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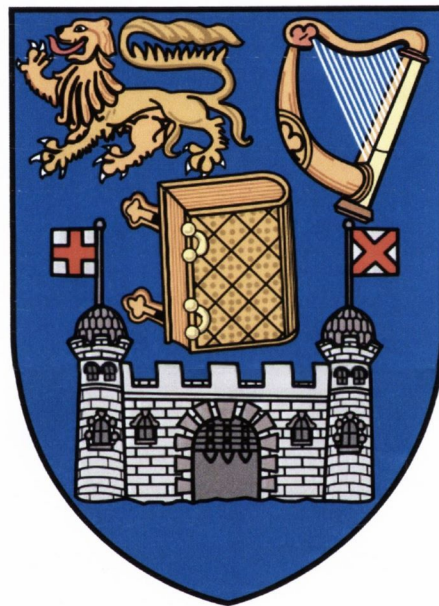
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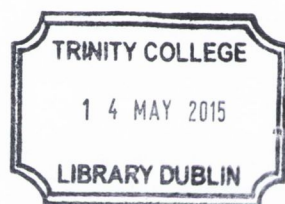
**Functional Investigation of
Neurodevelopmental Effects of *DISC1* and Related
Psychosis Risk Genes**

Stefania Bellini



**A thesis submitted to the University of Dublin
for the degree of Doctor of Philosophy**

**Department of Psychiatry
University of Dublin
Trinity College – 2015**



Thesis 10496

Declaration

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Summary

Schizophrenia (SCZ) is a severe neurodevelopmental disorder, which affects about 1% of the population worldwide, and whose symptoms start during early adulthood. SCZ is determined by the interaction of genetic and environmental factors, but its pathophysiology is largely unknown. Heritability was estimated at 0.8: recent advances in genetics approaches have made it possible to identify a number of genetic variants associated to SCZ; however, most of them also increase the risk for other mental conditions. Thus, the functional investigation of penetrant risk genes for SCZ might better clarify pathophysiological mechanisms and provide a molecular profile of the disorder. In this work we focus on two risk genes: *DISC1* and *PAK7*.

Disrupted in schizophrenia 1 (*DISC1*) is one of the best studied rare penetrant genes for SCZ: it encodes for a scaffolding protein involved in many aspects of neuronal development and function, through the interaction with different molecular partners at specific subcellular compartments. *PAK7* (p21-activated kinase 7) is a recently identified risk factor for SCZ: a rare tandem duplication in the gene is associated to the disorder. *PAK7* is a brain-restricted member of the PAK family, whose neurobiology is uncertain. For our analyses we used *Disc1*-L100P mutant mice, in which a missense mutation disrupts the interaction between *DISC1*, and the phosphodiesterase *PDE4b* (another SCZ risk factor).

For the first time here, we studied the neurodevelopmental pattern of expression of selected markers for synaptic transmission and neuronal physiology in *Disc1*-L100P mutant at three different postnatal ages: P8-10 (postnatal day 8-10: age of active synaptogenesis), P30-40 (age of synaptic pruning) and P>60 (adulthood). We measured protein levels of the selected markers in different cellular compartments (synaptoneurosome, cytoplasm or nucleus) from either brain or cerebellum (CRB) tissue at the three ages. We also measured levels of protein expression of selected markers in histological sections from P>60 *Disc1*-L100P mutant brains. Our histological analysis focused on prefrontal cortex (PFC), hippocampus (HP) and CRB, which are brain structures involved in SCZ. We show that synaptic alterations start at early postnatal stages when aberrant phenotypes are not yet detected, determining the onset of pathological features later in life probably through adaptive mechanisms; the cellular

mechanisms involved in the impairment are age-specific, and take place according to spatial-temporal patterns.

We carried out functional investigation of PAK7 and its interaction with DISC1 in the mouse brain. We find that PAK7 levels are developmentally regulated in *Disc1*-L100P mutant brains. Also, PAK7 and DISC1 interact in synapses as proven by co-immunoprecipitation from brain extracts and colocalization of immunostaining in brain sections of brain cortex and HP. Finally, protein levels of PAK7 are regulated in response to neuronal stimulation in primary neuronal cultures.

We propose a new molecular model in which synaptic and neuronal defects occur early during postnatal life and according spatial-temporal regulated patterns in *Disc1*-L100P mutant mice. Our functional characterization of PAK7 confirms that risk factors for SCZ do not act independently, they rather interact during development. Taken together our results contribute to elucidate the neurobiology of risk factors for SCZ, and to dissect out pathophysiological mechanisms underlying the disorder.

These findings provide insights on the biology of neuropsychiatric disorders like SCZ, pointing out the necessity to investigate these illnesses in a developmental prospective in order to provide early diagnosis and effective treatments.

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Statement of Work

This work was undertaken by the author with the Neuropsychiatric Genetics research group, Department of Psychiatry and Institute of Molecular Medicine, Trinity College Dublin.

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Table of Acronyms

A.U.	arbitrary units
aa	amino acids
AbI	primary antibody
AbII	secondary antibody
AC	adenilate cyclase
AKT	protein kinase B
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AraC	cytosine β -D-arabinofuranoside
ASD	autism spectrum disorder
ATF	activating transcriptional factor
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter (protein)
BBS	Bardet-Biedl syndrome (protein)
BD	bipolar disorder
BSA	albumin from bovine serum
CA1	cornu ammonis 1 (hippocampal area)
CA3	cornu ammonis 3 (hippocampal area)
CaMKIV	calmodulin kinase IV (protein)
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
chr	chromosome

CI	confidence interval
CMV	cytomegalovirus
CNV	copy number variant
Co-IP	Co-immunoprecipitation
CRB	cerebellum
CREB	cAMP dependent responsive element binding protein
CV	coefficient of variation
D ₂	dopamine receptor 2
DA	dopamine
DAPI	4',6-diamidino-2-phenylindole
DG	dentate gyrus
<i>DISC1</i>	disrupted in schizophrenia 1 (<i>Homo sapiens</i> , human gene)
<i>Disc1</i>	disrupted in schizophrenia 1 (<i>Mus musculus</i> , house mouse gene)
DISC1	disrupted in schizophrenia 1 (protein)
DIV	day <i>in vitro</i>
DKO	double knock out
DLPFC	dorsolateral prefrontal cortex
DMEM	Dulbecco 's modified Eagle's medium
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid

eNOS	endothelial nitric oxide synthase
ENU	N-nitroso-N-ethylurea
ERK	extracellular signal-regulated kinase (protein)
ES cells	embryonic stem cells
F ₁	first generation
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
FM	feeding medium
fMRI	functional magnetic resonance imaging
FRET	fluorescence resonance energy transfer
GABA	γ -amino-butyric acid
GAD65	glutamic acid decarboxylase 65 (protein)
GAD67	glutamic acid decarboxylase 67 (protein)
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GP	guinea pig
GPCRs	G protein-coupled receptors
GSK3 α	glycogen synthase kinase α (protein)
GSK3 β	glycogen synthase kinase β (protein)
GTP	guanosine-5'-triphosphate
GWAS	genome-wide association study
HC IgG	heavy chain of IgG

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HP	hippocampus
HRP	horseradish peroxidase (enzyme)
IgGs	immunoglobulins G
IHC	immunohistochemistry
IP	immunoprecipitation
IS	immunostaining
Kal-7	kalirin 7
kb	kilobases
kDa	kiloDaltons
KO	knock out
LB	lysogeny broth
LI	latent inhibition
LIS1	lissencephaly 1 (protein)
LOD	log of the odds ratio
LTD	long-term depression
LTP	long-term potentiation
LUT	look-up table
MAF	minor allele frequency
MAP2	microtubule-associated protein 2
MARK2	microtubule affinity-regulating kinase 2
MGB	minor groove binder

MRI	magnetic resonance imaging
MS	mouse
MSK	mitogen- and stress-activated protein kinase
mTOR	mechanistic target of rapamycin (protein)
MW	molecular weight
NA	numerical aperture
NDEL1	nudE neurodevelopment protein 1-like 1 (protein)
NLS	nuclear localization signal
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
<i>NRXN1</i>	neurexin 1 (<i>Homo sapiens</i> , human gene)
<i>Nrxn1</i>	neurexin 1 (<i>Mus musculus</i> , house mouse gene)
<i>Nrxn3</i>	neurexin 3 (<i>Mus musculus</i> , house mouse gene)
OF	open field
OR	odds ratio
<i>P</i>	<i>P</i> -value
P	postnatal day
P1	pellet 1 (nuclear fraction and heavy cellular membranes)
P2	pellet 2 (synaptoneurosomal fraction)
PAK	p21-activated kinase (protein)
<i>PAK7</i>	p21-activated kinase 7 (<i>Homo sapiens</i> , human gene)
<i>Pak7</i>	p21-activated kinase (<i>Mus musculus</i> , house mouse gene)

PALM	photoactivated localization microscopy
PBS	phosphate buffered saline
PCP	phencyclidine
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDE4b	phosphodiesterase 4b (protein)
PFA	paraformaldehyde
PFC	prefrontal cortex
PGC	psychiatric genomic consortium
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	protein kinase A (protein)
PPI	prepulse inhibition
PSD	postsynaptic density
PSD95	post-synaptic density 95 (protein)
PV	parvalbumin (protein)
PVDF	polyvinylidene difluoride
RB	rabbit
Res	resolution
RSK	ribosomal S6 kinase
RT	room temperature
RTK	tyrosine kinase receptor
S1	supernatant 1

S2	supernatant 2 (cytoplasmic fraction)
SCZ	schizophrenia
SDS	sodium dodecyl sulphate
SE	standard error
SF	serine-phenylalanine
SNP	single nucleotide polymorphism
SOC	catabolite repression medium
SSC	somatosensory cortex
STORM	stochastic optical reconstruction microscopy
TBE	Tris/Borate/EDTA
TBS	Tris-buffered saline
TEA	tetra-ethyl-ammonium chloride
TEMED	tramethylethylenediamine
TNIK	TRAF2 and NCK-interacting protein kinase
TTX	tetrodotoxin
VC	visual cortex
<i>VIPR2</i>	vasoactive intestinal peptide receptor 2 (<i>Homo sapiens</i> , human gene)
WB	Western blot
WT	wild type
α -CaMKII	α -calmodulin kinase II (protein)
Δ DISC1	<i>Disc1</i> L100P/L100P mutant

1. Introduction

1.1 Neurodevelopmental hypothesis of schizophrenia

Schizophrenia (SCZ) is a severe psychiatric condition, which affects approximately 1% of the population, and represents a major human and financial cost worldwide (Whiteford et al. 2013). This is a neurodevelopmental disorder characterized by positive symptoms (delusions and hallucinations), negative symptoms (avolition, alogia and decreased sociability) and disorganized behavior, which usually start in late adolescence or early adulthood (van Os & Kapur 2009). Schizophrenia is caused by a combination of genetic susceptibility factors and environmental insults (pre- and perinatal hazards, urbanization, abuse of substances); and its pathophysiology remains poorly understood. Drug discovery occurred by chance due to the lack of knowledge of the biological basis of the disorder. There have been few advances in therapeutics since that time. Neurotransmitter systems – including DA (dopamine), serotonin, GABA (γ -amino-butyric acid) and glutamate – have been related to SCZ to date. The most supported theories propose DA and glutamate alterations as neurochemical mechanisms responsible for SCZ symptoms. Pharmacological agents that modulate DA (amphetamines and cannabis) and glutamate (phencyclidine or PCP) receptors can produce SCZ-like symptoms in rodent models as well as in humans. DA transmission was found to be increased in the striatum and reduced in the prefrontal cortex of patients, suggesting DA hyperactivity as a main mechanism of the disorder. However, glutamate agonists can alleviate psychotic symptoms, which demonstrates a role for the glutamate deficit as well. Alterations of the two systems might not be independent; glutamate dysfunctions via NMDA (N-methyl-D-aspartate) receptors reported in prefrontal cortex of SCZ patients might lead to altered DA transmission at the level of the mesolimbic system (Laruelle 2014; Brisch et al. 2014; Seeman 2009). Current treatments for SCZ mainly target the DA system; they are partially effective for psychotic symptoms but not for other facets of the disorder (Leucht et al. 2012). Understanding the biology of the disorder is a pressing need if better therapies are to be developed. The neurodevelopmental theory of SCZ states that neurodevelopmental abnormalities, which start years before the onset of the symptoms, lead to the onset of the illness in early adulthood. This theory is supported by decades of literature which converge on a few main lines of evidence. First, epidemiological studies show association between pre- and perinatal complications (e.g. prenatal hypoxia) and SCZ susceptibility, although the

increase of the risk is relatively small (Cannon et al. 2002). Second, individuals who later in life develop SCZ tend to present with subtle social, cognitive and motor impairments during childhood, predating development of the disorder (Dickson et al. 2012). Third, neuroimaging studies demonstrate that schizophrenic patients have abnormalities of brain structure and connectivity at onset of the disease including cerebroventricular enlargement, reduction of gray and white matter, and abnormal laminar organization maybe due to abnormal neuronal migration and pruning (reviewed by Iritani, 2013). Finally, there is no sign of neurodegeneration and gliosis detected by post-mortem investigations on SCZ brains (Rapoport et al. 2012). Abnormal brain structure might cause altered brain function; functional magnetic resonance imaging (fMRI) and electrophysiological studies have shown altered network response to cognitive tasks in different brain regions of schizophrenic patients (Fusar-Poli et al. 2007). The absence of neuronal loss in SCZ suggests that brain volume reduction results from altered histological and molecular features where both projection neurons and interneurons are affected. Pyramidal neurons in DPFC (dorsolateral prefrontal cortex) and temporal cortex present reduced dendritic spine density (Glantz & Lewis 2000; Garey et al. 1998); also striatal spines are significantly impaired in size in SCZ brains compared to controls (Roberts et al. 1996). Pyramidal neurons in SCZ prefrontal cortex have smaller cell body (Selemon & Goldman-Rakic 1999), shorter and less branched dendritic protrusions (Black et al. 2004). These histological defects are accompanied in PFC (prefrontal cortex) and hippocampus by altered expression of transcripts and proteins of both pre-synaptic secretory machine (synapsin I/II, synaptotagmin, synaptojanin, synaptobrevin) and post-synaptic density like PSD95 (Ohnuma et al. 2000; Mirnics et al. 2000). Interneurons appear functionally altered: reduction in the expression of glutamic acid decarboxylase 67 (GAD67) enzyme has been consistently reported by multiple studies (Guidotti et al. 2000; Akbarian et al. 1995; Volk et al. 2000). Another consistent feature in SCZ is decreased parvalbumin (PV) -expressing subclass of interneurons in both PFC and hippocampus (Lewis et al. 2001; Beasley et al. 2002; Zhang & Reynolds 2002). Parvalbuminergic neurons are of particular interest as their fast spike-firing gives rise to the gamma oscillation (Lewis et al. 2005), which are necessary for pyramidal neurons synchronization that is impaired in SCZ. In conclusion, a significant body of literature shows that schizophrenia is a

neurodevelopmental disorder: the gene-environment interaction alters synapse development and brain structure throughout life from prenatal stages to the onset of the disease.

1.2 Heritability of schizophrenia

Most likely the etiology of SCZ involves pathologic processes caused by the interaction between genetic and environmental factors, which begins early during development. Pedigree observations, twin and adoption studies have long revealed SCZ to cluster in families, and heritability was estimated at 0.81 (Sullivan et al. 2003). In the last few years, with the advent of new genetic approaches, significant advancements have been achieved in our knowledge of the genetic architecture of neurodevelopmental disorders like SCZ. Because of ethical and practical limitations in investigating the human brain physiology, genetic information is particularly relevant in studying brain diseases as it provides molecular clues for functional investigation in model organisms. No Mendelian form of SCZ has been identified; instead, the available genetic results support a polygenic model for the disorder in which both rare and common variants contribute to the risk of disease (McCarroll & Hyman 2013).

1.2.1 Rare and common variation associated with SCZ

Rare but penetrant structural variations contribute to the genetic risk in a small proportion of SCZ patients. These rare variants are usually fairly large copy number variants (CNVs), in which portions of DNA are duplicated or deleted, and chromosomal rearrangements (translocations or inversions). At least eight rare CNVs of strong effect were identified for SCZ to date. These high-risk mutations occur more frequently in SCZ patient than in control subjects, conferring a 2-30 fold increased risk of schizophrenia depending on the individual event (Ripke et al. 2013). The size of DNA fragment varies for each CNV, and the expression of multiple genes can potentially be affected by single structural alterations. However, structural variants that affect single genes have been identified. Deletions within the neurexin1 gene (*NRXN1*) and duplications of the *VIPR2* or vasoactive intestinal peptide receptor 2 were associated with SCZ and other psychiatric conditions (Rujescu et al. 2009; Vacic et al. 2011).

Rare ch20p12.2 duplication was recently identified in our group. This mutation increases risk of schizophrenia and bipolar disorder, and overlaps to the first two exons, promoter and

enhancer regions of *PAK7* gene (Morris et al. 2014). Rare penetrant single gene structural alterations are of particular interest as they provide opportunity for downstream functional study. While the importance of rare copy number variants is well established in the genetic etiology of SCZ, exome-sequencing studies are now starting to be carried out to assess the role of *de novo* rare exonic variation in schizophrenia (Need et al. 2012); this type of study aim to identify functional mutations significantly associated with SCZ cases. However, larger studies are needed to meet this purpose.

The other component of the genetic risk associated with schizophrenia is represented by common variants. They are SNPs (single nucleotide polymorphisms), which arose in the ancient populations and expanded because of their small phenotypic impact. These common alleles are much more numerous than rare structural variations (they number in millions), and each of them individually confer much smaller effect to high polygenic disorders like SCZ. The increased power of genome-wide association studies (GWASs) together with the decreased cost of sequencing techniques, have made it possible to uncover several loci significantly associated with schizophrenia. Recent common-variant association studies, including a large GWAS mega-analysis conducted by the Schizophrenia Psychiatric Genomic Consortium (PGC), identified more than 20 loci associated with genome-wide significance. The proportion of susceptibility to SCZ covered by common SNPs was estimated at least 32% (Ripke et al. 2013); however it is likely that more SNPs will be identified as associated with schizophrenia as the sample population size increases (Lee et al. 2012).

1.3 Neurobiology of schizophrenia

Although advances in genomics have recently provided insights into the hereditary component of SCZ risk, none of the rare or common variants associated with the disorder is fully penetrant, and so cannot be used to predict the onset of the disease.

Interestingly, the genetics findings globally converge on specific molecular complexes and pathways and proteins, which are likely neurodevelopmentally regulated: results implicate common variants in voltage-gated calcium channels, and *de novo* CNVs in excitatory post-synaptic components in the pathogenesis of schizophrenia (Smoller et al. 2013; Kirov et al. 2012). Although some variants affect genes which are neurobiologically meaningful, a

comprehensive understanding of the molecular pathophysiology of SCZ is still far from being achieved. Some common variants have shown specific association with SCZ and map to regions with one or more genes, but not all of them are clearly protein changing (Winantea et al. 2006; McCarroll & Hyman 2013). This suggests that part of the variability increasing the risk of polygenic psychiatric diseases like SCZ does not result in rare protein-disrupting phenotypes; it might rather affect levels and pattern of expression of proteins in specific brain areas and/or cell types relevant to the disorder.

Moreover, most rare risk variants also increase risk of other neurodevelopmental disorders including bipolar disorder (BD), autism spectrum disorder (ASD) and mental delay (Ching et al. 2010; Guilmatre et al. 2009; Steinhausen et al. 2009). Shared genetic etiology between SCZ and other psychiatric disorders, different penetrance and variable expressivity of single mutations, make it difficult to match specific mutations to clinical symptoms. Therefore, there is an underlying necessity to unveil the neurobiology of schizophrenia by investigating the spatial and temporal regulation of the risk factors before adulthood including early postnatal stages. Importantly, SCZ risk factors are in many cases proteins that do not act independently; instead, evidences suggest that they play a synergistic role in the pathophysiology of the disease (Mitchell 2011). Thus, the interaction between risk factors is an additional fundamental aspect to explore.

Because of the genetic heterogeneity of the disease, functional investigation of high penetrant rare variants in animal and cellular models may be particularly important for understanding the neurobiology of schizophrenia (Karayiorgou et al. 2012). This approach might best allow dissecting out pathophysiological mechanisms starting from penetrant causative mutations in humans. One of the best studied rare penetrant mutations in a gene which interact with other SCZ risk genes is disrupted in schizophrenia 1 (*DISC1*).

1.4 Disrupted in Schizophrenia 1 (*DISC1*)

The gene disrupted in schizophrenia 1 (*DISC1*) is of interest as a prototypical candidate gene emerging from analysis of rare variation. It was first identified in a large Scottish pedigree with a high incidence of mental and behavioral disorders (schizophrenia, affective disorders, bipolar disorder and major depression), which co-segregate with the balanced t(1q42.1;11q14.3) chromosomal translocation (St Clair et al. 1990). This structural alteration

in the Scottish family was statistically associated with broad psychiatric illness (log of the odds ratio or LOD = 7.1), and linkage with SCZ alone was also significant with LOD = 3.6. However, the penetrance is incomplete; there are member of the family affected by a psychiatric disorder who present a normal karyotype, although none with a major mental illness (Blackwood et al. 2001). Of the disrupted genes at the chromosomal breakpoint, only *DISC1* on chromosome 1 was found to have open reading frame thus having protein coding potential, while *DISC2* on the other strand is an untranslated gene (Millar et al. 2000). *DISC1* consists of 13 exons; the Scottish translocation occurred between Exon 8 and 9, interrupting the coding sequence. Although the t (1; 11) is unique of the Scottish family, it is to date one of the most convincing causative mutations associated with major psychiatric disorders.

