n = 112$  for WT) that are unreachable for nose exploration (whisker exploration only). It was evident from our data that, for the short gap-distances, KO mice made significantly fewer whisker contacts (KO:  $4.6 \pm 0.28$  versus WT:  $6.7 \pm 0.55$ ;  $p = 0.001$ ; Fig. 8A); spent less time engaged in whisker sampling (Tactile Sampling Duration:  $295.5 \pm 18.78$  ms for KO and  $584.3 \pm 45.25$  ms for WT;  $p < 0.0001$ ; Fig. 8B); and had shorter average whisker touch-time (KO =  $77.2 \pm 7.1$  ms, WT =  $132.4 \pm 18.02$  ms;  $p = 0.0051$ ; Fig. 8C) than their WT littermates (unpaired  $t$ -tests). In contrast, the number of whisker contacts made was not statistically different between the two groups of mice for the long gap-distances (KO =  $6.7 \pm 0.53$ , WT =  $5.7 \pm 0.45$ ; unpaired  $t$ -test,  $p = 0.1727$ ; Fig. 8A). Nevertheless, we observed the same changes for the other two parameters (Tactile Sampling Duration:  $442.0 \pm 34.63$  ms for KO and  $632.6 \pm 41.32$  ms for WT;  $p = 0.0005$ ; and Touch-time:  $83.2 \pm 7.06$  ms for KO and  $158.7 \pm 13.46$  ms for WT, unpaired  $t$ -test,  $p < 0.0001$ ; Fig. 8B, C). Data for each gap-distance are presented in separate graphs (“Whisker Contacts” in Fig. 8D; “Tactile Sampling Duration” in Fig. 8E; “Touch-time” in Fig. 8F).

Reduced tactile sampling duration together with shorter exploration could indicate a shorter duration of sensory integration necessary to make a decision to cross, or not cross the gap. However, since we observed reduced whisker sampling but normal exploration duration in *Fmr1* KO mice, we interpreted the decreased whisking as a defect in the sensory processing of whisker information rather than that the animals need less tactile information to solve the task. Specifically, we hypothesized that information from one whisker may cause hyperexcitation in the sense that a larger cortical area is activated.

#### 4. Discussion

*In vivo* recordings from barrel cortex revealed that *Fmr1* KO mice show an enlargement in the cortical area activated by whisker deflections, i.e., an expansion of the somatosensory map in L2/3. Further recordings revealed impairments in frequency encoding of somatosensory tactile information. These findings highlight neuronal mechanisms that could contribute to the different exploratory behavior observed in *Fmr1* KO mice (Arnett et al., 2014; Santos et al., 2014). Adversity to touch, manifested as “tactile defensiveness” or “tactile sensitivity” (Baranek et al., 1997; Baranek et al., 2008; Miller et al., 1999; Reiss and Freund, 1990), is a common symptom of FXS (Hagerman et al., 1991), ASD and related diseases (Weber and Newmark, 2007). In mice, tactile information is received through deflections of their whiskers for further processing in the somatosensory barrel cortex (Diamond and Arabzadeh, 2013; Diamond et al., 2008; Feldmeyer et al., 2013). Whisking is an important part of mouse social interaction (Arai and Erzurumlu, 2015; Brecht and Freiwald, 2012; Sofroniew and Svoboda, 2015) and studies show an altered social behavior in *Fmr1* KO mice (McNaughton et al., 2008; Santos et al., 2014; Sorensen et al., 2015). The physiological alterations in the primary barrel cortex reported in this study are thus mechanisms that could contribute to deficits in touch perception present in FXS patients. Moreover, because *Fmr1* KO mice display similar deficiencies also in other sensory modalities, the deficiencies observed in the somatosensory system on the single cells/circuit level may share some common mechanisms with other sensory disruptions. Finally, not only FXS but also other autistic syndromes have been characterized by hyperexcitable cortical networks

(Markram and Markram, 2010), so attempts in understanding sensory processing mechanisms may bring us closer to common therapeutic strategies designed for a wider application.