1.4.1. Independent *DISC1* genetic studies

Since *DISC1* was discovered, multiple independent linkage and association analyses have been conducted in order to find genetic association with psychiatric disorders.

Several studies reported linkage between major psychiatric disorders and *DISC1* loci in population from Finland, Taiwan, Scotland and Britain/Iceland (Ekelund et al. 2004; Hwu et al. 2003; Macgregor et al. 2004; Curtis et al. 2003; Detera-Wadleigh et al. 1999).

Positive association was proposed for *DISC1*-spanning haplotypes and SNPs to major psychiatric disorders and SCZ in particular. A detailed revision of *DISC1* association data available can be found elsewhere (Chubb et al. 2008). *DISC1* gene variants were associated with impaired learning, memory and cognitive abilities in both schizophrenic patients and healthy individuals. A number of studies reported association between the common Ser704Cys *DISC1* polymorphism and abnormalities that include reduced working memory and alterations of gray matter (reviewed in Chubb et al., 2008). Impairment in cognitive functions is one of the most consistent and quite specific features recognized in patients affected by SCZ, consistently with altered function of PFC and hippocampus.

However, candidate gene studies for common genetic variations are small and lack of adequate quality control. In fact, the largest and more comprehensive genome-wide association studies and mega-analyses to date found no significant association of *DISC1* SNPs in schizophrenia cases (Anon 2011; Mathieson et al. 2012).

1.4.2 DISC1: gene, protein and expression pattern

In parallel with genetic studies, broad investigation has been carried out in order to clarify the structure of the gene, the regulation of its expression and its neurobiology. A comprehensive knowledge of the neurobiology of DISC1 is fundamental to understand the pathophysiological contribution of the gene to neuropsychiatric disorders like SCZ.

The human *DISC1* gene consists of 13 major exons, producing a transcript of about 7.5kb (kilobases). Notably, intron 9 consists in an exceptionally large portion of DNA that in human accounts for approximately one third of the full length gene. The structure of *DISC1* is evolutionary conserved; a very large intron 9 is present also in other species including *Mus musculus* (house mouse) and this similarity suggests that the intron might exert a functional role in the gene expression. However, the rodent gene (*Disc1*) lacks of the additional exon 9a that is present in the human one. Overall, the percentage of identity between the human and the mouse orthologous is 58% (reviewed by Chubb et al., 2008). Figure 1 illustrates the structure of *DISC1* human gene and *Disc1* mouse gene. The picture was modified from Chubb et al. paper published in 2008, and it was constructed based on previously published data (Millar et al. 2000; Ma et al. 2002).

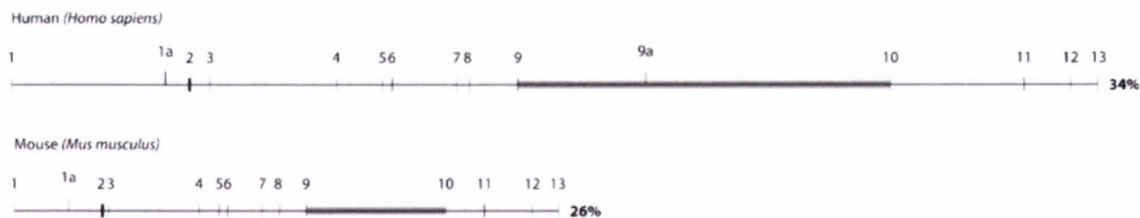


Figure 1. Genomic structure of disrupted in schizophrenia 1 in *Homo sapiens* and *Mus musculus* (*DISC1* and *Disc1* respectively). Full length of the genes is represented, exons are identified by numbers. Percentages on the right show the proportion of transcript associated to intron 9, which is underlined by thick line in both species.

Expression of the gene occurs in diverse human and rodent tissues - including brain - and in a very complex fashion, giving rise to four main splicing variants and thus to the following predicted protein isoforms: long (L, approximately 100 kDa), long variant (Lv, ~98 kDa), short (S, ~75 kDa) and extremely short (Es ~37 kDa). Correspondent molecular weight

protein species have been identified; the 100 kDa isoform is believed to reflect the entire translated product of the gene in both human and mouse. However, dozens of additional transcripts have been reported by mRNA screening of brain tissue, and multiple DISC1 protein species have been identified, which likely corresponds to post-translational and/or oligomerisation events.

The three-dimensional structure of the protein has not been revealed yet. The full-length (L isoform) human DISC1 protein structure was thus hypothesized based on literature information and using bioinformatics tools (Nakata et al. 2009). The L isoform consists of 854 aa (amino acids); the first 350 aa-residues are encoded by exons 1 and 2 of the gene and constitute the N-terminal globular head domain of the DISC1. Within the N-terminal region are located two conserved motifs: a nuclear localization signal (NLS) and a short SF (serine-phenylalanine) rich sequence. The C-terminal domain of DISC1, spanning approximately aa 326-854 and encoded by exons 3 to 13, is characterized by alpha-helical and coiled-coil conformations, which are believed to be necessary for the binding between DISC1 and putative protein interactors. Between N- and C-terminal domains, a short self-association core sequence is located, and considered to be required for DISC1 dimerization and cytoskeleton assembly (Leliveld et al. 2009). Figure 2 was modified from Chubb et al. (2008) and shows the predicted structure of human DISC1 protein, which highlights specific domains and motifs (the self-association core domain is not shown).

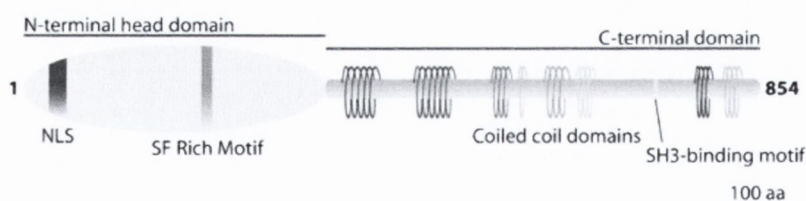


Figure 2. Predicted structure of the full-length (L) human DISC1 protein. The proteins consists of 854 amino-acids organized in a N-terminal globular head and a long coiled-coil C-terminal tail. Conserved nuclear localization signal (NLS) and SF rich motif are found at the N-terminal.

It is not clear how many transcripts are actually translated into functional proteins, although their number seems to decrease throughout development (Nakata et al. 2009). Numerous studies in mouse brain, reported neuronal DISC1 expression as spatial-temporal regulated

during brain development. It is expressed at the highest level in the dentate gyrus of the hippocampus, followed by cerebral cortex, olfactory bulb and cerebellum (Chubb et al. 2008). and shows two peaks of expression at embryonic day 13.5 (E13.5) and post-natal day 35 (P35); which correspond to the time of active neurogenesis and puberty pruning respectively (Schurov et al. 2004).

1.4.3 DISC1 brain function: subcellular localization and interactors throughout development

More than one hundred potential protein interactors of DISC1 were identified a few years ago by yeast two-hybrid screens: the so called DISC1 interactome (Camargo et al. 2006). Some of these putative interactions, mainly those proteins which engage neurophysiological roles have been investigated in other experimental systems and may be risk factors for neurodevelopmental disorders. To date, the functional studies of DISC1 protein interactors and complexes have provided some insight into the neurophysiological and pathophysiological role of DISC1. The protein exerts multiple functions at the neuronal level by association with diverse proteins specifically located in the various subcellular compartments. Moreover, the protein is required for diversified neuronal mechanisms throughout life, from neural precursor proliferation to migration, neuronal maturation, integration and neurosignaling in mature neurons (for a detailed review of DISC1 brain function see Thomson et al., 2013). Some of the best characterized interactions will be described below. Figure 3 was made using figures taken from two reviews (Brandon & Sawa 2011; Lonze & Ginty 2002) and illustrates the main protein interactions of DISC1 and how some of those might eventually influence gene expression via regulation of the transcriptional factor CREB.

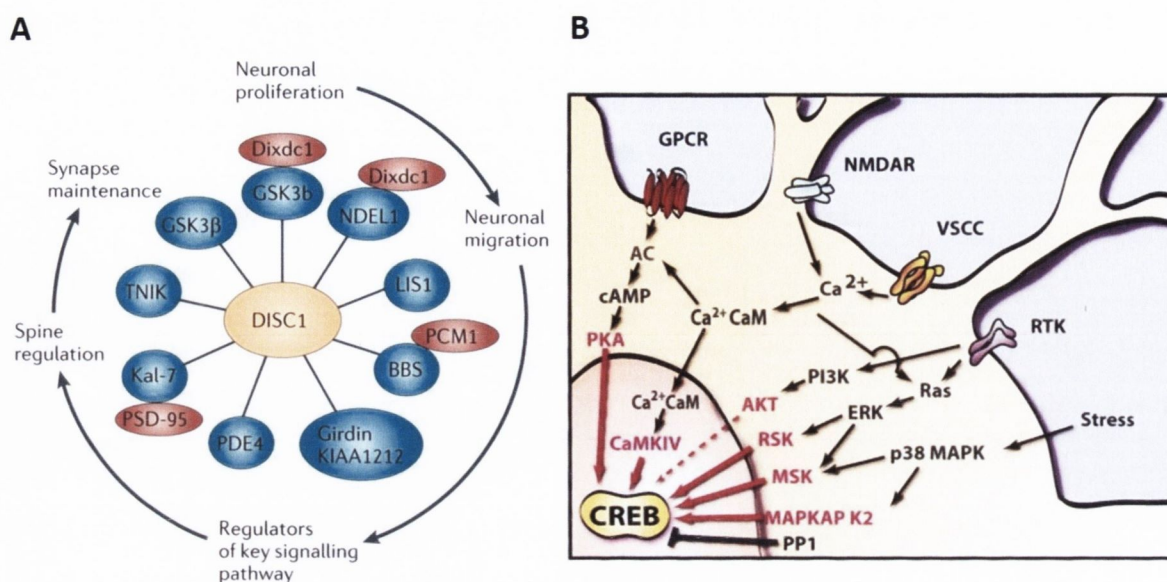


Figure 3. Main DISC1 protein interactions and regulation of CREB-dependent gene expression. The DISC1 protein engages multiple interactors participating to different neuronal processes such as proliferation, migration, synapse maintenance and spine regulation during development (A). DISC1 directly interacts with the PDE4 phosphodiesterases (involved the regulation of cAMP levels), AKT and GSK3 β . These molecules are key regulators of cellular pathways that directly affect the activation of the transcriptional factor CREB in the nucleus, linking DISC1 to CREB-dependent gene regulation processes (B).

DISC1 mediates different aspects of neurogenesis in both developing and adult brain. Neurogenesis is the process through which new neurons are generated from neural progenitor cells and they migrate to their final destination in the brain; the generation of newborn neurons is a common process in the entire developing brain, but in the adult brain it gets restricted to the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricle only. A peak of DISC1 expression between embryonic day 13.5 (E13.5) and E15 was demonstrated in mouse brain: this is a period of active neurogenesis within developing cortex and hippocampus. DISC1 participates to the maintenance of the pool of neural progenitors through its direct cytoplasmic interaction with glycogen synthase kinase β (GSK3 β). GSK3 β is directly involved in cell proliferation as a component of the canonical Wnt signaling pathway (Mao et al. 2009). DISC1 directly binds and thus inhibits the GSK3 β kinase; this inhibition results in a reduced β -catenin phosphorylation and degradation, which finally leads to increased cell proliferation and reduced differentiation (Mao et al. 2009). At

the level of the centrosome DISC1 interacts with various centrosome-associated proteins like LIS1 and NDEL1: this association is known to be required for migration of both pyramidal neurons and interneurons. In mouse the phosphorylation of DISC1 at Ser701 abrogates the interaction between DISC1 and GSK3 β , and triggers the recruitment of BBS, LIS1 and NDEL1 (Ishizuka et al. 2011). This modification of DISC1 is believed to be a key developmental switch from progenitor's proliferation to post-mitotic migration in mouse developing cortex. In association with NDEL1, DISC1 was also demonstrated to regulate neurite outgrowth in vitro (Kamiya et al. 2006). DISC1 was also demonstrated to contribute to neuronal maturation and integration in the adult hippocampus through the mTOR pathway: in fact, the protein directly interact with Girdin (or KIAA1212) preventing its binding to AKT (Kim et al. 2009). In addition to its role in neurogenesis-related processes such as proliferation of neural precursors, maturation, migration and integration to neural circuits, recent studies have to date reported important roles for DISC1 in neurosignaling events in mature neurons, being present in both pre- and post-synaptic terminals. DISC1 is located at the post-synaptic density (PSD) of dendritic spines (Bradshaw et al. 2008), where it directly modulates synaptic dynamics morphology and maintenance by direct interaction with Kalirin 7 (Hayashi-takagi et al. n.d.). Kalirin 7 (Kal-7) is a guanine nucleotide exchange factor (GEF), and it works as a fundamental regulator of spine maturation and remodeling in response to neuronal activity; these results are achieved by the activation of Rac1 and its downstream effectors of the PAK (p-21 activated kinase) family (Penzes & Jones 2008). Through its association with Kal-7, DISC1 regulates its access to Rac1 and thus the Rac1-dependent reorganization of spines morphology. These events were proven to be coupled with NMDA-dependent synaptic activation, and necessary for AMPA-receptor subunits trafficking the synaptic terminals (Hayashi-takagi et al. n.d.). It has been recently shown that DISC1 participates to processes of synaptic vesicle transport and glutamate release at the level of glutamatergic pre-synaptic terminals (Flores et al. 2011; Maher & LoTurco 2012). Additional evidence for the multiple roles played by DISC1 at glutamatergic synapses comes from the discovery of its interaction with TNIK at the level of the PSD. DISC1 interacts with TNIK kinase in order to regulate the composition and stability of post-synaptic density; this association is required for proper expression and trafficking of PSD95 and AMPA receptor subunit GluR1 to the synaptic terminal (Wang et al. 2011). These evidences suggest that

DISC1 is a determinant regulator of activity-dependent processes that underlie synaptic plasticity events. Another example of the DISC1 role in neurosignaling is provided by the discovery of its interaction with phosphodiesterases 4 or PDE4s (Millar et al. 2005; Murdoch et al. 2007). The PDE4s are a family of enzymes that hydrolyze cAMP in response to the upregulation of the cellular levels of this second messenger. Fine regulation of cAMP levels is crucial for the activation of the transcriptional factor CREB, which is required for the activity-dependent changes of the transcriptional activity in neurons. DISC1 binding to PDE4B inhibits the catalytic activity of the phosphodiesterase; in a negative feedback loop, increased cAMP cellular levels lead to dissociation of the two proteins and the subsequent termination of cAMP signaling cascade (Millar et al. 2005). The contribution of DISC1 to the regulation of gene transcription is not limited to its regulation of neurosignaling pathways triggered by neuronal activity. In fact, it has been shown that DISC1 is directly involved in nuclear transcriptional regulation, being partially distributed to the neuronal nucleus, where it is mainly located within promyelocytic leukemia nuclear bodies (Sawamura et al. 2008). It was shown that DISC1 might be potentially involved in the regulation of multiple transcriptional events by direct interaction with two related members of the CREB family of transcriptional factors: ATF4 and ATF5 (Morris et al. 2003). In conclusion, multiple independent lines of evidence have demonstrated that DISC1 is an essential player in the regulation of diverse biological processes throughout neurodevelopment by interacting with different binding partners, some of which have are reported as independent risk factors for schizophrenia: this observations support the epistatic interaction between susceptibility factors for SCZ (Hennah et al. 2007; Burdick et al. 2008; Millar et al. 2005).

1.4.4 *Disc1* mouse models

The structure of disrupted in schizophrenia 1 is evolutionally conserved and the homologous in mouse (*Disc1*) shares 58% of its sequence with the human gene (Chubb et al. 2008). *DISC1* risk variants have not only been reported in schizophrenia patients as discussed above, but also in cases of autism spectrum disorder, BD and depression (Hashimoto et al. 2006; Kilpinen et al. 2008; Song et al. 2010). Specific anatomical, neurological, behavioral and cognitive features have been observed in schizophrenic patients, which can be studied in animal to validate models of neuropsychiatric diseases, in spite difficulties in translating

human characteristics to rodents. Seven mouse models mutant for *Disc1* have been generated in order to study the neurobiology of the gene and to shed light on the pathophysiological contribute of the gene to neuropsychiatric disorders including SCZ. All of the genetic models show behavioural abnormalities relevant to SCZ; most of them have cognitive deficits and neuroanatomical features reminiscent of the ones described in schizophrenia patients (Jaaro-Peled 2009; Johnstone et al. 2011). The first mutant was identified in the 129S6/SvEv mouse strain, in which a 25 bp spontaneous deletion in exon 6 leads to a frame shift that creates a premature stop codon in exon 7 (Koike et al. 2006; Kvajo et al. 2008). Two independent *Disc1* models were created by N-nitroso-N-ethylurea (ENU)-induced mutagenesis; they both carry a single missense mutation in exon 2 of the gene: L100P and Q31L (Clapcote et al. 2007). Three transgenic models were generated in order to assess the effect of the expression of the human or murine gene under control of the α -calmodulin kinase II (α -CaMKII) in the brain: in these models express a N-terminal or C-terminal truncated protein, which in some cases compete with the endogenous DISC1 as a dominant-negative (Hikida et al. 2007; Li et al. 2007; Pletnikov et al. 2008). The last *Disc1* model expresses a truncated sequence (first 8 exons) of the mouse gene under the control of an endogenous promoter through a bacterial artificial chromosome (Shen et al. 2008).

1.4.4.1. The *Disc1*- L100P mutant mouse

The original Scottish family is the only example *DISC1* truncation associated to neuropsychiatric disorders; however, the investigation of other putative risk variants at the locus will also be important. The *Disc1*-L100P mutant mouse is a SNP mutant that was generated at the RIKEN Genomic Sciences Centre (Japan) and characterized for the first time by Clapcote and colleagues in 2007 (Clapcote et al. 2007); further behavioural investigation was later carried out by other groups (Walsh et al. 2012; Lipina et al. 2010; Lipina et al. 2011). The L100P model carries a missense mutation N-nitroso-N-ethylurea (ENU)-induced in exon 2 of *Disc1*: as a result, the amino acid leucine (L) in position 100 is replaced by a proline residue (P). Exon 2 is expressed by all the known *Disc1* isoforms and encodes for most of the N-terminal globular head domain in both human and mouse genes as described above. Association between major psychiatric disorders including SCZ and exon 2-spanning haplotypes has been reported (Cannon et al. 2005; Hennah et al. 2005). The L100P missense

mutation is located within the *Disc1* binding site for PDE4b, which is an independent risk factor for SCZ (Millar et al. 2005). For this reason, the expression of both DISC1 and PDE4b was compared between mutant and wild type brains: no difference between genotypes was found in terms of levels and cellular distributions for both proteins. However, it was shown that the mutation greatly reduces the binding between the two proteins (Clapcote et al. 2007). PDE4b is a phosphodiesterase required for cAMP second messenger turnover; association of DISC1 to PDE4b is believed to regulate the activity of the enzyme as described above (Millar et al. 2005). Thus, disruption of the DISC1-PDE4b interaction might affect the regular cAMP-dependent regulation of the transcriptional factor CREB. The N-terminal domain (amino acids 1-220) of DISC1 is required for its interaction with GSK3 (Mao et al. 2009); the protein binding between both GSK3 α and GSK3 β , as well as the level of GSK3 α activation are greatly reduced in striatal extracts of L100P mutants (Lipina et al. 2011). Behavioral tests conducted on *Disc1*-L100P mutants of 12-16 weeks of age showed that they have impaired PPI (prepulse inhibition) and LI (latent inhibition). PPI and LI test sensor and information processing, alteration of these functions are well established endophenotypes of schizophrenia. Additional behavioral abnormalities were identified in L100P mutants such as increased locomotion in the open field (OF) test (Clapcote et al. 2007; Lipina et al. 2010), disruption in exploratory and habituation pattern (Walsh et al. 2012), and impaired working memory that was assessed by T-maze test (Clapcote et al. 2007). The L100P mutants are more sensitive to the psychostimulant effect of amphetamine, and they were proven to express higher levels of striatal dopamine-2 (D₂) active receptors (Lipina et al. 2010). The D₂ receptor antagonist haloperidol normalizes hyperactivity, impaired PPI and LI and the psychostimulant effect of amphetamine in L100P mutants (Lipina et al. 2010). The same results is achieved by pharmacological treatment with the GSK3 inhibitor TDZD-8 (Lipina et al. 2011), and the PDE4 inhibitor rolipram (Clapcote et al. 2007). These data suggest that the altered interaction between DISC1 and both PDE4B and GSK3, due to the missense mutation in exon 2 of *Disc1*, are certainly related to L100P mutant phenotype; the supersensitivity to dopamine might also contributes to phenotype. Adult *Disc1*-L100P mutants show neuroanatomical abnormalities when compared with wild type mice: MRI analyses revealed a 13% reduction in overall brain volume and tissue contraction was detected in cortex, thalamus and cerebellum (Clapcote et al. 2007). These macroscopic

abnormalities are accompanied by defective proliferation, maturation and migration of newly generated neurons to their final position within the dentate gyrus of the hippocampus (Chandran et al. 2014). The *Disc1* missense mutation produces subtle histological and neuronal changes earlier during development. In fact, the mutants show altered dendritic morphology in frontal cortical pyramidal neurons and reduced hippocampal spine density, accompanied by disrupted neurogenesis and neuronal migration that lead to defective location of the mature neurons within the cortex during embryonic stages and in early postnatal life (Lee et al. 2011). Recently, altered interneuronal development and positioning was also observed in *Disc1*-L100P embryos (Lee et al. 2013). Expression profiles of *Nrxn1* and *Nrxn3* in L100P mutants differ significantly from the wild types throughout neurodevelopment (Brown et al. 2011). Neurexins (*NRXNs*) are synaptic modulators implicated in crucial neuronal and synaptic proteins; *NRXN1* was widely recognized as an independent risk factor for SCZ, bipolar disorder and autism (Südhof 2008; O'Dushlaine et al. 2011). This finding provides a further evidence of functional correlation between risk factors for SCZ. Studies on *Disc1*-L100P mutants have so far revealed behavioral, anatomical and histological abnormalities relevant to SCZ. However, a comprehensive characterization of the molecular phenotype is missing. Peculiar feature of this *Disc1* model is the impaired interaction between the SCZ risk factors *DISC1* and *PDE4B*, which provides opportunity for investigating the molecular consequences of the hypothetical dysregulation of the cAMP-dependent phosphorylation of the transcriptional factor CREB in the brain throughout development.

1.5 Neuronal pathways affecting CREB-dependent gene expression

In the nervous system, a variety of physiological and developmental processes, including neurogenesis, progenitor migration and differentiation, survival, learning, memory formation and consolidation, and the activity-dependent reorganization of synaptic connections, require changes in gene expression. The transcriptional factor CREB (cAMP response element binding protein) is critically involved in the stimulus-inducible regulation of gene transcription. In neurons, CREB activation occurs through the action of different cellular kinases that take action as part of diverse signaling pathways. Kinases are enzymes that transfer the terminal phosphate of an ATP molecule to a specific amino acid of a target

protein; this catalytic reaction is named “phosphorylation” and allows conformational changes in the target that induce change of its activity or localization. Kinases are classified in serine-threonine kinases and tyrosine kinases based on the specific amino acid targeted by their action (Alberts et al. 2002). The activation of CREB occurs as a result of its phosphorylation at Serine133, and can be triggered in response to a wide range of extracellular stimuli. Among these, neuronal growth factors stimulation during development of the nervous system; membrane depolarization and synaptic activity occurring in the context of physiological neuronal function. All these forms of neuronal stimulation can be transduced into the cell by activation of a number of signaling pathways that converge upon CREB, leading to Ser-133 phosphorylation and to subsequent changes in the gene transcription (Lonze & Ginty 2002). A considerable crosstalk exists among these neuronal pathways, which do not act independently in determining CREB activation. In fact, a single stimulus can trigger several parallel pathways. The main pathways and kinases mediating CREB activation are described below:

- a) cAMP-PKA. In the nervous system, a number of neurotransmitters and neuropeptides, bind to G protein-coupled receptors (GPCRs). G proteins stimulations lead to adenylate cyclase (AC) activation and increase in levels of the second messenger cAMP (cyclic adenosine monophosphate). cAMP works by activating protein kinase A (PKA), which can activate CREB by phosphorylation. PKA activation can also occur in response to increased cellular calcium.
- b) Ca^{2+} -CaM. In neurons, levels of calcium can be raised through voltage or ligand-gated channels. After membrane depolarization or during glutamatergic synaptic transmission, Ca^{2+} flows into the neuron, where it interacts with Ca^{2+} binding protein calmodulin (CaM). CaM in turn can activate CaM kinase IV (CaMKIV), which can phosphorylate CREB. Calcium can also activate the Ras-ERK pathway.
- c) Ras-ERK. Many growth factors can interact with tyrosin-kinase receptors (RTKs), inducing their dimerization and activation. RTK binding results in the activation of a variety of signaling cascades. In particular the ERKs (extracellular stimuli-regulated kinases) can be phosphorylated/activated via Ras. The Ras-ERK phosphorylation of CREB can be eventually carried out by a kinase member of the RSK and MSK families.

d) PI3K-AKT. This is the second most important signaling pathway activated by RTKs. Under phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) activation, pAKT controls CREB activation in neurons, although it is not clear if it is directly responsible for the phosphorylation of the transcriptional factor.

1.6 PAK7: a brain specific member of the p21-activated kinases (PAKs)

PAK7, also known as PAK5, is the last identified and less characterized member of the p21-activated kinases (PAKs) (Pandey et al. 2002).. The PAKs are a family of serine-threonine kinases effectors of the small Rho GTPses Rac and Cdc42, which have a well-established role in determining neuronal morphology and migration as regulators of actin and microtubule reorganization (reviewed by Govek et al., 2005). The PAKs are divided in group I (PAK1-3) and group II (PAK4-6), whose members are structurally related. The two subfamilies share an N-terminal GTPase binding domain (50% of homology) and a C-terminal kinase domain, group I PAKs are activated upon Rac and Cdc42 binding, while the mechanisms of activation of the group II members remain to be elucidated (Jaffer & Chernoff 2002). PAK7 it is highly expressed in the central nervous system and in a few other tissues – including adrenal gland, pancreas, prostate and testes – at significantly lower level. Within the brain, PAK7 is expressed everywhere at the neuronal level but not in glial cells (Li & Minden 2003; Pandey et al. 2002). PAK7 was detected in different cellular compartments within the neuron – including cytoplasm, mitochondria, nucleus and synapses - where it contributes to various functions as described below. The expression of activated PAK7 was shown to lead to filopodia formation and neurite outgrowth in N1E-115 neuroblastoma cells (Dan et al. 2002a), and to the inhibition of MARK2, a regulator of microtubule dynamics (Timm et al. 2006) Additionally, PAK7 was found to phosphorylate p-120 catenin, another protein involved in cytoskeletal reorganization; PAK7 and p-120 catenin colocalize at the cytoplasmic level in vitro, and their interaction was demonstrated both in cell lines (HEK293) and in the mouse brain (Wong et al. 2010). PAK7 is preferentially bound by Cdc42 upon GTP activation; differently from group I PAKs, this interaction is not required for PAK7 kinase activity, but essential for proper subcellular targeting of the protein (Pandey et al. 2002; Wu & Frost 2006), suggesting that the protein might also be involved in a number of neuronal process not directly related to cytoskeletal

reorganization. In vitro studies show that PAK7 is located primarily at mitochondria, where it contributes to anti-apoptotic signaling through phosphorylation and subsequent inhibition of BAD; PAK7 translocation to the nuclear compartment abolishes protection mediated by pro-apoptotic stimuli (Cotteret & Chernoff 2006). Regulatory domains within the C and N-terminal domains of PAK7 are targeted by multiple Rho proteins: these interactions are believed to be involved in subcellular localization of PAK7 (Wu & Frost 2006). Two substrates have been recently identified for PAK7 at synaptic terminals in the mouse brain - named pacsin1 and synaptojanin1 - which are involved in synaptic vesicles trafficking (Strochlic et al. 2012). The synaptic localization of PAK7 suggests that it might be involved in pathophysiological mechanisms relevant to neurodevelopmental disorders. Members of the PAK family have been involved in spine dynamics mechanisms being regulated by the SCZ risk factor DISC1, as a downstream effector of the DISC1/Kal-7/PSD95/Rac1 signaling as described above (Hayashi-takagi et al. n.d.). Spine dynamics and plasticity have been implicated in learning and memory which are important cognitive functions impaired in psychiatric (Kasai et al. 2010). The mechanisms of activation of PAK7 have not been fully clarified. Therefore, the possibility of a similar regulatory control needs to be investigated in order to gain insights on the pathophysiological role of PAK7.

1.6.1 *PAK7* as a risk factor for psychosis

We recently identified a rare duplication of about 149 kb mapping on *PAK7* gene associated with schizophrenia (SCZ) and BD or bipolar disorder (Morris et al. 2014). The duplication was identified as described below. A gene-based analysis of large (>100 kb) rare [$<1\%$ MAF (minor allele frequency)] CNVs was carried out in an Irish dataset of 1,564 schizophrenic cases and 1,748 controls. The analysis was extended with 5,196 UK controls in order to better determine the population frequency, and showed evidence of association ($P = 0.007$) at chr20p12.2, a locus containing *PAK7*. Association was replicated in an independent European population of 3,111 cases and 2,267 controls from UK, Portugal, Sweden and Bulgaria; in this replication sample 6 duplication events were found in cases (all from UK) and none in controls ($P = 0.052$). A second replication sample included bipolar disorder (BD) samples ($n = 2,243$) and independent controls ($n = 10,769$) from UK and provided further evidence of association ($P = 0.047$). A combined analysis of all Irish/UK subjects, which

included additional independent psychosis cases and controls, gave a P -value = 2×10^{-4} (OR = 11.3, 95% CI = 3.7, ∞), with 22 carriers in 11,707 psychosis cases and 10 carriers in 21,204 controls. This result represents an estimate of the pooled risk ratio for all discovery and replication cohorts included in the study, under the assumption of a fixed effects model, obtained using a Cochran-Mantel-Haenszel test. Nineteen of 22 case carriers and 8 of 10 control carriers showed very similar duplication size and start positions; haplotype analysis and sequencing of flanking regions identified a tandem duplication of about 149 kb overlapping the gene *PAK7*, which was in linkage disequilibrium with local haplotypes ($P = 2.5 \times 10^{-21}$), providing support for a single ancestral duplication event. The analysis of pedigrees of two Irish patients who carried the duplication, revealed co-segregation of the duplication with psychosis in two additional family members of one of the affected carriers.

1.6.2 *Pak7* knock out (KO) mice

Group II *Pak* knock out (KO) mice were generated - in which the *Pak* genes were inactivated - in order to investigate the biological function of each member of the family. While *Pak4* knock-outs (KO) embryonic lethal, *Pak7* and *Pak6* KO mice are viable and fertile. *Pak7* KO mice were generated by targeted disruption of *Pak7* gene as described below (Li & Minden 2003). The technique of homologous recombination was used to generate a targeting vector in which a 13-kb genomic region – including exon 3 and flanking intronic sequences of *Pak7* – was replaced by a 1.8-kb Neomycin resistance gene that contained a stop codons in each reading frames. The targeting vector was electroporated in embryonic stem cells (ES cells); Neo-resistant ES clones were screened by Southern blot, where the wild-type allele resulted in a 12-kb band and the KO allele resulted in a 7.7-kb band. *Pak7* heterozygous (*Pak7*^{+/-}) clones were expanded and injected into C57BL/6 blastocysts to generate chimeric mice. Chimeric males with more than 50% ES cells contribution were bred with C57BL/6 females. F₁ generation was genotyped using both Southern blot and genomic polymerase chain reaction (PCR) from tail DNA; heterozygous F₁ pups were crossed to generate homozygous KO mice that were obtained at the expected Mendelian rate (25% of total offspring). *Pak7* KO brains didn't show gross abnormalities as by histological analysis (Li & Minden 2003). However, subsequent behavioral tests revealed that *Pak7* KO animals exhibit worse coordination and balance measured by rotarod test, and lower levels of aggression measured

in a resident/intruder paradigm compared to wild type littermates (Furnari et al. 2013). *Pak7/Pak6* double knock-outs (DKOs) displayed wider deficits when compared to *Pak7* KO mice: additionally to impaired balance, coordination and aggression behaviors, DKOs showed lower open field activity, reduced sociability, and deficits in learning and memory evaluated performing active avoidance tests (Furnari et al. 2013). Taken together these results suggests a certain extent of functional redundancy among subfamily-II PAK members.

2. Working hypothesis and aims of the study

Schizophrenia is a neurodevelopmental disorder. The manifest disease is believed to be the end stage of abnormal development of the nervous system that begins years before the onset of the symptoms. Genetic findings show that SCZ is a highly heritable polygenic disorder. Interestingly, some genetic findings converge on synaptic proteins which are likely neurodevelopmentally regulated and might synergistically contribute to pathophysiological mechanism throughout life (Walsh et al. 2008; Elia et al. 2010; Guilmatre et al. 2009; Glessner et al. 2010; Kirov et al. 2012). Functional investigation of rare high risk variants in animal and cellular model might help to clarify mechanisms of disease based on causative penetrant mutations. Genetic rodent models of *Disc1*, a high risk gene for SCZ, show behavioral and anatomical features relevant to SCZ. In this study a SNP mutant was used, in which the interaction between the interaction of DISC1 and PDE4b, another risk factor for SCZ, is disrupted. Since the late onset of the human illness, studies on rodent genetic models of risk factors for SCZ to date have mainly focused on adult animals. The systematic study of cellular mechanisms at different developmental stages has not been carried out and might be important in order to gain insight on the neurobiology of SCZ genes. Our hypothesis is that behavioral impairment and brain abnormalities observed in adult *Disc1* mutants, are determined by disruption in the expression of neuronal and synaptic markers that starts at early developmental stages and that specific alterations occur in different brain areas of mutants during development. We also hypothesize that different susceptibility factors for SCZ play synergistic role in the pathophysiology of the disease in a spatial-temporal regulated manner. Based on these assumptions, the aims of this work are:

- 1- To identify changes in the protein expression of molecular markers involved in neuronal function, between *Disc1* L100P mutants and wild type, at different postnatal stages.
- 2- To clarify if the identified changes occur differentially at selected brain areas, known to be involved in SCZ, such as PFC (prefrontal cortex), HP (hippocampus) and CRB (cerebellum).
- 3- To characterize in vitro and in the mouse brain the SCZ risk factor *PAK7*, and explore its neurophysiological interaction with DISC1 at the protein level.

3. Materials and Methods

3.1 Animals, tissue collection and storage

CD1 wild type, *Disc1*-L100P and *Pak7* KO mice of four different postnatal stages were used for the experiments described in this thesis. *Disc1*-L100P and *Pak7* KO have a C57Bl/6 genetic background; while mating within our *Pak7* KO colony were performed among animals homozygous for the mutant allele, the *Disc1* mutant colony was maintained by crossing heterozygous animals. Therefore, *Disc1* litters were mixed, and animals were genotyped since only mutants L100P-homozygous were used for WB (Western blot) and IHC (immunohistochemistry) experiments reported. For WB and IHC, *Disc1*-L100P mutant mice and C57Bl/6 WT (wild type) littermates at postnatal day 8-10 (P8-10), P30-40 and P>60 were used; for co-IP (co-immunoprecipitation), *Pak7* mutants and C57Bl/6 WT at P30-40 and P>60 were used. The animals described as P>60 were in a range of age between P60 and P80. For primary cell culture, subsequent transfection and/or immunocytofluorescence CD1 WT and *Pak7* KO pups at P0-1 were utilized. Animals of either sex were used in all cases, and sex ratio was not monitored in this study. All procedures were carried out according to the institution regulations for animal handling.

3.2 DNA extraction and real time-PCR

Disc1-L100P mice used for WB experiments were euthanatized by decapitation (P8-10) or cervical dislocation followed by decapitation (P30-40 and P>60), while *Disc1* mice used for IHC were deeply anaesthetized and perfused with PBS (phosphate buffered saline) followed by a solution containing 4% paraformaldehyde (Thermo Scientific, 28908) made in PBS. In both cases, tail tips were cut following euthanasia procedure and collected in single labelled tubes, then stored at -20°C for DNA extraction and genotyping. In order to determine mice genotype (wild type, heterozygous or homozygous for the L100P mutation), allelic discrimination assay was performed by real time-PCR starting from mice tail tissue. DNA was extracted from each tail using Wizard SV Genomic DNA Purification System (Promega, A2361). Tissue samples were singularly placed in 0.5 ml labelled tubes; approximately 1.5 cm of tail tissue was immersed in 275µl of lysis buffer containing 200µl Nuclei Lysis Solution, 50µl 0.5M EDTA pH=8, 20µl Proteinase K (Promega, V3021) and 5µl RNase solution. Samples were then incubated for 18 hours at 55°C using a PCR thermal cycler.

DNA isolation from lysed tissue samples was performed at RT (room temperature) according to the manufacturer instructions and consisted on a few steps of samples filtering and washing, followed by DNA elution with nuclease free water. DNA concentration for each sample was determined by spectrophotometric reading (Nanodrop, ND8000). DNA samples were stored at -20°C. Genotypes were established by multiplexed real time-PCR performed using the Applied Biosystems 7900HT sequence detector (SDS 2.3 software): a primers/probe specific for the wild type allele and a primers/probe specific for the mutant allele, which target the SNP (single nucleotide polymorphism) site, were used in each reaction. Samples were loaded in triple into 96-well plates and contained: 10ng DNA, TaqMan Universal Master Mix no AmpErase UNG (Applied Biosystems, 4324018), and a premade TaqMan assay primers/probes mix purchased from Applied Biosystems. The primers/probes mix contained forward/reverse *Disc1* primers (*Disc1_F*: CCA CTG CCA AGC CTC ACT; *Disc1_R*: GGC CAC AGC AGG GAC AA) and genotype-specific MGB (minor groove binder) probes, which were conjugated to different reporter dyes (*Disc1* wild type allele: AGC TCT TGA GAA AAG, VIC-conjugated; L100P mutant allele: AGC TCT TGG GAA AAG, 6-FAM-conjugated). Nuclease free water was used as a negative control and genotypes were established by evaluation of the amplification curves generated by the machine and plotted as reporter fluorescence against PCR cycle. Figure 4 shows typical amplification plots generated by allelic discrimination assay on representative wild types and animals heterozygous and homozygous for the L100P single nucleotide mutation. Only homozygous wild type and L100P mutant animals were selected for WB and IHC experiments described in this thesis.

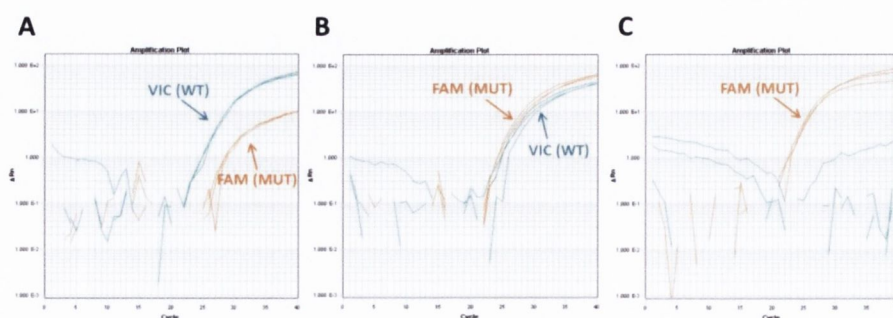


Figure 4. Typical amplification plots generated by allelic discrimination assay on representative animals: wild type (WT) (A), WT/L100P heterozygous (B) and L100P/L100P homozygous (C).

3.3 Total protein extraction and synaptoneurosomal preparation

For co-IP (co-immunoprecipitation) and WB (western blot), *Disc1*-L100P at P8-10, P30-40, P>60 and *Pak7* KO at P30-40 and P>60 were euthanatized as above, brain and separated CRB (cerebellum) tissue were dissected from each animal, collected in single labelled tubes and stored at -80°C for further processing. Total proteins were extracted from wild type P30-40 and P>60 brains without CRB: glass-glass hand homogenization was performed in ice using daily fresh lysis buffer containing 10mM Tris-HCl pH=8, 0.1M sodium chloride, 4mM EDTA, 0.1% nonidet P40 and added with Protease Inhibitor Cocktail (cOmplete Mini, EDTA-free, Roche Diagnostic, 11836170001); homogenates were centrifuged for at 4°C for 20 minutes at 13,000g and supernatants stored at -80°C for preliminary co-IP experiments. For WB and co-IP, a specialized protein extraction procedure was performed in order to obtain synaptoneurosomal-enriched, cytoplasmic/synaptoneurosomal-depleted and nuclear enriched protein extracts from whole forebrain or CRB tissue. Synaptoneurosomes are particles consisting of presynaptic- (synaptosome) and attached postsynaptic-elements (neurosomes). The procedure was adapted from classical cell fractioning (Hollingsworth et al. 1985) as described below.

All steps were performed in ice to minimize protein degradation processes. Full forebrain or cerebellum tissue from each animal was glass/glass homogenized in ice-cold protein extraction buffer with about 20 strokes or until complete lysis. The buffer contained: HEPES 10mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2mM EDTA, 2mM EGTA (ethylene glycol tetraacetic acid), 150mM sodium chloride to extract protein destined for direct western blot analysis; or 10mM Tris Base, 0.1M sodium chloride, 4mM EDTA, 0.1% nonidet P40 to make protein extracts to be used for immunoprecipitation. In both cases, protein extraction buffer was made daily fresh, added with Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail 3 (Sigma, P0044). The volume of buffer used for total protein extraction or synaptoneurosomal preparation depended on the age of the brain and varied between 500 and 1000µl. In all cases, synaptoneurosomal preparation was carried out according to the protocol below. Homogenate was centrifuges at 1000g for 10 minutes at 4°C, in order to separate heavy cellular membranes and nuclei which precipitated as a first pellet or P1 fraction. The resulting supernatant (S1) contained cytosolic proteins, lighter organelles and subcellular formations, including synaptoneurosomal entities. S1

fraction was moved to a clean tube for further processing, while P1 was resuspended in protein extraction buffer and kept in ice. S1 was subsequently passed through two 105µm polypropylene mesh (Amazon US) and a 5µm pore nitrocellulose filter (Millipore, SVLP01300) with the aid of a small syringe and spun at 1000g and 4°C for 15 minutes. After centrifugation, the supernatant (S2), which had been depleted of synaptoneurosomal entities, was moved to a clean tube and kept in ice; the pellet (P2) contained synaptoneurosomes and was resuspended in protein extraction buffer. All samples were subjected to medium-power sonication at 10 seconds intervals for a total time 2 minutes, with the exception of samples dedicated to co-IP, as ultrasonic waves would have disrupted most native protein-protein interaction. Protein concentration in P1, S2 and P2 fractions was determined by spectrophotometric reading (Nanodrop, ND8000). Samples were stored at -80°C for co-IP and WB experiments. Figure 5 provides a schematic description of the main steps of the cellular fractionation protocol.