##### 4.1. Network excitability and UP states

Previous *in vivo* multiunit recordings from somatosensory cortex in *Fmr1* KO mice under urethane anesthesia have shown that L4 and L5 excitatory neurons have longer duration of spontaneous UP states (Hays et al., 2011). Although only the duration but not the spiking frequency was increased, the extended UP states may cause a general increase in neuronal excitability. On the other hand, based on *in vivo* whole-cell recordings from the unanesthetized *Fmr1* KO mice, Gonçalves et al. (2013) reported that duration of the UP states in L2/3 was not increased. However, they report fewer silent UP states in KO mice, with a significant increase in a firing probability during the active UP states. Therefore, it seems that the duration and the frequency of firing during the active UP states can change independently. Additionally, Motanis and Buonanno (2015) in their whole-cell recordings on organotypic slices from *Fmr1* KO mice found no change in duration but a delay in the emergence of spontaneous UP states. In conclusion, the connection between network excitability manifested as UP states and the impact of UP states on activity in somatosensory barrel cortex remains to be investigated. Although our experiments did not reveal any changes in the average Spontaneous Activity in L2/3 of *Fmr1* KO mice, there were some neurons firing with a much higher rate than most of the cells (“outliers”). Some neurological diseases have a population component, meaning that although the activity is not abnormal in every cell, there are sufficiently many cells that are affected to change the network activity, thus contributing to the disease symptoms.

##### 4.2. Increased inter-columnar activation in the somatosensory cortex

To unravel the physiological mechanisms underlying reduction in whisking behavior, we analyzed cortical activity in the somatosensory barrel cortex of *Fmr1* KO and WT mice. Previous *in vivo* recordings from somatosensory cortex in *Fmr1* KO mice have shown that L4 cells have longer duration UP states. Furthermore, it was argued that these extended UP states may cause a general increase in neuronal excitability (Hays et al., 2011). In *Fmr1* KO mice the prolonged duration of UP states in L4 and the diffuse L4 to L2/3 axonal projections (Bureau, 2009) can be expected to affect responses in L2/3. *In vivo* studies from L2/3 in *Fmr1* KO mice accordingly showed higher firing rates in these cells during both UP and DOWN states (Gonçalves et al., 2013). In normal conditions, the main spread of excitation between cortical barrel columns occur at the level of L2/3 rather than L4 (Adesnik and Scanziani, 2010; Feldmeyer, 2012; Schubert et al., 2007). In accordance with these data, we found that excitatory cells activity was increased in response to SW deflection specifically in L2/3 but not in L4 (Fig. 2C, D). Additionally, *in vitro* data from Bureau et al. (2008) provide evidence for the abnormal diffusion of L4 to L2/3 axonal projections into neighboring barrels that may explain the change in the WSI observed in L2/3 in KO mice (Fig. 3A, B) but also a specific decrease of the SW response latency (Fig. 2E, F). Although the alterations in the synapse formation reported by Bureau et al. (2008) were transient in nature, their consequences may persist into adulthood, as has been shown for deficiencies in the expression of other developmentally crucial proteins (Greenhill et al., 2015a). It is during development that a functional balance between excitatory and inhibitory synapses is established (Turrigiano and Nelson, 2004), and it is maintained throughout life due to homeostatic plasticity processes (Turrigiano and Nelson, 2000). In recent years many studies have revealed a very exciting link between disruptions in synaptic homeostasis and various diseases of the nervous system, e. g. FXS, ASD, but also schizophrenia or epilepsy (Eichler and Meier, 2008; Wondolowski and Dickman, 2013). Because neuronal firing rate is one of the key modulators in homeostatic plasticity, the changes observed

in our studies on the mouse model for FXS, may be also considered in this wider context.