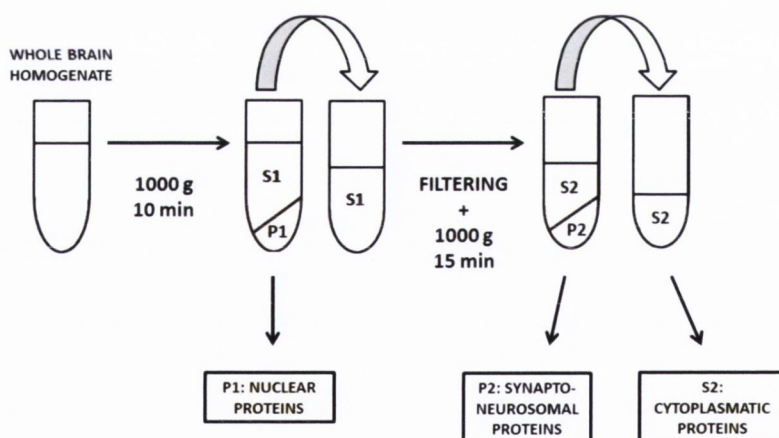


Figure 5. Schematic representation of the synaptoneurosomal preparation protocol. Whole brain homogenate was centrifuged to pellet the nuclear fraction (P1). The supernatant S1 was filtered and further centrifuged to pellet the synaptoneurosomal fraction (P2); the resulting supernatant (S2) represents the cytoplasmic/synaptoneurosomes-depleted fraction.

Figure 6 shows a representative Western blot for MAP2 (cytoplasmic neuronal protein) and PSD95 (synaptic protein) in S2 and P2 extracts obtained from P8-10 and P>60 brains.

Western blot protocol is described in section 3.4: for this experiment, antibody anti-MAP2 (Millipore MAB3418, made in mouse) was diluted in 5% milk/TBS-Tween at the concentration of 1:2500 and antibody anti-PSD95 (Cell Signaling #2507, made in rabbit) was used at the dilution of 1:2500 in 5% BSA/TBS-Tween. Incubation with primary antibodies was followed by incubation with a secondary antibody anti-mouse conjugated to horseradish peroxidase or HRP (Cell Signaling, #7076) and anti-rabbit-HRP-conjugated (Cell Signaling, #7074) used both at 1:10,000; immunodetection of β -actin was achieved as described below.

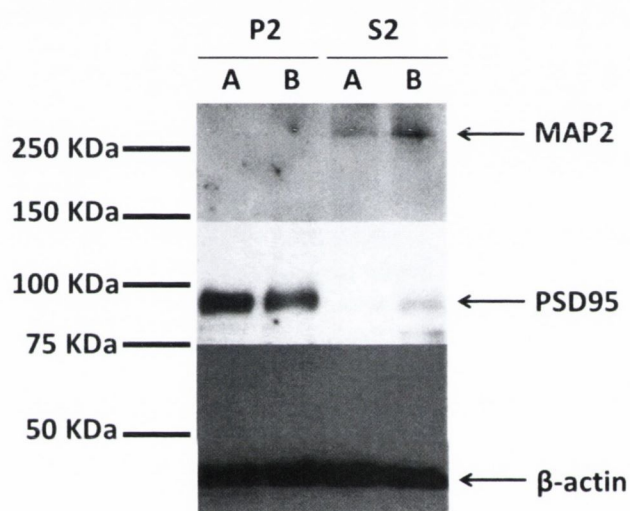


Figure 6. Representative Western blot for MAP2 and PSD95 in S2 and P2 protein extracts obtained from P8-10 (A) and P>60 (B) brain tissue. Cytoplasmic synaptoneurosomal-depleted protein extracts (S1) are enriched in cytoplasmic neuronal protein MAP2. β -actin immunolabeling indicates equal load of total proteins for each lane.

3.4 Western blotting immunodetection

For WB experiments, 50-70 μ g of P1, S2 or P2 extracts were added with reducing sample buffer (Invitrogen, NuPAGE NP0007, LDS sample buffer 4X), reducing agent (Invitrogen, NuPAGE NP0009, reducing agent 10X) and deionized water. Samples were then heated at 100°C for 5 minutes (in order to achieve protein reduction and denaturation necessary for their electric current-mediated migration) and run through 10% vertical acrylamide gel (containing Tris-HCl pH=8.8, SDS (sodium dodecyl sulphate), ammonium persulphate and TEMED (tramethylethylenediamine) as a polymerizing agent, along with colored molecular

weight marker (Bio Rad, 161-0374), for about 70 minutes at 200V. Gels were blotted onto PVDF (polyvinylidene difluoride) membranes (Sigma, P2688) previously activated in methanol and rinsed in TBE (Tris/Borate/EDTA) buffer. Successful protein transfer to PVDF membrane was confirmed by red Ponceau staining: the solution was made in deionized water with 5% acetic acid and 0.1% (w/v) Ponceau S powder (Sigma, P3504). After destaining with water and 0.5% tween20/TBS (Tris buffered saline), membranes were incubated for 1 hour at RT in a buffer made of 5% nonfat milk/0.1% tween20/TBS for aspecific binding sites blocking probed overnight at 4°C with primary antibody mix.

The primary antibody mix consisted in a blocking buffer made as above added with a primary antibody. Milk was replaced by BSA (albumin from bovine serum, Sigma, A9418) when specifically required by the antibody manufacturer. After 16-18 hours probing membranes were washed 3 times for 5 minutes with washing buffer (0.5% tween20/TBS) in order to remove unbound antibody molecules, and incubated for 1 hour at RT in a secondary antibody mix. Secondary antibody mix was prepared with 5% nonfat milk/0.1% tween20/TBS added with a secondary antibody either conjugated with HRP or biotinylated (when amplification of signal was required). One hour later membranes were washed 3 times for 5 minutes with washing buffer and incubated in amplification buffer if necessary: this buffer was made by diluting 1:2500 both solution A and B of the ABC Vectastain kit (Vector, PK-6100) in 0.1% tween20/TBS. Detail about antibodies used, concentrations and dilutions is reported by Table 1; Table 4 reports selected literature references of previous use of the antibodies when available. All membranes were probed for one or more antigens of interest and for a loading control (β -actin for S2 and P2 samples; Lamin A/C for P1 samples), which is needed during the analysis to establish the amount of protein of interest relatively to the amount of total protein loaded in each lane. For each experiment, antigen of interest and loading control results were obtained separately as the original membrane was cut after blotting and the two immunoreactions carried out independently according to the procedure described above.

Immunodetection of the band signal was realized using a chemiluminescent HRP substrate (Millipore, WBKLS0500) and exposing photographic film (Sigma, Z370398) on the membrane typically between 10 seconds and 10 minute time. Films were developed using AGFA CP1000 equipment and scanned using a Chemidoc system (Bio Rad). Alternatively, chemiluminescence light was captured using a Fusion FX system (Vilber Lourmat), and

digital images produced by the Fusion Capture Advance software. These systems produced 8-bit images which can display 256 (2^8) shades of gray. In these images each photo site or pixel has a grey intensity value (or density) included between 0 and 255: 0 is pure black and 255 is pure white. When the band is not saturated, the intensity of the signal is directly proportional to the amount of antigen in the sample. Image pixels with a density of 255 represent overexposed sites saturated by light, as these are the values of maximum intensity that can be displayed. Thus, band saturation does not allow to estimate the amount of target protein in a sample. Here ImageJ was used in order to check minimum and maximum gray values of each blot image. All images used for analysis presented an intensity range higher than 0 and lower than 255. Additional criteria of sample exclusion from measurement of band intensity were used as follows. Ponceau solution was applied onto freshly blotted membranes in order to visualize the distribution of protein samples: Individual experiments were included into the analysis regardless intensity of the red staining as long as this was comparable among samples and did not show abnormalities as below. Samples that showed uneven staining or white spots were excluded as a result of incomplete protein transfer from the acrylamide gel to the membrane. Incomplete bands resulting from either incomplete protein transfer or from imperfect HRP-substrate reaction were not included in the analysis, as well as bands that showed up at a different molecular weight when compared with the other samples within the same experiment. Figure 7 reports examples of blots that were considered not suitable and so excluded from subsequent analysis according to the criteria above.

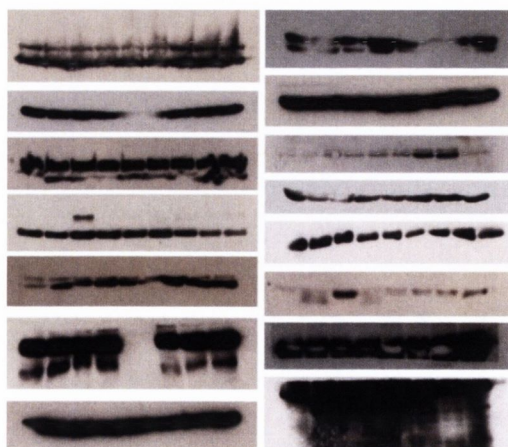


Figure 7. Examples of blot images considered not suitable for signal quantification analysis. The images shows images from blots that were excluded from quantification analysis due to diverse defects such as saturation, incomplete or defective transfer of the protein to the membrane, improper ECL reaction, impossibility to distinguish individual bands.

Intensity of blot bands was measured using ImageJ software as described below. For each experiment/image a selection box was created; the box was sized to include the whole area of each band and to exclude any portion of adjacent bands for each single measurement; when this was not possible, the band was not included in subsequent measurements. In general we aimed at obtaining a signal that allowed the easy detection of separation between lanes; where the borders were not distinguishable we placed the measuring box at the limit of the distinguishable border. Signal where both borders were not distinguishable were excluded from the analysis. The same box so designed was moved across the image as a selection area to measure the integrated density value of each band.

The integrated density was calculated by ImageJ as a product of area and mean gray area; because the selection area was maintained across lanes, the obtained value for each band reflected the mean gray value of each band making it possible to compare intensity values. For each sample in each experiment, the integrated density of the band belonging to the antigen of interest was divided to the integrated density of the correspondent loading control. Within the same blot, the raw ratios between antigen and loading control were converted to relative values by dividing each raw ratio by the average of the ratios of the wild type group: by doing this the density of each sample was expressed in wild type-fold, which allowed us to pool together density values from different experiments.

At least two independent experiments were carried out for each antigen at each age. Differences between groups (at least 6 animals per group, from at least two independent blots) were statistically assessed by Mann-Whitney test, and considered significant when P value was ≤ 0.05 .

ANTIGEN	PRIMARY ANTIBODY				SECONDARY ANTIBODY		
	HOST	PROD#	DILUTION	CONCENTR- -ATION	DESCRIP- -TION	PROD#	CONCENTR- -ATION
Synapsin	GP	SYSY 106 004	M	1:3000	α -GP-BIOT	Vector BA-7000	1:7000
PSD95	RB	Cell Signaling 2507	B	1:1000	α -RB-BIOT	Vector BA-1000	1:2000
Gephyrin	RB	Abcam AB25784	M	1:500	α -RB-BIOT	Vector BA-1000	1:1000
GAD67	MS	Millipore MAB5406	M	1:3000	α -MS-HRP	Cell Signaling 7076	1:7000
GAD65	GP	SYSY 198 104	M	1:2000	α -GP-BIOT	Vector BA-7000	1:5000
pAKT	RB	Cell Signaling 4058	B	1:500	α -RB-BIOT	Vector BA-1000	1:1000
pGSK3 β	RB	Cell Signaling 9323	B	1:1000	α -RB-BIOT	Vector BA-1000	1:2000
ERK1/2	RB	Cell Signaling 4370	B	1:500	α -RB-BIOT	Vector BA-1000	1:1000
pCaMKIV	RB	Santa Cruz SC-28443	M	1:1000	α -RB-BIOT	Vector BA-1000	1:2000
PAK7	RB	Sigma SAB-3500335	M	1:1000	α -RB-HRP	Cell Signaling 7074	1:10,000
nNOS	RB	Cell Signaling 4236	B	1:1000	α -RB-BIOT	Vector BA-1000	1:2000
CREB	MS	Cell Signaling 9104	M	1:2000	α -MS-HRP	Cell Signaling 7076	1:4000
pCREB	RB	Cell Signaling 9191	B	1:500	α -RB-BIOT	Vector BA-1000	1:1000
DISC1	RB	Invitrogen 40-6900	M	1:1300	α -RB-BIOT	Vector BA-1000	1:3000
β -actin	RB	Cell Signaling 4967	B	1:4000	α -RB-HRP	Cell Signaling 7074	1:8000
Lamin A/C	MS	Cell Signaling 4777	M	1:2500	α -MS-HRP	Cell Signaling 7076	1:7000

Table 1. Details about primary (AbI) and secondary (AbII) antibodies used in western blot. AbI: host, brand, product number, dilution buffer and working concentration are listed. AbII: conjugated molecule, brand, product number, working concentration in milk are listed. Abbreviations: GP = guinea pig; RB = rabbit; MS = mouse; M = milk buffer; B = bovine serum albumin (BSA) buffer; BIOT = biotin; HRP = horseradish peroxidase.

3.4.1 Characterization and validation of antibodies used for WB

The antibodies used for the experiments presented here are listed in Table 1, and previous uses of the antibodies found in literature are referenced in Table 4 in the methods chapter. The majority of WB experiments (synaptic and cytoplasmic antigens) employed β -actin as a loading control. The antibody anti- β -actin was made in rabbit, and consistently produced a single band at the expected MW correspondent to 43 kDa (Figure 8). For nuclear protein measurement, the nuclear structural proteins lamin A/C were used as a loading control; the antibody used was made in mouse, and produced two bands at the expected MW of 74 kDa and 63 kDa respectively (Figure 9).

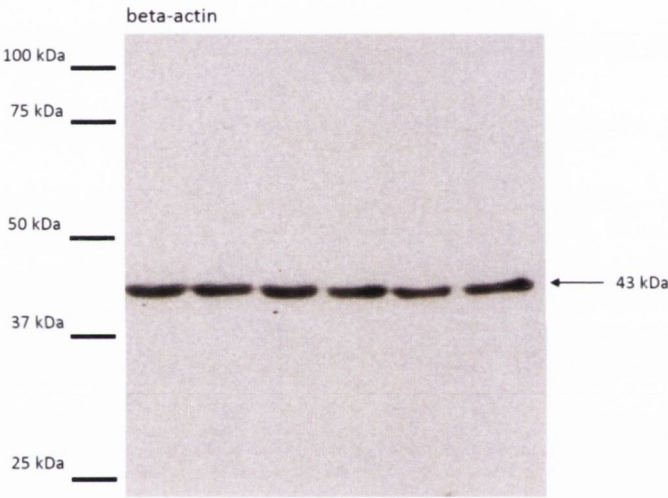


Figure 8. Representative blot for β -actin. The full-size blot shows an individual band (black arrow) resulting from incubation with the antibody anti- β -actin (Cell Signaling, #4967, produced in rabbit).

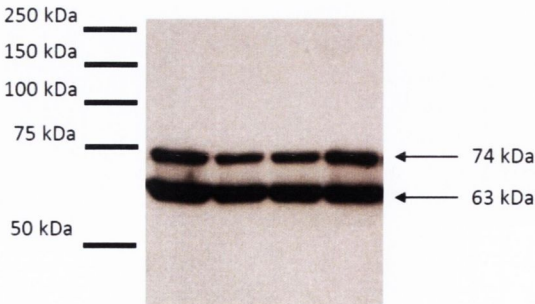
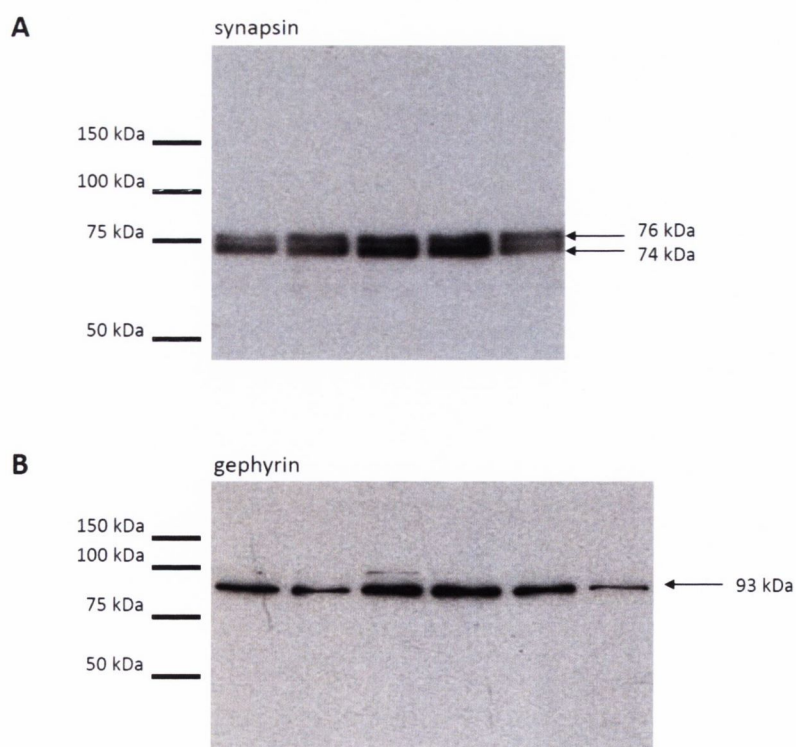


Figure 9. Representative blot for lamin A/C. The full-size blot shows two bands (black arrows) resulting from incubation with the antibody anti- lamin A/C (Cell Signaling, #4777, produced in mouse).

Antibody variability was observed within individual experiment performed for the same antigen: differences of intensity and size of the bands and occasional appearance of extra-bands of different molecular weight (MW) were present despite of keeping constant experimental conditions. Factors determining such variability might include significant changes of atmospheric temperature during any of the protocol steps (protein transfer from the gel to the membrane, primary and secondary antibody incubation, HRP-substrate reaction), and the different batches of antibody used over the duration of the project.

The antibody anti synapsin I/II produced two bands detectable at 74 and 76 kDa, corresponding to the expected MW (molecular weight) of Ia and IIa isoforms of the protein (Figure 10-A). The antibody anti-gephyrin produced a single band at the expected MW of 93 kDa (Figure 10-B); while the antibody anti-PSD95 produced two visible bands at the expected MW of 95 kDa and another one at approximately 70 kDa (Figure 10-C). Since the 95 kDa band is almost undetectable in synaptoneurosomal-depleted protein extracts while the 70 kDa is still visible in these extracts, we conclude that the 70 kDa band was aspecific, and probably given by reaction of the antibody with a peptide different from PSD95 containing an epitope similar to the PSD95 epitope used to produce the antibody.



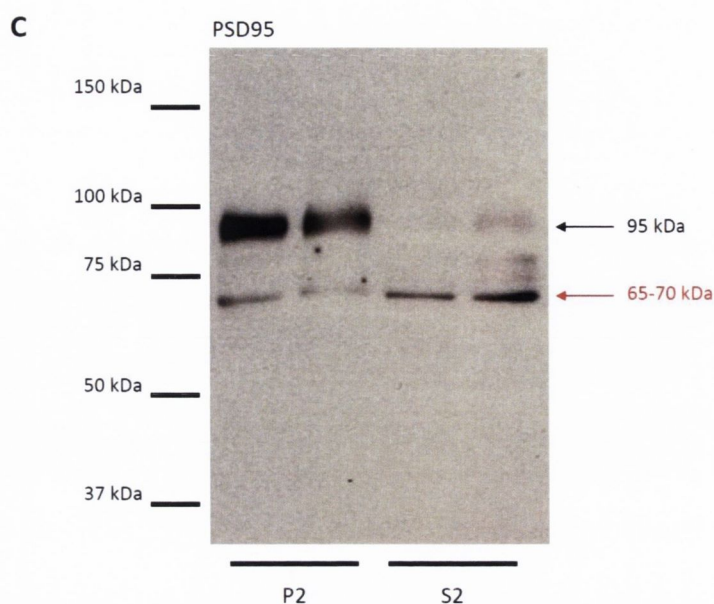


Figure 10. Representative blots for synapsin I/II, gephyrin and PSD95. The full-size blots show specific (black arrows) and aspecific (red arrows) bands resulting from incubation with the following antibodies. A: guinea pig anti-synapsin I/II (SYSY, 106 004); B: rabbit anti-gephyrin (Abcam, ab25784); C: rabbit anti-PSD95 (Cell Signaling, #2507). Specificity of the 95 kDa band was further demonstrated by its absence in synaptoneurosomal-depleted protein extracts (S2). P2 = synaptoneurosomal-enriched protein extracts.

Antibodies anti-GAD67 and anti-GAD2 (GAD65), produced a single band signal detectable at 67 kDa and 65 kDa respectively (Figure 11-A and 11-B).

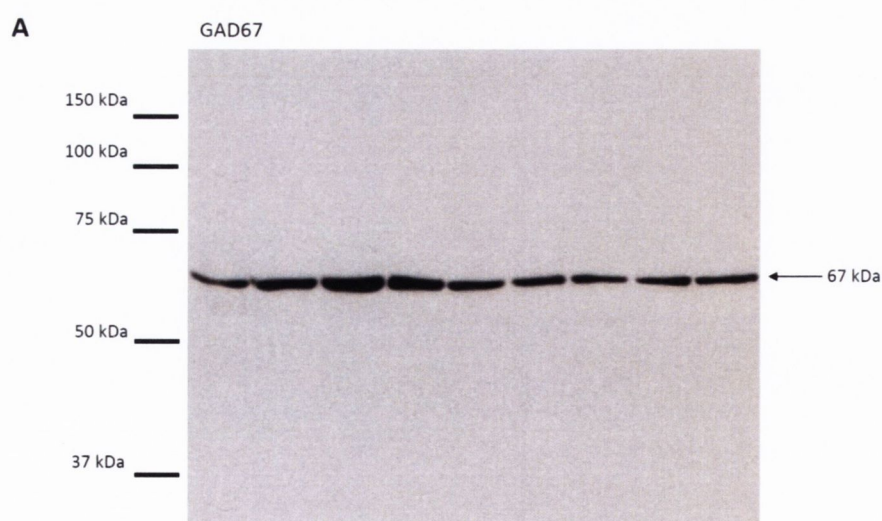




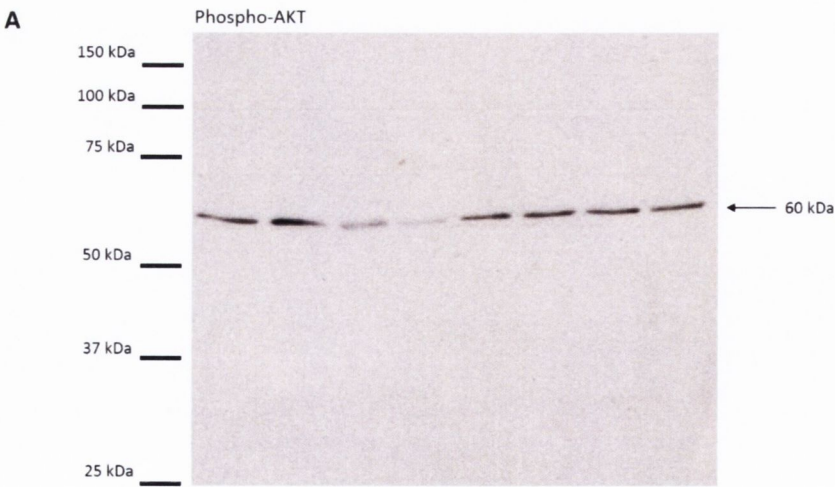
Figure 11. Representative blots for GAD67 and GAD65. The full-size blots show specific bands (black arrows) resulting from incubation with the following antibodies. A: mouse anti-GAD67 (Millipore, MAB5406); B: guinea pig anti-GAD2/GAD65 (SYSY, 198 104).

The antibody anti- total CREB produced a single band at the expected MW of 43 kDa (Figure 12). Full size blot images are not available for pCREB as portion of membrane were cut and incubated with different antibodies in all experiments; the possibility of detection of aspecific bands in a broader range of molecular weights cannot be excluded for this protein, the identified band for the portion of membrane incubated with the used antibody shows a band at the expected pCREB MW (43 kDa). Although a band of the expected MW was detected in pCREB blots, a strong background staining was also present due to unavoidable contamination of the nuclear protein fraction (P1) with heavy cellular debris.



Figure 12. Representative blot for total CREB. The full-size blot show a specific band (black arrow) at the expected MW (molecular weight) of 43 kDa resulting from incubation with mouse anti-CREB (Cell Signaling, #9104).

The antibody anti- pAKT produced a band at the expected MW of 60 kDa, as well as the antibody anti-pERK1/2 that produced two specific bands of 42 kDa and 44 kDa respectively (Figure 13-A and 13-C). Levels of pERK1/2 were combined during measurement of intensity; individual levels of pERK1 and pERK2 were not established. The antibody anti-pCaMKIV produced a band of the expected MW (60 kDa) and a band of approximately 95 kDa that was considered aspecific based on the expected MW. The 95 kDa band might result from low specificity of the antibody that might recognize a similar epitope of an unrelated brain protein. The possibility that the aspecific band corresponds to pCaMKII isoform as the predicted MW for this antigen would be 50 kDa (Figure 13-D). The antibody anti- pGSK3 β produced a band at the predicted MW of 46 kDa; however, an additional band, which was not individually distinguishable appeared at about 48 kDa. The 48 kDa was in some cases less intense and in others more intense than the 46 kDa one. The possibility that the 48 kDa band corresponds to the alternative kinase isoform pGSK3 α cannot be excluded, although the predicted MW for the isoform α would be 51 kDa and it should then appear above the 51 kDa step of the MW ladder. The 46 kDa and 48 kDa bands were combined for the quantification of the intensity, due to the impossibility to distinguish the borders of the individual band using the same selection box for the analysis of lanes in the same blot (Figure 13-B).



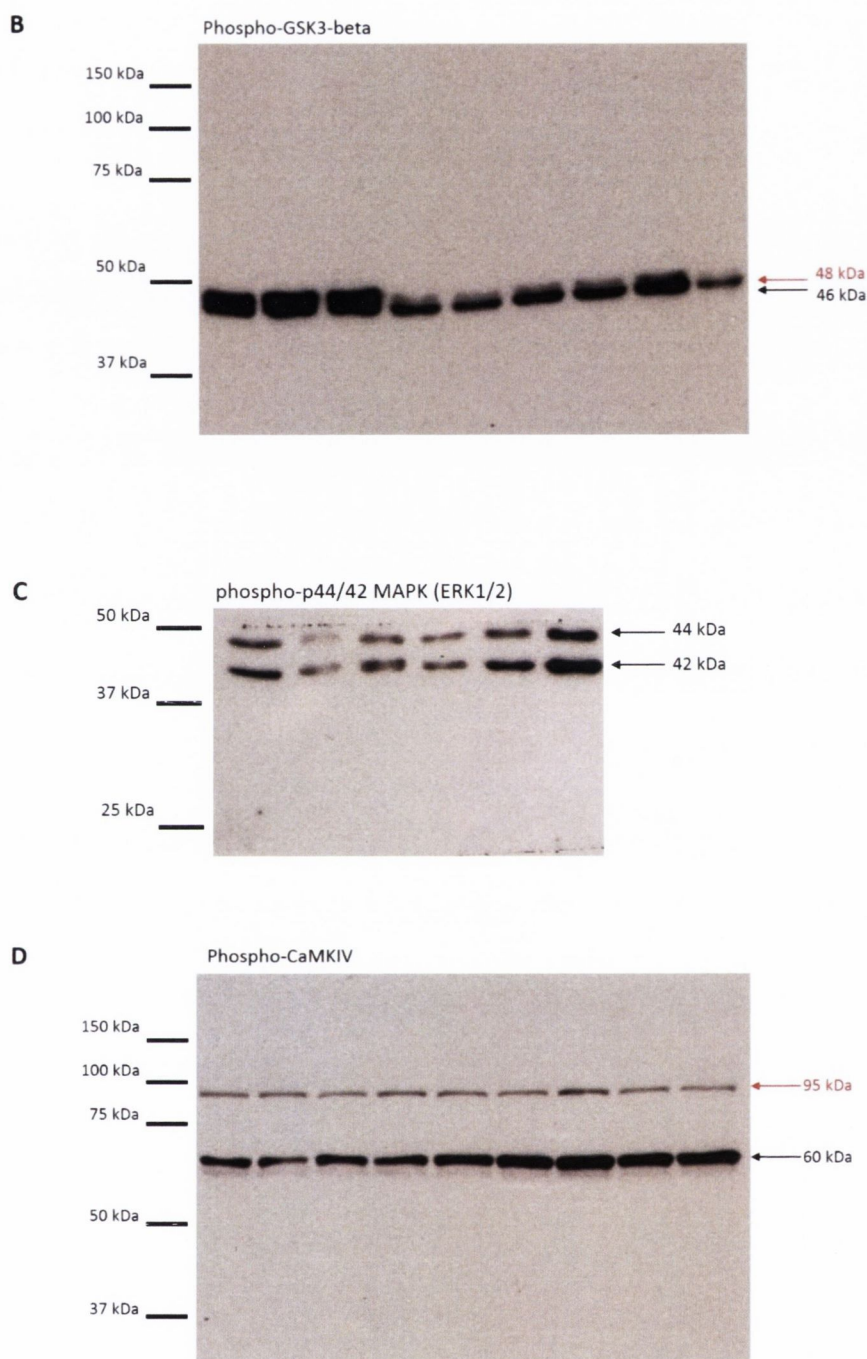


Figure 13. Representative blots for pAKT, pGSK3 β , pMAPK (pERK1/2) and pCaMKIV. The full-size blots show specific (black arrows) and aspecific (red arrows) bands resulting from incubation with the following antibodies. A: rabbit anti-pAKT (Cell Signaling, #4058); B: rabbit anti-pGSK3 β (Cell Signaling, #9323); C: rabbit anti-pERK1/2 (Cell Signaling, #4370); D: rabbit anti-pCaMKIV (Santa Cruz, sc-28443).

The antibody anti- nNOS produced two bands: a band of higher intensity was observed at the expected MW of 160 kDa, and a band of lower intensity with the observed MW of 140 kDa. One possible reason for the presence of the lower MW band is the incomplete specificity of the antibody and its subsequent cross-reaction with another isoform of NOS enzyme: the best candidate for the aspecific reaction was the endothelial isoform of the NOS (eNOS) with an expected MW 140 kDa. Only the 160 kDa band was considered specific and used for intensity measurements (Fig 14-A). The antibody anti- PAK7 produced in rabbit that was used for WT experiments produced a band at the expected MW of 81 kDa; specificity of this band was supported by its absence in total brain extracts from *Pak7* KO animals. A couple of additional bands were visible at MW of 100 kDa and approximately 60 kDa, which were considered aspecific because of their presence in both WT and *Pak7* KO brain extracts. In some cases, an additional band slightly heavier than the 81 kDa band was observed (approximately 83 kDa). The 83 kDa band was not attributed to cross-reactivity with other PAK-family members that have expected MW ranging from 58 to 75 kDa; moreover, the expected MW of the phosphorylated PAK7 isoform is 90 kDa. PAK7 might be subjected in theory to alternative forms of post-translational modification other than phosphorylation, which might explain the presence of an additional band of 83 kDa (Fig 14-B). When two band were present in PAK7 blots they were combined for the analysis of intensity.



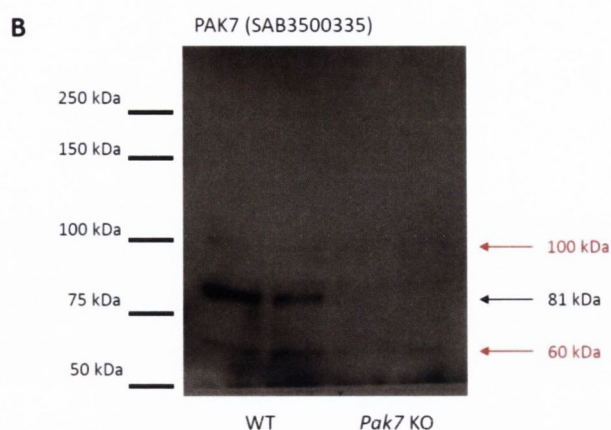


Figure 14. Representative blots for nNOS and PAK7. The full-size blots show specific (black arrows) and aspecific (red arrows) bands resulting from incubation with the following antibodies. A: rabbit anti-nNOS (Cell Signaling, #); B: rabbit anti-PAK5 (PAK7) (Sigma, SAB-3500335). The 81 kDa band correspondent to the PAK7 protein in WT brain extracts was not detected in extracts made from *Pak7* KO.

3.5 Co-Immunoprecipitation (Co-IP)

Co-immunoprecipitation experiments were performed in order to assess protein interaction between DISC1 and PAK7, on both whole brain extracts and synaptoneurosomes-enriched (P2) extracts, from P30-40 and P>60 brains. For experiments testing P2 extracts, *Pak7* KO brains were used as a negative control.

All steps were performed at 4°C to minimize protein degradation processes. Whole brain extracts were prepared from P30-40 and P>60 by glass/glass homogenization (until complete lysis of the tissue), using a non-denaturing/low ionic strength protein extraction buffer containing 10mM Tris Base, 0.1M sodium chloride, 4mM EDTA, 0.1% NP40 with protease inhibitors. The homogenates were spun at 1000g for 20 minutes to pellet down undigested tissue and debris that were discharged.

Synaptoneurosomal extracts were prepared as discussed in section 3.3 using whole brain of either wild type or *Pak7* KO tissue. Protein extraction preceding co-IP experiments were mild in order to preserve native protein-protein interactions.

Immunoprecipitation of a target protein in the sample was achieved by using an antibody specifically raised against the target; in order to exclude the possibility of aspecific antibody binding, samples were prepared in double, and immunoprecipitated with either antibody anti-target (immunoprecipitation/IP sample) or normal immunoglobulins of the same animal species that produced the antibody anti-target (IgG sample). Thus, for each tested interaction,

two 500µl similar samples containing 1000µg proteins diluted in protein extraction buffer were made from the same protein extracts. All samples underwent a pre-clearing step, in which 20µl of agarose beads (A/G PLUS-agarose, Santa Cruz Biotechnology, sc-2003) were added in order to clear up the protein extract from aspecific molecular binders that would have low affinity for the support. Tubes were spun at 2500rpm for 5 minutes to pellet agarose beads and possible aspecific binding molecules; supernatant was moved to clean tubes. Cleared samples were incubated overnight on a rocking platform with either 4µg of antibody anti-PAK7 (polyclonal goat, S-16, Santa Cruz Biotechnology, sc-22155) or 4µg of antibody anti-DISC1 (polyclonal goat, N-16, Santa Cruz Biotechnology, sc-47990); in both cases normal goat IgG (Sigma, I5256) was used as a negative control (IgG sample). During this step, the antibodies established specific molecular interaction with the target proteins in the extracts; normal IgG only minimally bound to the target protein due to unavoidable aspecific interactions. After approximately 16 hours, 20µl of agarose beads were added to all samples that were returned to the rocking platform for 4 hours. A/G agarose beads have high affinity for goat immunoglobulins: because of this property during the 4 hours the beads bound to the antibodies anti-PAK7 or anti-DISC1, or to normal goat IgG in the samples. This step resulted in formation of complexes consisting of: target antigen and its interactors, antibody anti-target and agarose bead. Taking advantage of the bead's density, complexes were purified from each sample through 12 washing steps. Each wash was carried out as follows: IP and IgG samples were spun at 2500rpm for 5 minutes to pellet beads and bound molecules, supernatant (containing unbound proteins) was discarded and pellet was resuspended by gentle tube inversion for about 1 minute using non-denaturing washing buffer (5mM Tris-HCl pH=7.5, 2mM EDTA, 2mM EGTA pH=8, 0.1M sodium chloride, 0.2mM sodium orthovanadate, 0.1% NP40, added with protease inhibitors). After the last wash, supernatant was discarded and beads resuspended with 20µl denaturing loading buffer for subsequent western blot. Tubes were heated at 80°C for 5 minutes in order to improve the elution of the protein complexes from the beads. Eluate from each sample contained antibodies/immunoglobulins fragments, target antigen and protein interactors of the target, and was further heated at 100°C for 5 minute for protein denaturation as for western blot. Details about primary and secondary antibodies used in western blot after

immunoprecipitation are listed in Table 1. Table 4 reports selected literature references of previous use of the antibodies when they exist.

3.6 Immunofluorescence on tissue, imaging and analysis

Free floating-immunofluorescence was performed on brain sections from wild type animals at P8-10, P30-40 and P>60, and adult *Disc1*-L100P mutants. Animals were perfused; brains were dissected and stored in sucrose solution as described in section 3.2. Coronal 50µm-thick brain sections were obtained using a vibratome (Leica), and post-fixed in a PBS solution containing 4% paraformaldehyde for 20 minutes (P30-40 and P>60) or 2 hours (P8-10). Sections were then washed in PBS three times for 10 minutes and stored in PBS at 4°C before use. For quantification experiments in adult (P>60) WT and *Disc1* mutants, we selected sections at the level of brain areas previously involved in the pathogenesis of SCZ (prefrontal cortex/PFC, hippocampus/HP and cerebellum/CRB); for colocalization experiments we used sections at the level of brain cortex and hippocampus from WT animals at P8-10, P30-40 and P>60. Figure 15 shows the localization of these structure in the mouse brain, it was made using a 3-dimensional model obtained selecting the brain areas of interest in the Allen brain atlas online.

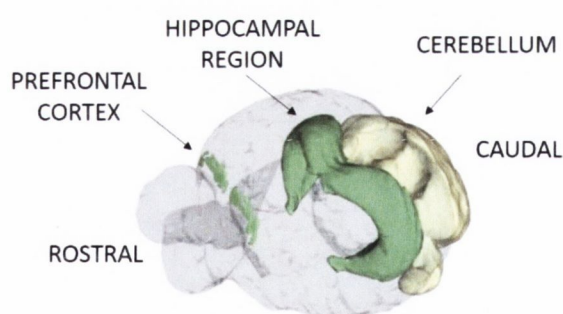


Figure 15. Localization of SCZ-related brain structures in the mouse brain. The 3-dimensional model created using the Allen brain atlas online provides approximate shape localization of prefrontal cortex (PFC), hippocampus (Hp) and cerebellum (CRB).

For each of the three studied regions, sections were selected using a mouse brain atlas as a reference (The mouse brain in stereotaxic coordinates, fourth edition, Elsevier, by Paxinos G. & Franklin K.B.J). Specific coronal plans of section are identified by a bregma value.

Bregma is a reference point on the skull defined as the intersection between coronal (or bregmatic) and sagittal sutures (Figure 16). Bregma value is positive if the plan of section is in a frontal position and negative when the plan is in caudal position with respect to bregma. Here we used sections within the following bregma intervals: 3.17/2.65 (PFC), -1.67/-2.53 (HP), -5.79/-6.11 (CRB).

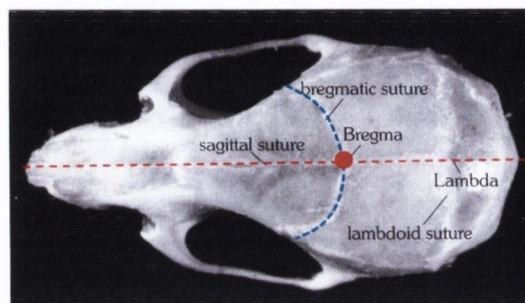


Figure 16. Dorsal view of adult mouse skull. The red dot marks the position of bregma: a point located at the intersection between bregmatic/coronal suture (underlined by blue dashed line) and sagittal suture (red dashed line) of the skull. The bregma projection of the brain provides a reference useful to establish a universal system of brain coordinates.

For quantification experiments, immunostaining procedures were carried out as follows. All protocol steps were carried out in gentle agitation at RT unless otherwise specified. Between one step to another, sections were gently moved from a well to another in the plate using a glass hooked Pasteur pipette. Sections were incubated in fresh made hydrogen peroxidase solution (0.1% H_2O_2 /PBS) for 5 minutes in order to deactivate endogenous peroxidases that could cause aspecific staining. Tissue was washed 4 times in PBS for a total time of 20 minutes and incubated for 1 hour in a solution of PBS containing 10% goat serum (Sigma, G9023) and 0.3% triton X-100 (Sigma, T8787). Triton X-100 was used to achieve tissue permeabilization for proper antibody penetration, and goat serum was used for covering aspecific binding sites. Sections were then probed overnight with two or three primary antibodies specific for different antigens, which were diluted in a PBS buffer containing 1%goat serum and 0.3% triton X-100: this step was performed at 4°C, in static conditions, and lasted approximately 48 hours in order to guarantee optimal primary antibody binding throughout the thickness of the tissue. A primary antibody (AbI) directed against NeuN

(neuronal specific protein) was added to AbI buffer when required, and used in phase of imaging as a marker of neurons. The excess of primary antibody was washed off with several changes of PBS for a total time of 2 hours. Washes were followed by incubation with secondary antibody mix, in which fluorophores-conjugated immunoglobulins were diluted in 1%goat serum/0.1%triton X-100/PBS for 2 hours: during this step, plate was covered with thin foil in order to preserve fluorescence. Fluorophores used included DyLight488 (green light emission), Cy3 (red light emission) DyLight649 (far red/purple light emission). For pCREB staining, which required signal amplification, a secondary antibody conjugated to biotin was used, followed by 1 hour incubation with ExtrAvidin-FITC conjugated. Fluorescein isothiocyanate (FITC) is a fluorophore that emits green light and can be used in alternative of DyLight488. Detail about primary and secondary antibodies used for free floating immunohistochemistry, concentrations, dilutions and amplification is reported by Table 2. Table 4 reports selected literature references of previous use of the antibodies when they exist. Tissue sections were finally washed 3 times for 10 minutes with PBS, and mounted onto glass microscope slices with the help of a small paintbrush, and using non-hardening mounting medium containing DAPI (Vector, H-1200). DAPI (4',6-diamidino-2-phenylindole) is a blue fluorescent dye with strong affinity for DNA: it was used to stain cellular nuclei. Slides were protected from light and kept at 4°C until they were imaged. For DISC1/PAK7 colocalization experiments, sections were stained as described above with the following specification: both anti-PAK7 (sc-22155, made in goat) and anti-DISC1 (Invitrogen 40-6900, made in rabbit) were used as primary antibodies, followed by anti-goat-DyLight488 (green staining) and anti-rabbit-DyLight649 (purple staining). These two secondary antibodies were chosen to be used for co-localization experiments because of their excitation/emission wavelength spectra; in fact the spectra of the two fluorophores only minimally overlap (Figure 17), excluding the possibility of a bleeding-through effect that would have otherwise affected conclusions on PAK7/DISC1 co-localization. For graphic purposes the representation of anti-PAK7 and anti-DISC1 staining were set as green and red respectively by using a LUT (look-up table); this allowed the overlapping puncta staining to appear yellow.