#### 4.3. Impairments in encoding of somatosensory information

The resulting imbalance in excitation-inhibition dynamics, present in FXS, has been suggested to underlie described deficits in brain development and cognitive functions (Bear et al., 2004; Gibson et al., 2008; Gonçalves et al., 2013; van der Molen et al., 2014). The increased activation evident in *Fmr1* KO mice may be the result of stronger excitatory inputs (Olmos-Serrano et al., 2010) and/or a reduced activity or number of GABA-releasing interneurons (D'Hulst et al., 2006; Gibson et al., 2008; Paluszkiwicz et al., 2011). Arguing for an involvement of GABAergic interneurons is also that blocking GABA receptors in the barrel cortex has been shown to cause a similar broadening of the receptive field in wild-type rodents (Kyriazi et al., 1996a; Kyriazi et al., 1996b; Petersen and Sakmann, 2001). Although a cortical change in inhibition is likely to contribute to the map expansion reported for the *Fmr1* KO mice, it is not likely to be the sole cause of the effect. This is because blocking inhibition in wild-type mice not only increases the response to SW deflection, but also increases the PW response *per se* (Foeller et al., 2005; Kyriazi et al., 1998), and an increase of the PW response was not seen in *Fmr1* KO mice, but the effect was rather an increase only in the SW response. Furthermore, morphological changes are observed in the length and shape of dendritic spines of excitatory neurons (Galvez et al., 2003; Hinton et al., 1991; Irwin et al., 2000) as well as in the size of the soma, number and immunoreactivity of GABA-releasing interneurons (Selby et al., 2007).

Somatosensory information is encoded by the changes in the mean cortical response delay (latency) and firing rate (frequency) (Ahissar et al., 2001; Kleinfeld et al., 2006). In addition, the whisker deflection frequency determines the spatial extent of cortical activation, such that there is a sharpening of the receptive field with increased stimulation frequency (Moore, 2004). We studied these spatio-temporal encoding mechanisms and found that in both *Fmr1* KO and their WT littermates (Fig. 5E, F) the latency increased with stimulation frequency. Furthermore, in the WT control group we observed an increase in L2/3 pyramidal cell spiking for whisker stimulations up to 4-Hz, and then a subsequent decrease at 8- and 10-Hz whisker stimulation (Fig. 5A, B). In contrast in *Fmr1* KO mice, the whisker-evoked response did not change between 1-Hz and 4-Hz whisker stimulation (Fig. 5C, D). This result implies that fundamental mechanisms underlying encoding of sensory information from the whisker are impaired. The changes observed in cortical processing may be underlying mechanisms contributing to the abnormalities seen in the whisker-dependent gap-crossing task (Figs. 7, 8).

#### 4.4. Abnormal receptive fields in *Fmr1* KO mice

In addition to impaired spike frequency modulation with increasing stimulation frequencies the cortical areas activated by whisker stimulation were larger in *Fmr1* KO mice. This observation was quantified by calculating a whisker selectivity index (WSI). The decrease in WSI evident in *Fmr1* KO mice (Figs. 3, 6) indicates that the specificity with which deflection of a given whisker activates cortex has decreased. It should be noted that the WSI was determined for different stimulation frequencies, and for different durations of whisker deflection. This fact, that the WSI decreased over a range of stimulation parameters, would imply that one factor underlying the increased cortical excitability is a shift in the whisker response threshold (Zhang et al., 2014). Correct processing of whisker-mediated touch information requires the formation of receptive fields in the somatosensory cortex (Simons, 1978; Simons and Carvell, 1989). Development of intra-cortical connections plays a key role in the formation of the receptive fields and depends on sensory experience (Allen et al., 2003; Bender et al., 2006). Therefore, the abnormal receptive fields in *Fmr1* KO mice (Figs. 3 and 6A) may be a direct