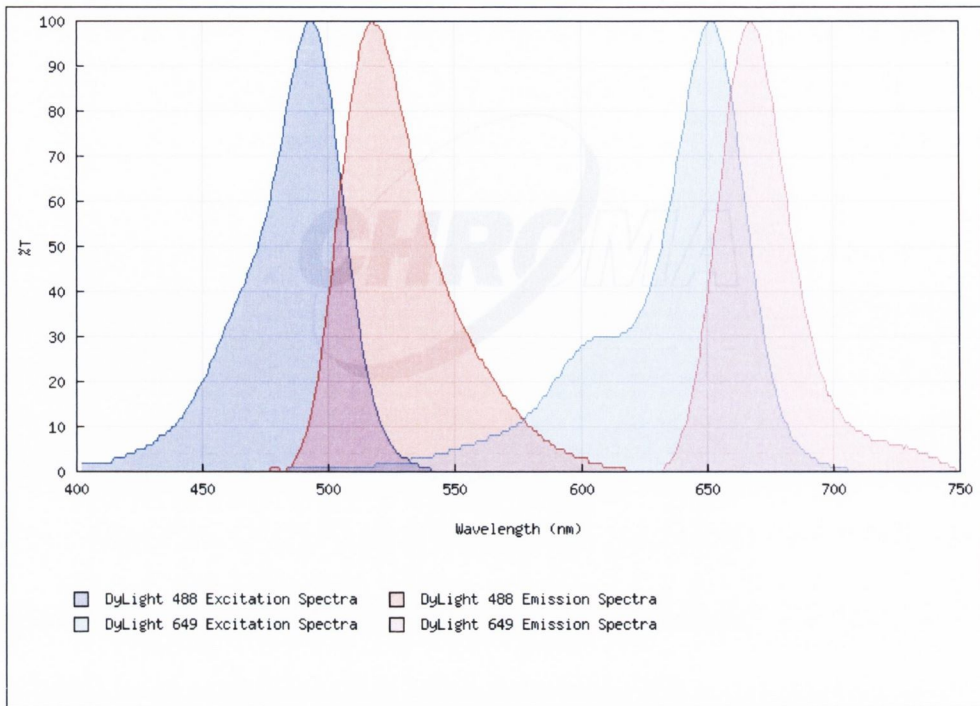


Figure 17. Excitation and emission spectra of DyLight 488 and DyLight 649 fluorophores. The image was obtained using the Croma Technology Corp. spectra viewer available online, and shows peaks and distribution of radiant energy (T = transmittance) of the two fluorophores at each given wavelength in the light spectrum. The emission spectrum of DyLight 488 only minimally overlap with the excitation spectrum of DyLight 649.

A control was performed for each experiment in order to evaluate the level of fluorescence produced by aspecific secondary antibody binding and the autofluorescence of the tissue: control sections were subjected to the same protocol of the test ones (described above), except that primary antibodies were omitted from the primary antibody mix, which only consisted in 1%goat serum and 0.3% triton X-100 diluted in PBS. The antibodies were added at the same concentration used for test sections in each given experiment. For the co-localization experiment, additional “single primary/double secondary antibodies” controls were performed on $P>60$ cortical sections as follows. Independent sections were incubated with primary antibody against either PAK7 or DISC1, following by incubation with secondary antibodies both anti-goat (PAK7) and anti-rabbit (DISC1) performed on all sections. All control sections for quantification as well as co-localization experiments were mounted onto microscope slides using DAPI-containing mounting media in order to ensure integrity of the tissue and to be able to detect the position of cellular nuclei.

For quantification experiments in WT and *Disc1* mutant P>60 brains, at least two individual brains were tested and at least two different sections at the level of each studied brain area were stained for each brain. For DISC1/PAK7 colocalization experiments during postnatal development, two sections from both cortex and hippocampus (dentate gyrus) were selected from single brains at P8-10, P30-40 and P>60.

Imaging of the stained sections was performed by confocal microscope (Zeiss). Areas of interest (PFC, CA1 and/or CA3/or dentate gyrus of hippocampus, CRB) were identified on each brain section and a single field was selected within the area of interest. In all cases, a Zeiss 63X magnification oil-immersion objective (numerical aperture/NA = 1.4) was used for confocal microscope imaging. The high NA of this objective allows an average resolution of about 200 nm for the wavelengths of excitation that were used according to the Abbe model of resolution in transmitted light microscopes ($\text{Res}_{x,y} = \lambda/2\text{NA}$), where λ is the wavelength of the light used. For reference, Chapter 1 of the following book may be viewed: “Imaging, a laboratory manual”, edited R. Yuste, CSH Press). Since the average diameter of a synaptic spine was measured to be about 400 nm (Schikorski & Stevens 1997) and the pixel size at 63X magnification is about 139 nm, the objective used was chosen in order to obtain a good visualization of the puncta resulting from immunostaining of synaptic proteins. For each field of view, a variable number of Z stacks were imaged through the thickness of the tissue, with a distance of 5 μm between two consecutive stacks; from the majority of the fields four consecutive stacks were imaged, and no more than five. Z stacks scanning imaging allows to acquire immunostaining signal from individual 5 μm thick focal plans, and visualize the punctate staining typical of synaptic proteins throughout the thickness of a tissue section; this made it possible to distinguish more clearly specific signal versus background staining. Intensity of immunostaining was measured for individual Z stacks and these measurements were averaged for each field of view: average values were incorporated in the Mann Withney test in order to assess differences in the expression of the markers in the same brain area between genotypes.

Following IS quantification, sequential Z stacks correspondent to the same field of view were collapsed into a composite single image or “projection” using the Zeiss software LSM Image Browser. Projections are presented here as a graphic representation of the results; projections result from combining multiple focal plans, therefore the punctate staining in these images is

sometimes not as recognizable as it is in single Z stacks.

In order to avoid image saturation and to assure unbiased image detection across experimental groups in all experiments, imaging was performed in blind by an expert operator as follows:

- 1) All the experimental groups for each experiments were imaged during the same session to avoid bias due to the intrinsic variability of the microscope (i.e. time of laser operativity), and all microscope parameters were selected so that no staining was present in the no primary antibody-control sections.
- 2) Sampling imaging for each experimental group was used to set up the superior and inferior thresholds of the parameters. The parameters considered were: laser intensity, pinhole, detection gain, offset. Laser intensity and pinhole were kept constant throughout each imaging sessions. In order to set amplification gain and offset we used the HiLow scale, which shows the level of saturation and understimulation for each pixel: this allowed signal detection to be in the linear range of stimulation-emission. Once conditions were set for one group, sample imaging was done on the other experimental groups to ensure that the conditions set were in the right range also for the other groups. Once the parameters were set, they were kept constant for all the images of each and every experimental group.

Co-localization of the two proteins in the same tissue sections was further investigated by two-photon microscope (Olympus), which was used to assess co-immunolabeling DISC1 and PAK7 throughout the thickness of the tissue in three dimensions.

As in all procedures of immunodetection, when signal is not saturated, intensity is directly proportional to the amount of antigen in the sample. Measurement of immunostaining (IS) intensity was carried out in blind using ImageJ and following different criteria of analysis, depending on brain area analyzed and subcellular localization of the antigen of interest. For quantification of IS intensity, single cells or broader tissue surface were selected: the sum of the value of bright pixels in the selected surface (or integrated density) was normalized to the measure of the area. Also, three surfaces of background were measured with the same criteria and average value was calculated. For each field, normalized background value was subtracted from normalized IS value, so as to obtain an IS value net of background. In all

cases differences between groups were statistically assessed by Mann-Whitney test, and considered significant when P value was ≤ 0.05 .

ANTIGEN	PRIMARY ANTIBODY			SECONDARY ANTIBODY		
	HOST	PRODUCT#	CONCENTRATION	DESCRIPTION	PRODUCT#	CONCENTRATION
Synapsin	GP	SYSY 106 004	1:1000	Cy3-conj GT anti-GP	JIR 106-165-003	1:500
PSD95	RB	Cell Signaling 2507	1:400	DL488-conj GT anti-RB	JIR 111-485-003	1:500
GAD65	GP	SYSY 198 104	1:500	Cy3-conj GT anti-GP	JIR 106-165-003	1:500
PDE4b	RB	Abcam ab14612	1:500	DL488-conj GT anti-RB	JIR 111-485-003	1:500
cAMP	MS	Abcam ab70280	1:500	DL649-conj GT anti-MS	JIR 115-495-003	1:500
CREB	MS	Cell Signaling 9104	1:500	DL649-conj GT anti-MS	JIR 115-495-003	1:500
pCREB	RB	Cell Signaling 9191	1:200	1-GT anti-RB biotinylated	Vector BA-1000	1:200
				2-ExtrAvidin FITC-conj	Sigma E2761	1:300
PAK7	GT	Santa Cruz PAK5(S-16) sc-22155	1:1000	DL488-conj DK anti-GT	JIR 705-485-003	1:500
DISC1	RB	Invitrogen 40-6900	1:500	DL649-conj GT anti-RB	JIR 111-495-003	1:500
NeuN	MS	Millipore MAB377	1:500	DL649-conj GT anti-MS	JIR 115-495-003	1:500

Table 2. Details about primary (AbI) and secondary (AbII) antibodies used in immunofluorescence on tissue sections. AbI: host, brand, product number, and working concentration are listed. AbII: host, conjugated fluorophores, specificity, brand, product number and concentration are listed. Abbreviations: GP = guinea pig; RB = rabbit; MS = mouse; GT = goat; DK = donkey; DL = DyLight; JIR = Jackson ImmunoResearch.

3.7 Primary neuronal cultures

Primary neuronal cultures were made from brain cortices of CD1 and *Pak7* KO pups at postnatal day 0 (P0) or P1. Animals were euthanatized by decapitation, brains were dissected right after in ice-cold sterile DPBS (Dulbecco's phosphate buffered saline, gibco, 14190-094) and finely minced in 1mm³ pieces. Tissue was moved to sterile tubes in a volume of 200 µl DPBS, added with 2 units DNase (DNase I RNase-free, New England BioLabs, M0303S) and 20µl trypsin-EDTA (solution of 0.025%trypsin and 0.01%EDTA in PBS, gibco, R-001-100) for cell dissociation. Tubes were incubated at 37°C and 5%CO₂ for 10 minutes, enzymatic reaction was then blocked by adding a double volume of DMEM media (Dulbecco's modified Eagle's medium-high glucose, Sigma, D5796) containing 10% FBS (foetal bovine serum GOLD, PAA, A15-101). Cells were mechanically dissociated by gentle pipetting and kept at room temperature to settle down for 5-15 minutes. Supernatant was collected from each sample and moved to clean tubes that were spun for 5 minutes at 10°C and 800rpm to pellet dissociated cortical cells. Supernatant was this time discharged; pelleted cells were resuspended in 10%FBS/DMEM and counted using a haemocytometer under a light microscope with 10X magnification. Cells were plated at the density of 1X10⁶ cells/ml, into 24well plates onto glass coverslips, previously coated with poly-L-lysine (Sigma, P4707). Plates were kept at 37°C/5%CO₂ and fed 2 hours later with feeding medium (FM) to achieve a volume of 500µl/well. Feeding medium contained: 95% neurobasal medium (neurobasal medium [-]-L-glutamine, gibco, 21103-049) containing 1% P/S (penicillin/streptomycin, 50 units/ml, gibco, 21103-049), 2mM L-glutamine (gibco, 15140-163), 0.45% glucose (D-(+)-glucose solution 45%, Sigma, G8769) and 2% B27 (50X B27 serum-free supplement, gibco, 17504-044). After 24 hours incubation at 37°C and 5%CO₂, cells were fed by replacing half FM volume with fresh FM containing 7µM AraC (cytosine β-D-arabinofuranoside, Sigma, C1768) in order to achieve a final concentration of 3.5µM AraC in each well. Cytosine β-D-arabinofuranoside or AraC is a mitotic inhibitor, which is used at low concentrations as an inhibitor of glial growth. Cells were kept at 37°C and 5% CO₂, checked using a light microscope and fed every second day by replacing half medium with fresh FM containing 3.5µM AraC. Cells were grown as above until 6 days in vitro (6DIV), when they were treated as described in detail below. At 8DIV cells were fixed for

20 minutes using a PBS solution containing 4%PFA (paraformaldehyde), washed with PBS and kept at 4°C until immunostaining experiment were carried out.

3.8 Chemical treatments on primary neuronal cultures

Neuronal primary cultures made from CD1 pups and grown as described in section 2.7, were treated with different protocols of chemical stimulation at either 6DIV or 8DIV. Protocol for chemical induction of LTP (long-term potentiation) and LTD (long-term depression) was adapted from (Roth-Alpermann et al. 2006; Solger et al. 2005), while rapid potassium depolarization, TTX and KCl treatments were performed using a protocol modified from (Wierenga et al. 2005; Righi et al. 2000). Stimulation procedures were changed and carried out as described below. All chemicals for stimulation were diluted using sterile deionized water in order to make master solutions used for treatments. Solution made with TTX (tetrodotoxin, Ascent Scientific, Asc-055), NMDA (N-methyl-D-aspartate, Sigma M3262) and TEA (tetra-ethyl-ammonium chloride, Sigma, T2265) were stored at -20°C and D-glucose solution (Sigma, G8644) was kept at 4°C. The other solutions were made using Sigma powders and kept at room temperature. Some cultures underwent 48 hours stimulation starting from 6DIV, in which either 20 nM KCl (potassium chloride) or 50µM TTX was added to each well of 24-well cell culture plates. Other cultures were treated at 8DIV with either 20nM KCl for 20 minutes (inducing rapid neuronal depolarization), long term potentiation (LTP) mix for 10 minutes, or 20µM NMDA for 3 minutes (which induce long term depression). LTP mix contained 124mM NaCl (sodium chloride), 5mM KCl (potassium chloride), 1.25mM KH₂PO₄ (monopotassium phosphate), 5mM CaCl₂ (calcium chloride), 24mM NaHCO₃ (sodium bicarbonate), 25mM TEA (tetra-ethyl-ammonium chloride), 10mM D-glucose; pH was adjusted at 7.2 using 1M HCl solution, the mix was filtered in sterile conditions and warmed up to 37°C before use. In all experiments, control cultures treated with sterile deionized water were included.

48 hours treatments reproduce feedback mechanisms of plasticity, aimed to stabilize circuit activity in response to stimulation. Short stimulations induced at 8DIV mimic feed-forward forms of neuronal plasticity, by which local and fast strengthening or weakening of synapses are achieved. All cultures were fixed at 8DIV after stimulation using 4%PFA/PBS and kept at 4°C in PBS for subsequent immunostaining.

3.9 Plasmid amplification and purification

3.9.1 Transformation of plasmids in JM109 competent cells

The cloning expression vector pCMV6-A-GFP was obtained as a purified cDNA plasmid from OriGene: the plasmid was 5.8 kb long and contained the full sequence of *Mus musculus* *Pak7* gene, tagged with C-terminal GFP (green fluorescent protein). A control vector GFP-tagged (EX-EGFP-M03) was purchased from Gene Copoeia. Both plasmids contained CMV (cytomegalovirus) promoter for strong constitutive expression of the cDNA insert, and an Ampicillin resistance gene for antibiotic selection in *Escherichia coli*. Plasmids were diluted at 15 ng/μl using nuclease-free water and stored at -20°C; this concentration was used for transformation procedure. JM109 bacteria (JM109 competent cells, Promega, L2001) were used as described below in order to obtain the amount of plasmid necessary for subsequent transfection experiments. Tubes containing 50μl of JM109 cells were prepared in ice and added with either 1μl *Pak7*-GFP fusion plasmid or 1μl GFP plasmid. Nuclease-free water was used as a negative control. Tubes were placed in a water bath at 42°C for 45 seconds; during this step JM109 cells underwent heat shock required for plasmid incorporation. Immediately after tubes were placed in ice for 2 minutes; after this time tubes were added with 400μl 37°C-warm SOC medium and kept in a water bath at 37°C for 1 hour for recovery. Super optimal broth with catabolite repression (SOC) medium was made using deionized water and contained 2% w/v tryptone (Fluka, T9410), 0.5% w/v yeast extract (Y1625), 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride and 20mM glucose; it was autoclaved and added with 0.1μg/ml carbenicillin. Carbenicillin is an analogue of ampicillin and it is widely used in antibiotic selection when *E.coli* cells are transformed with expression vectors carrying resistance to ampicillin. After 1 hour incubation at 37°C, individual agar plates were inoculated with 50μl of either *Pak7*-GFP, GFP or control transformation media and incubated at 37°C overnight. To prepare agar plates, LB (lysogeny broth) agar medium was made with deionized water with 1% w/v tryptone, 0.5% w/v, 1% w/v sodium chloride and 1.5% bacteriological agar (Sigma, A5306); it was autoclaved, added with 0.1μg/ml carbenicillin and poured into 10cm Petri dishes in a vacuum hood to minimize contamination. After agar medium hardening, plates were inverted to avoid moisture formation on agar surface and stored at 4°C; they were allowed to reach room temperature before inoculation with transformation media.

3.9.2 Post transformation and growth of transformants' clones

After 16 hours agar plates were checked for bacterial colonies growth. Since plasmids contained a gene for antibiotic resistance, and carbenicillin was added to both SOC and agar LB media, only cells transformed with the plasmids (transformants) were able to grow and single transformants originated isolated colonies (clones) on agar surface. Absence of growth was confirmed for plates inoculated with negative control transformation media. Three colonies were selected from both *Pak7*-GFP and GFP agar plates and each colony was individually pre-amplified into SOC medium-containing wells of a cell culture plate. The plate was kept into a shacking incubator at 150rpm and 37°C for a few hours, or until SOC media in the wells appeared cloudy as a sign of bacterial growth. 200µl of these starting cultures were then added into individual conical flasks containing 200ml of warm SOC medium, which were placed back into a shacking incubator at 37°C and 150rpm overnight for further clonal amplification. Remaining positive cultures were stored at a temperature of 4°C as a back-up.

3.9.3 Plasmid purification and quantification

After 16 hours, content of the flasks appeared were cloudy confirming massive bacterial growth; it was then centrifuged for 10 minutes at 4000g at RT in order to pellet transformed bacterial cells and supernatants were discharged. Bacterial cell lysis was performed with a Promega kit (Pure Yield Plasmid Maxiprep System, Promega, A2393): cell resuspension solution, cell lysis solution and neutralization solution were subsequently used according to manufacturer's instructions. Lysates were centrifuged for 45 minutes at 4000g and RT in order to pellet bacterial debris; supernatants were kept for plasmid isolation and pellets were discharged. Plasmid purification step was carried out using filters provided by the Promega kit (A2393) with the help of a vacuum pump according to the manufacturer's instructions as follows: supernatant resulting from each clone was subsequently passed through a clearing column and a DNA binding column, which bound plasmidic DNA with high affinity. Each binding column was washed with 5ml endotoxin removal solution and 20ml column washing solution; plasmids were then eluted into 1.5ml tubes using nuclease-free water. Each purified plasmid was quantified by spectrophotometric reading (Nanodrop, ND8000) and A260/A280 ratios were checked to assess purity of samples. Purified *Pak7*-GFP plasmid was run through

a 0.8% agarose gel containing ethidium bromide in order to assess plasmid quality and purity (Figure 18): 100ng, 50ng and 25ng of purified plasmid DNA were added with 3µl loading dye and loaded into the gel aside with a molecular weight DNA ladder (λ DNA-HindIII Digest, New England BioLabs, N3012).

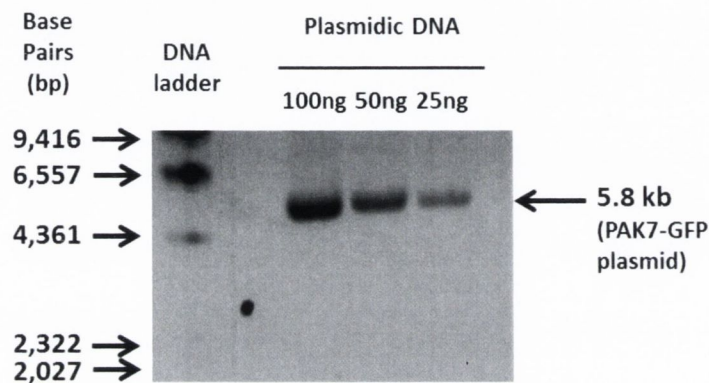


Figure 18. Purified *Pak7*-GFP plasmidic DNA (5.8kb). 100ng, 50ng and 25ng of purified plasmidic DNA were run through 0.8% agarose gel. A single band between 4361bp and 6557bp is visualized.

3.10 Transfection of primary neuronal cultures

For transfection experiments, primary neuronal cultures at 6DIV, made from *Pak7* KO pups as described in section 3.7, were used. Transfection was performed using lipofectamine as follows. For each experiment, at least one sample/cover slip underwent either transfection with *Pak7*-GFP plasmid or GFP plasmid. All coverslips were moved into plate wells containing 600µl of antibiotic-free feeding media (FM, made as above) pre-warmed at 37°C, and kept at in incubator at 37°C and 5%CO₂ for 30 minutes. During this time, independent tubes containing 50µl of 10mM HEPES/DMEM (h-DMEM, pH=7.0) were prepared for each coverslip, added with either 6µg *Pak7*-GFP plasmid, 3µg GFP plasmid or 5µl of nuclease-free water, and kept at RT for 5 minutes. A master mix containing 8% lipofectamine made in 10mM HEPES/DMEM was also made. Lipofectamine was purchased from Invitrogen (lipofectamine 2000 reagent, Invitrogen, cat#11668-022). All h-DMEM/DNA tubes were added with 54µl lipofectamine master mix and incubated at 37°C and 5% CO₂ for 20 minutes: this allowed the formation of liposomes containing plasmidic DNA. Content of each tube was then added to each well of the initial plate, which was kept at 37°C and 5% CO₂ for 4 hours to allow transfection; the process was stopped by moving glass coverslips into wells

containing regular FM. Samples were kept in incubator at 37°C and 5% CO₂ for about 36 hours to allow plasmid expression, fixed with 4%PFA/PBS for 20 minutes, washed with PBS and kept cover with foil at 4°C for subsequent immunostaining. A lipofectamine only-control was included in the experiment, which was subjected to the same procedure except that no plasmid was added: this was done in order to evaluate any possible effect due to lipofectamine treatment and independent from plasmid.

3.11 Immunocytofluorescence, confocal imaging and analysis

Immunofluorescence protocol was carried out using fixed neuronal cultures grown as described in section 3.7 and treated as in section 3.9 or 3.10. All immunostaining steps were performed at RT; plates underwent gentle shaking all the time except during primary antibody incubation, when plates were kept motionless. After a few washes with PBS, cultures were incubated for 1 hour in a solution made in PBS containing: 10% goat serum for aspecific binding sites-blocking, and 0.1% triton X-100 for cell permeabilization. Cultures were then incubated overnight in a primary antibody solution, which was made as the blocking solution described above and added with two or three different primary antibodies. After about 16 hours, cultures were rinsed 3 times with PBS for a total time of 15 minutes in order to remove unbound primary antibody, and incubated for 2 hours with secondary antibodies diluted in a solution of 0.05%triton X-100/PBS. Secondary antibodies used were conjugated to fluorescent dyes; plates were kept covered by thin foil from this step onward in order to avoid fluorescence decay. Details about primary and secondary antibodies used for immunocytofluorescence and concentrations are listed in table 3. Table 4 reports selected literature references of previous use of the antibodies when available. Cultures were finally washed with PBS and coverslips were mounted onto glass microscope slides using Vectashield Hard Set with DAPI (Vector, H-1500). A “no primary/secondary antibodies only” control was performed for each chemical treatment experiment, according to the same principle explained in section 3.6, in order to visualize possible aspecific signal given by cellular autofluorescence or by the background; controls were performed using secondary antibodies anti-rabbit/DL488-conjugated and anti-mouse/DL649-conjugated, while primary antibodies against PAK7 (made in rabbit) and NeuN (made in mouse) were omitted. PFC and VC cultures were made from at least three different animals for chemical treatments

experiments. For each animal/treatment one or two coverslips were stained, and at least 10 fields were imaged from each coverslip. Culture samples were imaged using a Zeiss confocal microscope with 63X oil immersion-objective using non-saturating settings as described in section 3.6. Only images showing neurons clearly distinguishable from the background staining were carried forward for analysis. Images were analyzed using ImageJ as follows. For the experiment involving chemical treatments, area and integrated density were measured for both whole cell body and nucleus of each neuron of all images included in the analysis. Cytoplasmic area was calculated as the difference between whole cell area and nuclear area; the integrated density values were used as a measure of the IS. Background fluorescence was measured and subtracted from cellular IS signal.

Immunostaining values of each control and treated cell was normalized to the average IS value of the control cells. Resulting values for each cell were now expressed in control IS-fold, which allowed us to combine measures from multiple biological replicates. Normalized values of single cells in each treatment group were first used for statistical analysis, and not the mean values of treatment groups from independent biological replicate. This choice was considered more appropriate in order to represent biological variability for the following reason: the variability among independent cells in each treatment group, calculated as standard error, was smaller than the one among averages of cells in different biological replicates. All groups were tested for normality in order to determine the most appropriate test to use for statistical analysis. Some groups of data did not result to be follow normal or Gaussian distribution; therefore, non-parametric tests were used for statistical analysis of data. The existence of statistically significant differences among groups was assessed using the Kruskal-Wallis test (the non-parametric equivalent of the one-way analysis of variance), followed by a Mann-Whitney to assess for differences between each treatment and the control. Although in molecular studies which use primary neuronal culture it is common practice to use single entities – individual cells or dendrites – as single data points for the analysis (Righi et al. 2000; Yoshii & Constantine-Paton 2007), we also performed additional statistical assessment using mean values of each treatment from each biological replicate.

For analysis of images from transfection experiments, a line was drawn along the perimeter of each neuronal primary dendrite using the segmented line-selection tool; integrated density within resulting areas were used as a measure of IS intensity of individual dendrites;

background fluorescence was measured and subtracted as above. In order to measure dendritic length a segmented line was drawn along each primary dendrite and its length was measured. No specific scale was set for length measurement in ImageJ as the final goal of this measurement is to establish the length of dendrites from transfected neurons relatively to the length of those belonging to control neurons. Since all images included in the same experiment are comparable with regard to magnification and imaging conditions, it is possible to perform a relative measurement. However, because the image size and pixel number are known, it would be possible to calculate the pixel size of the images: this would allow to set a scale in ImageJ, and to eventually determine the real dendritic length in nanometers or micron.

ANTIGEN	PRIMARY ANTIBODY			SECONDARY ANTIBODY		
	HOST	PRODUCT#	CONCENTRA-TION	DESCRIPTION	PRODUCT#	CONCENTRA-TION
PAK7	RB	Sigma SAB-3500335	1:5000	DL488-conj DK anti-GT	JIR 705-485-003	1:500
				Cy3-conj GT anti-RB	JIR 111-165-003	1:2000
DISC1	RB	Invitrogen 40-6900	1:1000	Cy3-conj GT anti-RB	JIR 111-165-003	1:500
NeuN	MS	Millipore MAB377	1:500	DL649-conj GT anti-MS	JIR 115-495-003	1:500

Table 3. Details about primary (AbI) and secondary (AbII) antibodies used in immunocytofluorescence. AbI: host, brand, product number, and working concentration are listed. AbII: host, conjugated fluorophores, specificity, brand, product number and working concentration are listed. Abbreviations: RB = rabbit; MS = mouse; GT = goat; DK = donkey; DL = DyLight; JIR = Jackson ImmunoResearch.

ANTIGEN	PRODUCT INFO	REFERENCES
Synapsin I/II	SYSY, 106 004	– Corvin et al., 2012; – Herrera-Molina et al., 2014
PSD95	Cell Signaling, 2507	– Neveklovska et al., 2012; – Um et al., 2012
Gephyrin	Abcam, ab25784	– Duarte et al., 2012; – Ke, Fujimoto, & Imai, 2013)
GAD67	Millipore, MAB5406	– Calu et al., 2013; – Shi et al., 2012; – Ziemińska et al., 2014
GAD65	SYSY, 198 104	– Cordeiro et al., 2011; – Dazzi et al., 2014; – Herrera-Molina et al., 2014
pAKT	Cell Signaling, 4058	– Kleiman et al., 2011; – Luckert et al., 2012; – Sykes et al., 2011
pGSK3 β	Cell Signaling, 9323	– Chiang et al., 2014; – Guertin et al., 2009; – Tran et al., 2012
pERK1/2	Cell Signaling, 4370	– Hutchins & Szaro, 2013; – Moritz et al., 2010; – Schwechter et al., 2013
nNOS	Cell Signaling	– Schallner et al., 2013
pCaMKIV	Santa Cruz, sc-28443	– Kong et al., 2009; – Tai et al., 2008
PAK7 (rabbit)	Sigma, SAB-3500335	No previous use found in literature
CREB	Cell Signaling, 9104	– Bodenshtein et al., 2010; – Hashimoto et al., 2009

pCREB	Cell Signaling, 9191	– Almeida et al., 2009; – Gehani et al., 2010
DISC1	Invitrogen, 40-6900	No previous use found in literature
DISC1	Santa Cruz, sc-47990	– Steinecke et al., 2012 – Faulkner et al., 2008
B-actin	Cell Signaling, 4967	– Cai et al., 2013; – Novaira et al., 2012
Lamin A/C	Cell Signaling, 4777	– T. Hashimoto et al., 2013; – Nicholson et al., 2012
PDE4b	Abcam, ab14612	No previous use found in literature
cAMP	Abcam, ab70280	– Corredor et al., 2012; – Ogata et al., 2012
PAK7 (goat)	Santa Cruz, sc-22155	No previous use found in literature
NeuN	Millipore, MAB377	– Codeluppi et al., 2014; – Nieland et al., 2014
MAP2	Millipore, MAB3418	– Guo et al., 2013; – Hevner et al., 2001; – Lee et al., 2012

Table 4. Literature references of primary antibodies used. Selected references of previous published research papers using antibodies utilized here are provided when available. References were selected by matching application of the specific antibodies (western blot and immunolabeling of tissue), and by matching research subject when possible.

4. Results

DISC1 is a prototypical example of high risk factor for neuropsychiatric disorders. Previous research shows that the expression of *Disc1* is developmentally regulated in mouse, with peaks of expression at E13.5 and P35, which represent periods of active neurogenesis and synaptic pruning respectively. DISC1 participates to important neuronal processes in different stages of life, including neuronal proliferation and migration; the protein is also a crucial regulator of cellular signaling in mature neurons, and actively takes part to mechanisms of synaptic reorganization and plasticity.

We used the *Disc1*-L100P mutant mouse, in which the interaction between the two SCZ risk factors DISC1 and PDE4b is disrupted, to investigate the neurobiology of risk genes involved in SCZ. *Disc1*-L100P mutant mice present altered brain morphology and neurogenesis during adulthood, and defective neuronal development at the embryonic level and during early stages of life: these defects include aberrant neuronal branching and positioning, accompanied by a decreased number of dendritic spines in cortical and hippocampal regions. Previous studies show that DISC1 acts as a scaffold that – located at synaptic terminals – plays important roles in synaptic reorganization and dynamics (Hayashi-Takagi et al. 2010). We hypothesize that the expression of crucial synaptic and neuronal markers is altered as a consequence of the *Disc1* mutation, with different trends depending on the postnatal stage and brain area. To test this hypothesis, we carried out a systematic analysis of cellular mechanisms in *Disc1*-L100P mutant brains and cerebella during postnatal development.

We used animals at P8-10 (postnatal day 8-10), P30-40 and P>60 mutant mice and WT littermates, corresponding in mouse to ages of active synaptogenesis, pruning and adulthood (Holtmaat & Svoboda, 2009; Cowan W.M., *Studies In Developmental Neurobiology*, Chapter 15, Oxford University Press, 1981). In fact, in rodents synaptogenesis starts during embryonic life and continues through the first three weeks of postnatal development, peaking during the second week (P7-10); around P20 the brain reaches 90-95% of the adult weight and synaptic connections attain their maximum density (50% higher number of synapses than adult brain). At approximately one month of age, and up to P49, a pruning phase occurs in the brain of rodents that leads to reduced synaptic density, resulting from activity-dependent refinement of neural circuitry; this process reaches a plateau at P60 when adult levels of synaptic density and neurotransmitter are established (Semple et al., 2013).

Furthermore, we sought to assess the hypothesis of a synergistic developmental interaction between risk factors for SCZ; therefore, we investigated the developmental interaction of DISC1 and PAK7 in WT animals as well as in *Disc1* mutants.

Table 5 reports the number of wild type (WT) and *Disc1* mutant mice used for western blot (WB) experiments, age range and average age for each group. For these experiments animals of both sexes were used. Although sex ratio was comparable in the two genotypes, sex was not taken in account in the experiments described. Therefore, it is not possible to establish if the same ratio of females and males was used for individual experiments, and conclusions cannot be drawn regarding putative sex specific differences.

WB	AGE RANGE	MEAN AGE	EXPERIMENT CODE	WT	Disc1-L100P/L100P
	P8-10	P9	A	3	3
			B	3	3
			C	3	3
			D	3	3
			E	3	3
	P30-40	P35	A	4	4
			B	4	3
			C	3	3
			D	3	3
	P>60	P70	A	3	3
			B	3	3
			C	3	4
			D	3	3
			E	3	3
ALL AGES		ALL EXPERIMENTS	44	44	

Table 5. Age range, average age and number of wild type (WT) and *Disc1*-L100P/L100P mutant mice used for each western blot (WB) experiment (A, B, C, D or E). Total number of mice used for each genotype is also shown at the bottom of the table.

4.1 Protein levels of selected neuronal and synaptic markers in brain and cerebella of *Disc1*-L100P mutant mice during postnatal development

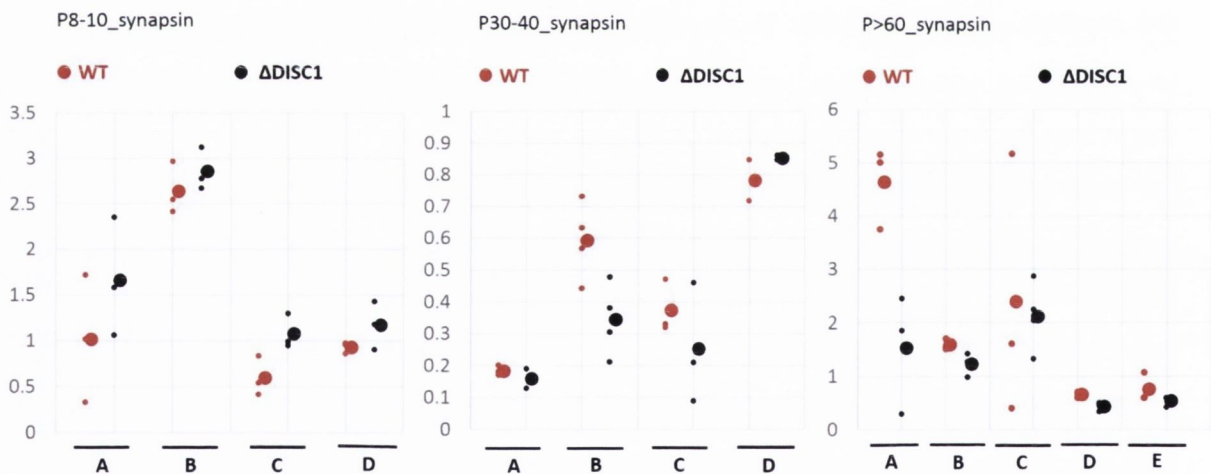
We measured by WB the expression of crucial markers for brain function (including pre- and post-synaptic proteins, the transcriptional factor CREB and the main kinases involved in its activation) in protein extracts from P8-10 (postnatal day 8-10), P30-40 and P>60 mutant mice and WT littermates; these ages correspond in mouse to synapse formation, synaptic pruning and adulthood respectively.

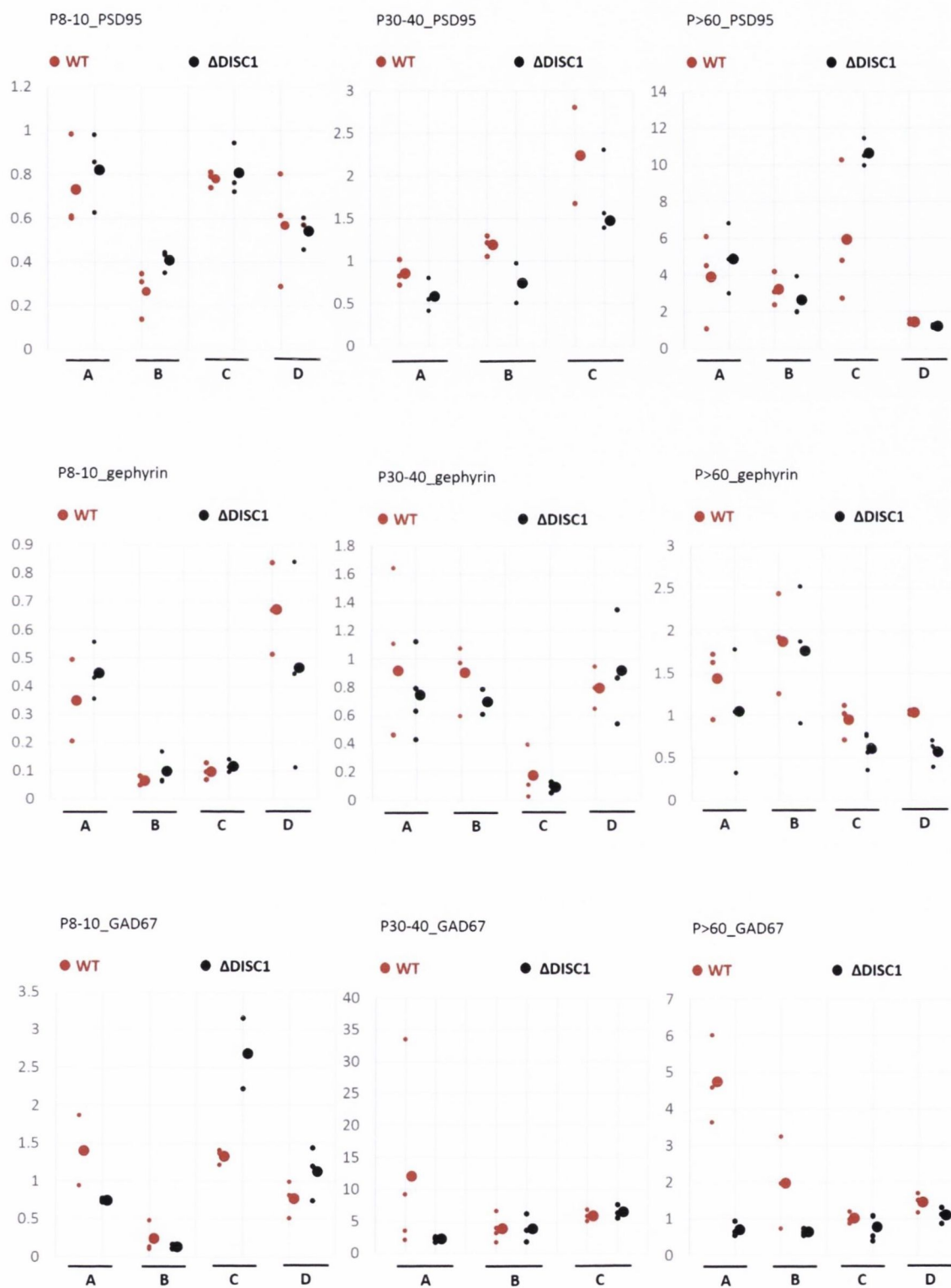
When different WB experiments need to be combined for analysis a standard protein sample should be run. For this work, brain tissue of different ages used for this work was collected and processed over the course of two years. In order to avoid the experimental results to be biased, adequate standard samples should have the same characteristics of test samples (same protein extraction procedure and same starting tissue), and should be chosen as average samples among the control condition. Therefore, it was not possible to establish the adequate standard samples for each cellular fraction and postnatal age from the beginning of the project. In order to be able to pool together measurements of multiple blots in our analysis, we performed normalization as described in the material and methods chapter. Each target/loading control ratio within the same blot was divided to the average of the target/loading control ratios of the control samples in the blot itself. Figure 19 reports plots for each antigen measured in WB from brain and CRB extracts at the three considered postnatal ages before normalization; in each graph data points representing antigen of interest/loading control ratio of each sample have been singularly plotted in order to visually show gel to gel variability. For each single experiment (A, B, C, D or E), target/control ratios of WT (red) and *Disc1* mutants (black) are represented by individual points.