consequence of altered sensory integration already in the early postnatal weeks. It is likely that sensory processing of tactile information cannot shape developing circuits in the appropriate way, taking into account, in addition to the impaired UP states dynamics (Gibson et al., 2008; Motanis and Buonomano, 2015), a decreased spatial precision of whisker-stimulation-evoked response in all tested frequencies (Fig. 6A), and disruptions in the temporal spike precision (Fig. 6B). The altered cortical activity may interfere with the formation of connections in the early somatosensory circuit affecting neuronal plasticity mechanisms throughout life (Harlow et al., 2010; Meredith and Mansvelder, 2010; Nimchinsky et al., 2001). To sum up, changes in cortical connectivity, cell morphology and neural plasticity may be caused by abnormal developmental processes occurring during the pre-natal or early post-natal periods (Braun and Segal, 2000; La Fata et al., 2014; Padmashri et al., 2013; Patel et al., 2014).

#### 4.5. Tactile information processing in a whisker-dependent task

In mice, tactile information is received through deflections of their whiskers for further processing in the somatosensory barrel cortex (Diamond and Arabzadeh, 2013; Diamond et al., 2008; Feldmeyer et al., 2013). We used a whisker-dependent task, the gap-crossing task (Harris et al., 1999; Hutson and Masterton, 1986; Papaioannou et al., 2013), to analyze somatosensory processing in *Fmr1* KO mice. These mice appeared to have no problems solving the task, i.e., crossing the gap between the platforms (Fig. 7); nevertheless, when exploring the gap area, they made significantly fewer whisker contacts at shorter gap-distances, in addition to reduced sampling duration time and whisker touch-time at all gap-distances (Fig. 8). In catch trials, trials where the platform is out of reach from the animal's whiskers, the mice do not cross the gap. Touching the platform is thus a crucial prerequisite for attempting to cross the gap. Our finding that KO mice performed the task as well as WT mice despite significantly reduced use of their whiskers may suggest that KO mice require less tactile information to adaptively explore their environment. Indeed, some evidence suggests that there is a redundancy in the need for sensory information, since single-whisker and multi-whisker WT mice reach the same max gap distance (Celikel and Sakmann, 2007). If *Fmr1* KO mice needed less sensory information to solve the task, one would expect that they would show reduced Exploration Duration (Fig. 7E); however, this was not observed. Instead, our data are more consistent with the view that *Fmr1* KO mice, to avoid oversampling, engage in less whisking behavior. Further arguing against the view that *Fmr1* KO mice show improved efficiency in information processing, is that when tested in more complicated behavioral paradigms than the gap-crossing task, *Fmr1* KO mice show deficits in attentional processing and mild locomotor impairments (Kramvis et al., 2013; Padmashri et al., 2013; Santos et al., 2014; van der Molen et al., 2014; Zhang et al., 2008).

#### 4.6. Potential explanations of the deficits in a whisker-dependent task

Self-injurious behavior constitutes one of the main traits of FXS patients (Tranfaglia, 2011) and it may be related to alterations in the pain processing pathways (Symons et al., 2010). *Fmr1* KO mice show decreased nociceptive sensitization (Busquets-Garcia et al., 2013; Price et al., 2007) but normal acute nociceptive responses (Price et al., 2007; Zhao et al., 2005). They also do not show any self-injurious behavior (Busquets-Garcia et al., 2014). Hence, deficits in whiskers-behavior of *Fmr1* KO mice, their preference to engage in fewer whisker contacts when solving the gap-crossing task, is not likely to be caused by alterations in pain pathways. Since the *Fmr1* KO mice can reach the same maximum gap distance, it appears unlikely, at least in the learning paradigm used in the present study that the differences in solving the gap-crossing task are due to motor impairments or anxiety. Consequently, alongside the known human phenotype of sensory hypersensitivity in FXS, the contribution of hypersensitivity to whisker touches is one



possible explanation of the whisking behavior differences observed in our study. Decision-making based on tactile information is a complicated process that involves not only the primary sensory cortical area, but also other brain areas such as the basal ganglia and the prefrontal cortex. Although our data are consistent with the view that an increased neo-cortical excitability in *Fmr1* KO mice contribute to the observed alterations in sensory sampling using the whiskers, deficiencies in other brain areas are thus also likely to contribute. Experiments where, e.g., the time from the initial whisker contact to the final gap-crossing is analyzed may shed some light on the decision-making process and deficiencies in other brain areas.

In the gap-crossing test we removed all but one whisker on each side of the mouse snout during the 8-day testing period. The rationale for this was to enable accurate whisker tracking and the analysis of single whisker contacts. Although the whiskers were only plucked during the behavioral testing phase removing whiskers may bring some concerns about plasticity. In the whisker system it has been shown in many studies (Glazewski et al., 2007; Glazewski and Fox, 1996; Greenhill et al., 2015b; Jacob et al., 2012; Kaliszewska et al., 2012; Marik et al., 2010), that deprivation induced plasticity is age- (less plasticity with animal age) and time-dependent (the longer the deprivation the robust the effect). In our experiments we used relatively old animals (9–13 weeks compared to most studies that use 4–8 weeks old animals) and the duration of sensory deprivation was relatively short. Furthermore, in our study animals were tested using a protocol (see Methods) where the average gap-distance was shorter in the first 4 days, thus approximately 60% of the results (Fig. 8) for “short gap-distance” were collected during day 1–4, where, if any, time-dependent plasticity effects on animal behavior are considerably smaller. Taken together, we think that the observed behavioral changes cannot be best explained by a difference in the susceptibility to sensory deprivation. Additionally, it has been shown that mice learn the gap-crossing task similarly with a single whisker or multiple whiskers (Celikel and Sakmann, 2007); therefore, sparing only single whiskers does not invalidate the use of the gap-crossing task. And finally, when *Fmr1* KO mice are trained with all whiskers intact they show impaired learning in the gap-crossing task (Arnett et al., 2014).

#### 4.7. Sensory processing changes and clinical relevance

Individuals with FXS often display autistic-like behaviors in reaction to normal sensory stimulation (Hagerman and Cronister, 1996). In fact hyperarousal of FXS patients may be directly linked to strong reactions to sensory stimuli (Hagerman and Cronister, 1996). If that is the case, then it is reasonable to think that also other behavioral symptoms are related to sensory sensitivity in a similar manner, e.g. poor eye contact and sensitivity to visual stimuli (Belser and Sudhalter, 1995; Merenstein et al., 1996) or avoidance in greeting behaviors and sensitivity to tactile stimuli (Musumeci et al., 2000; Wolff et al., 1989). Correspondingly FXS mouse model (*Fmr1* KO) displays deficiencies in sensory processing, not only in the whisker system (Arnett et al., 2014) but also in other sensory modalities such as hearing (Rotschafer and Razak, 2014), vision (Rossignol et al., 2014) and tactile stimulation of the hind paw (Zhang et al., 2014). This alteration in sensory processing, noticeable in various sensory modalities, appears to be a universal problem. Therefore, we may consider FXS as a disease causing impairment in processing and encoding of many types of sensory information. In this context, our study can be treated as an attempt to discover universal pathological mechanisms that affect all the senses.

The comparatively well characterized whisker system may prove to be a suitable experimental model where knowledge of fundamental neuronal mechanisms can provide insights to disease mechanisms. Considering that autism is highly co-morbid with FXS (Bagni et al., 2012), it is tempting to hypothesize that the experimental approach described in this study to investigate sensory processing can be informative if applied to mouse models of ASD (Crawley, 2012). Autistic people tend to

focus too much of their attention on a single task, eliminating other relevant cues from their scope of interest. This may be their strategy to avoid “overstimulation” if the system is hyperexcited. The other extreme, observed in ADHD patients, is an inability to focus attention that also may be a consequence of dysregulated excitatory-inhibitory balance in processing of sensory information. In all these three disorders FXS, ASD and ADHD we can thus observe problems with processing and encoding of sensory information. Therefore, information gained from the study of somatosensory processing in the whisker system of *Fmr1* KO mice may bring us new knowledge applicable to other diseases involving sensory processing deficits.

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