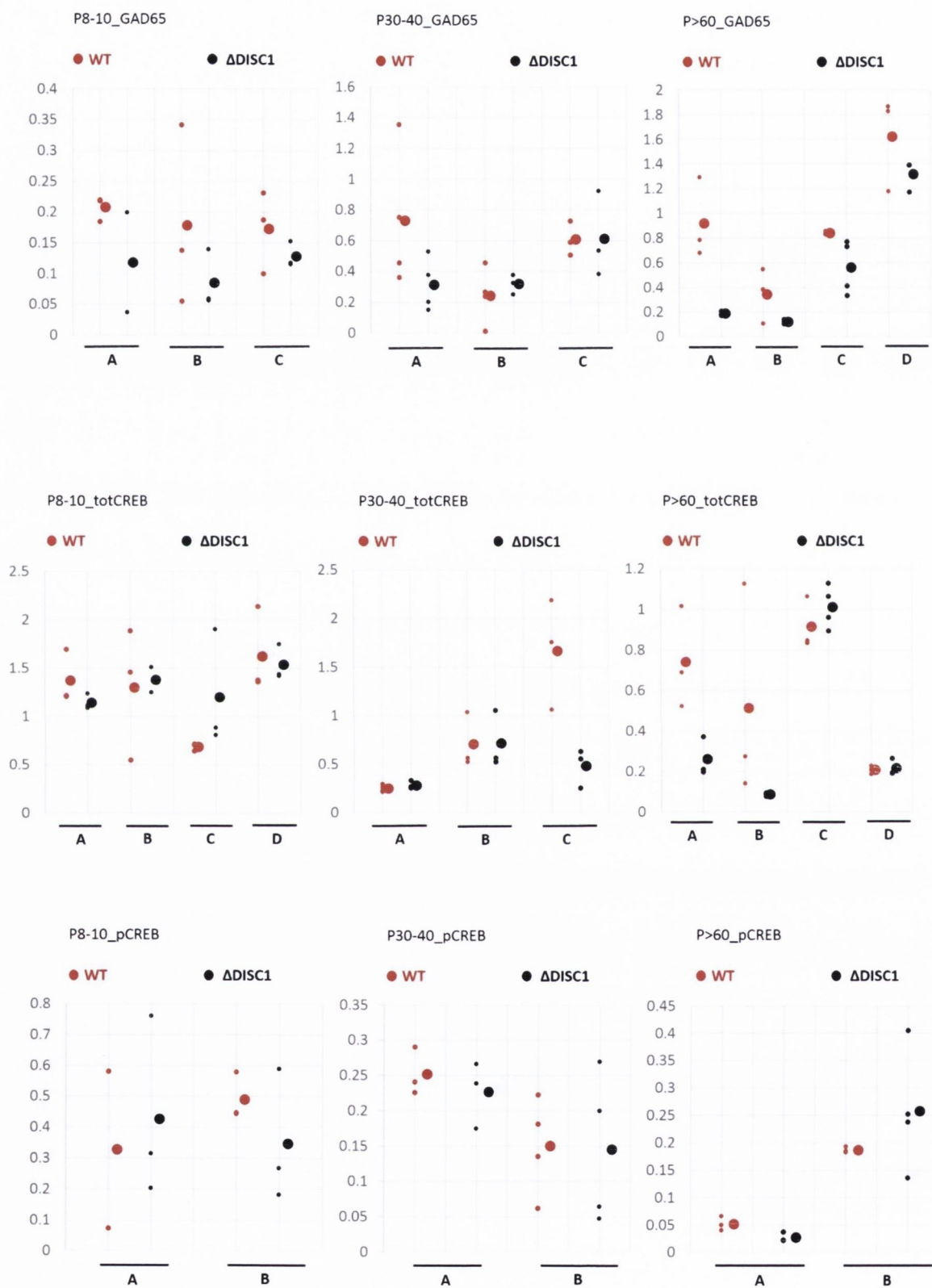
The lack of a standard sample to be run in each single experiment performed for the same antigen does not allow to establish the absolute amount of target protein present in each biological replicate. The degree of variability within the WT group for each experiment performed for the same protein in the same cellular compartment was calculated was evaluated by calculations of the coefficient of variation (CV), which is a statistical measure that allows to compare variability among groups when the average value is different in each group (Hallgrimson 2005). Although variability within WT groups appears not always constant, in general the plots show similar distribution of points in the two genotypes within

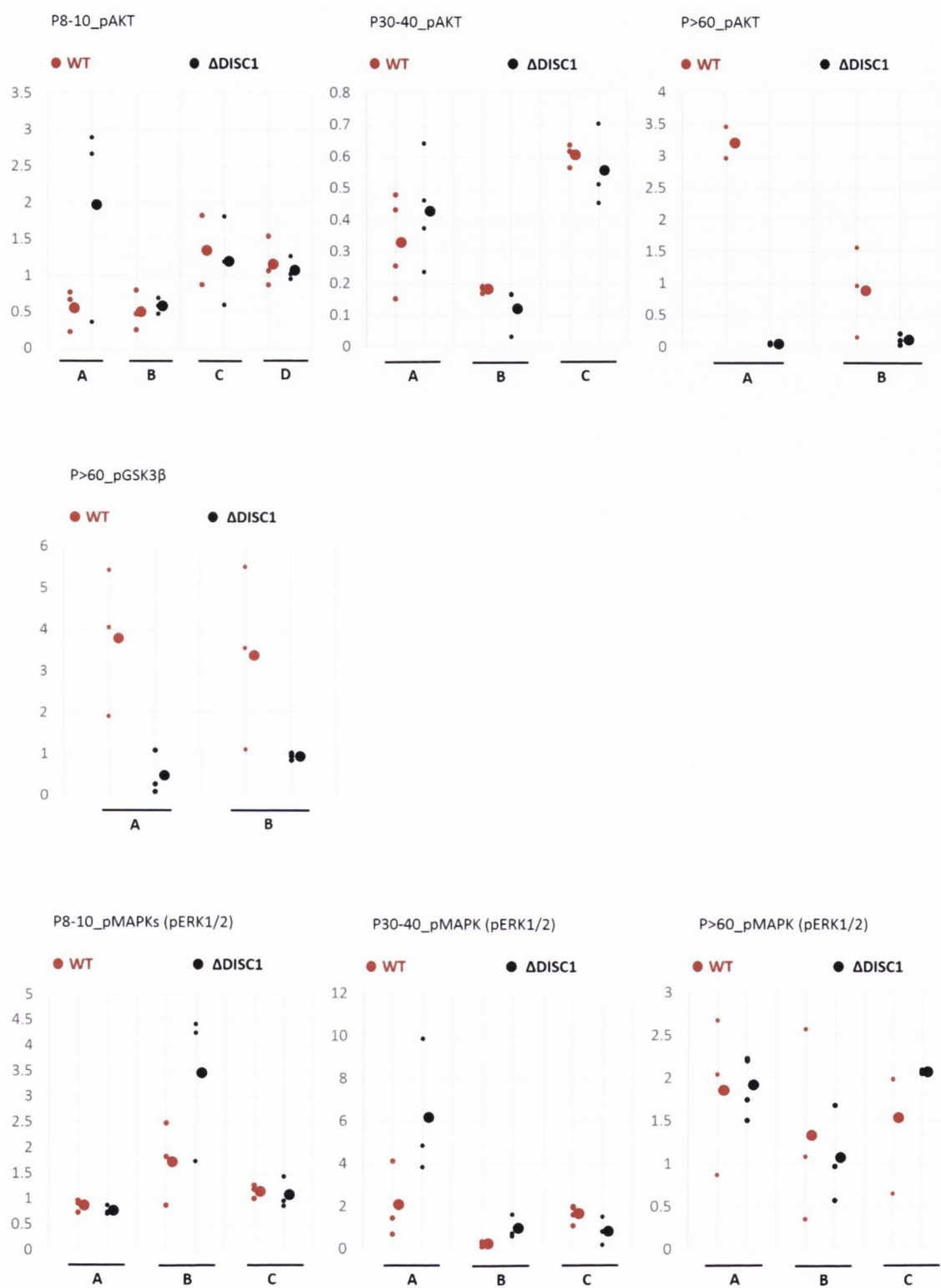
the same experiment: this made it possible to identify comparable trends across individual experiments, and made it reasonable to normalize our measurements as described. With the only exception of technical artifacts, none of the data points was excluded from subsequent normalization and analysis: this choice was considered correct in order to allow complete representation of the biological variability upon advice of an expert biostatistician. However, different degree of variability observed among WT groups might lead for some measured antigens to an underestimation of changes in protein expression between genotypes.

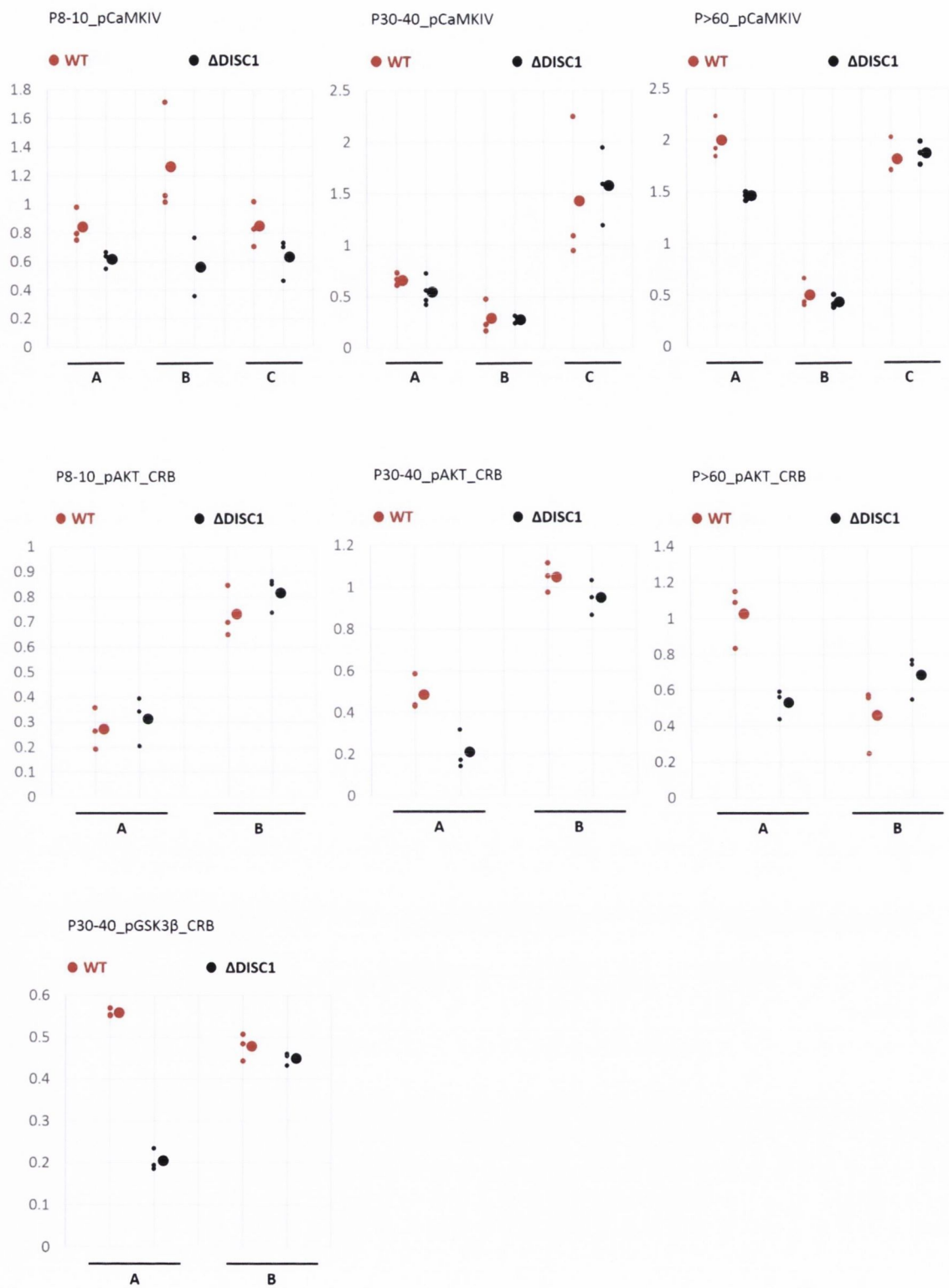
Figure 19 reports plots for each antigen measured in WB from brain and CRB extracts at the three considered postnatal ages; each graph contains single data points representing target/loading control ratios of WT (red dots) or *Disc1* mutant (black dots) samples before normalization to the WT average for each experiment performed (A, B, C, D or E). Average values for each group are also shown. These plots are useful to visualize the distribution of the single data points in each group and compare gel to gel variability.

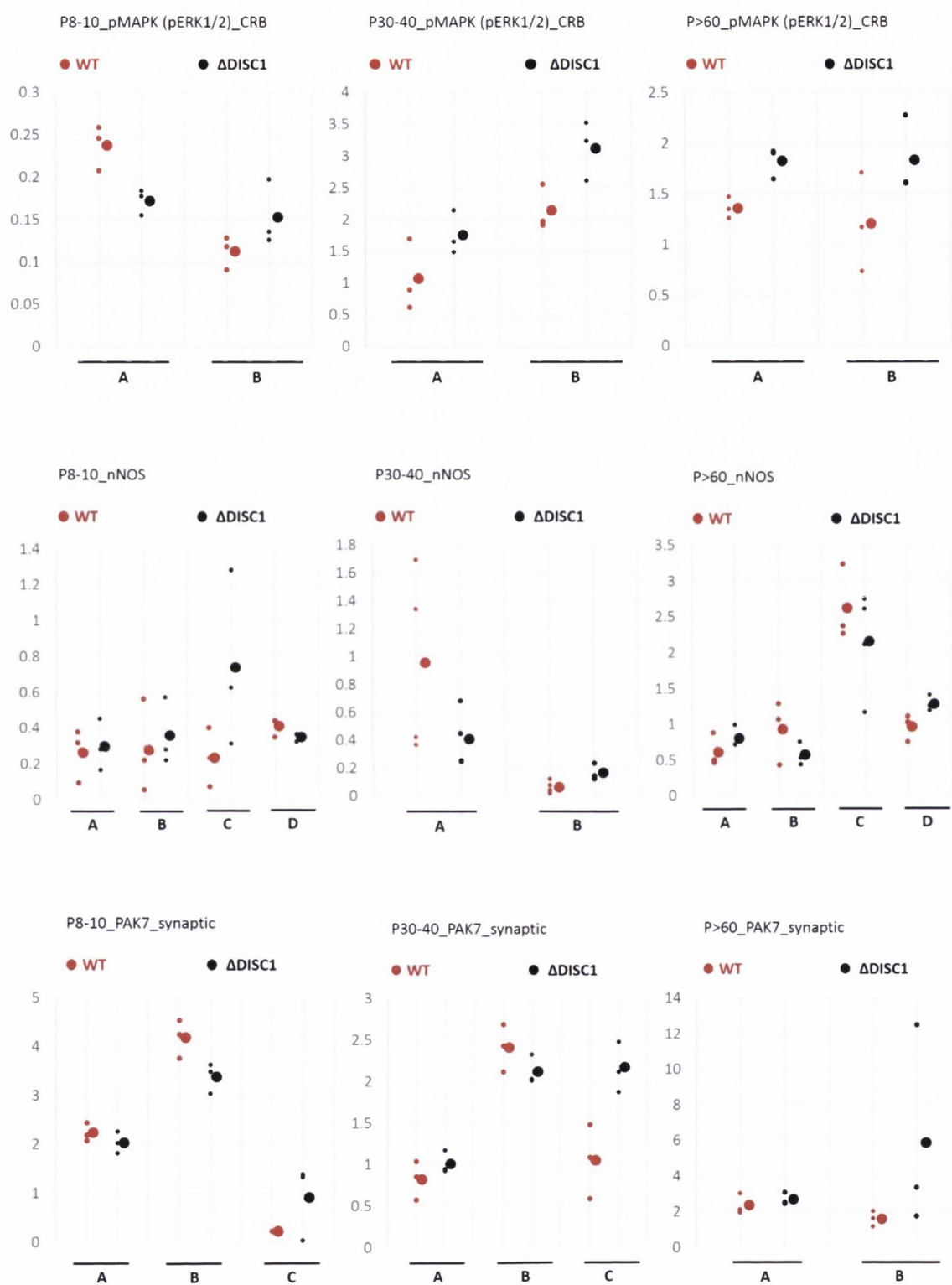












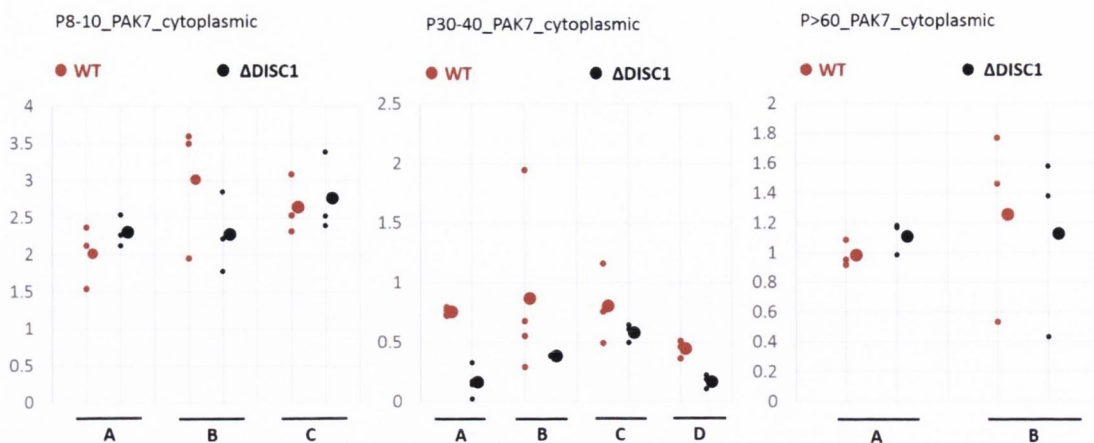


Figure 19. Graphic representation of the distribution of data points obtained from WB experiments. The figure reports individual graphs for each antigen measured in brain and cerebellum at the three postnatal stages studied (P8-10, P30-40 and P>60). Each graph contains single data points representing target/loading control ratios of WT (small red dots) and mutant (small black dots) samples before normalization to the wild type for each experiment performed (A, B, C, D or E). Average values for each group are shown beside (big dots).

4.1.1 Levels of synaptic proteins are altered in *Disc1*-L100P mutant brains during postnatal development

In order to investigate possible disruption of synaptic mechanisms during postnatal development, we compared levels of synapsin I/II, PSD95 and gephyrin in synaptoneurosomal or cytoplasmic extracts from *Disc1*-L100P homozygous and WT mice at P8-10, P30-40 and P>60. Synapsins are a family of synaptic vesicles-associated proteins, whose expression correlates with the number of both excitatory and inhibitory presynaptic terminals. Post synaptic density 95 (PSD95) is a key postsynaptic NMDARs-associated protein scaffold at the level of excitatory postsynaptic terminals, and used as a selective marker for spines; while gephyrin marks the majority (95%) of postsynaptic terminals in inhibitory synapses that locate on cell bodies of the inhibited neurons.

Our data show that at P8-10 protein levels of synapsin I/II are increased by more than 40% in *Disc1* mutants brains compared with age-matched WT (Table 6, Figure 20-A). On the opposite trend of what observed at the age of synapses formation, we find a significant ($P<0.05$) decrease of 30% and 44% in the levels of synapsin I/II in P30-40 and P>60 mutants respectively (Table 6, Figure 20-B and 20-C). In order to investigate both excitatory and inhibitory contribution to the identified presynaptic dysregulation, we measured protein

levels of PSD95 and gephyrin in synaptoneurosomal and cytoplasmic extracts respectively: at P8-10 we find a modest (more than one third), but not significant, increase in levels of both postsynaptic markers in mutants compared with WT (Table 6, Figure 20-D and 20-G); while at the age of pruning we show significantly reduced levels of PSD95 in *Disc1* mutants (42%), and a slight but not significant decrease of gephyrin levels (Table 6, Figure 16-E and 16-H). P>60 *Disc1* mutant and WT brains express comparable levels of PSD95 (Table 6, Figure 20-F); however, we find significantly reduced levels of gephyrin of about one third in mutants compared with WT littermates (Table 6, Figure 20-I).

In summary, we these results demonstrate an overexpression of synapsin I/II in mutant brains at P8-10, which suggests increased number of presynaptic entities in early postnatal life. Moreover, we find decreased levels of synapsin and PSD95 at P30-40, which taken together suggest reduced number of excitatory synaptic connections in mutant brains at this age. Finally, reported decreased levels of synapsin I/II and gephyrin at P>60, suggesting an overall decreased number of inhibitory synaptic entities in adult *Disc1* mutant brains compared to WT.

Post-natal Stage	Antigen	Number of Animals		Relative Protein Level ± Standard Error (SE)		<i>P</i> value
		WT	ΔDISC1	WT	ΔDISC1	
P 8-10	Synapsin	12	12	1 ± 0.1	1.44 ± 0.13	0.01
	PSD95	12	12	1 ± 0.1	1.34 ± 0.17	NS
	Gephyrin	10	12	1 ± 0.13	1.17 ± 0.38	NS
P 30-40	Synapsin	12	12	1 ± 0.04	0.73 ± 0.1	0.02
	PSD95	10	9	1 ± 0.06	0.63 ± 0.06	0.0004
	Gephyrin	14	12	1 ± 0.14	0.82 ± 0.1	NS
P > 60	Synapsin	15	16	1 ± 0.18	0.56 ± 0.09	0.04
	PSD95	12	13	1 ± 0.12	1.15 ± 0.13	NS
	Gephyrin	12	13	1 ± 0.06	0.71 ± 0.09	0.03

Table 6. Details about Western blot for synapsin, PSD95 and gephyrin on brain extracts from wild type (WT) and *Disc1* mutant (ΔDISC1) animals: post-natal stage, antigen, number of animals/genotype, relative protein level and standard error (protein of interest normalized to loading control and expressed as WT fold for each group) are listed. Statistical significance and *P* values were determined with Mann-Whitney test (NS = Not Significant).

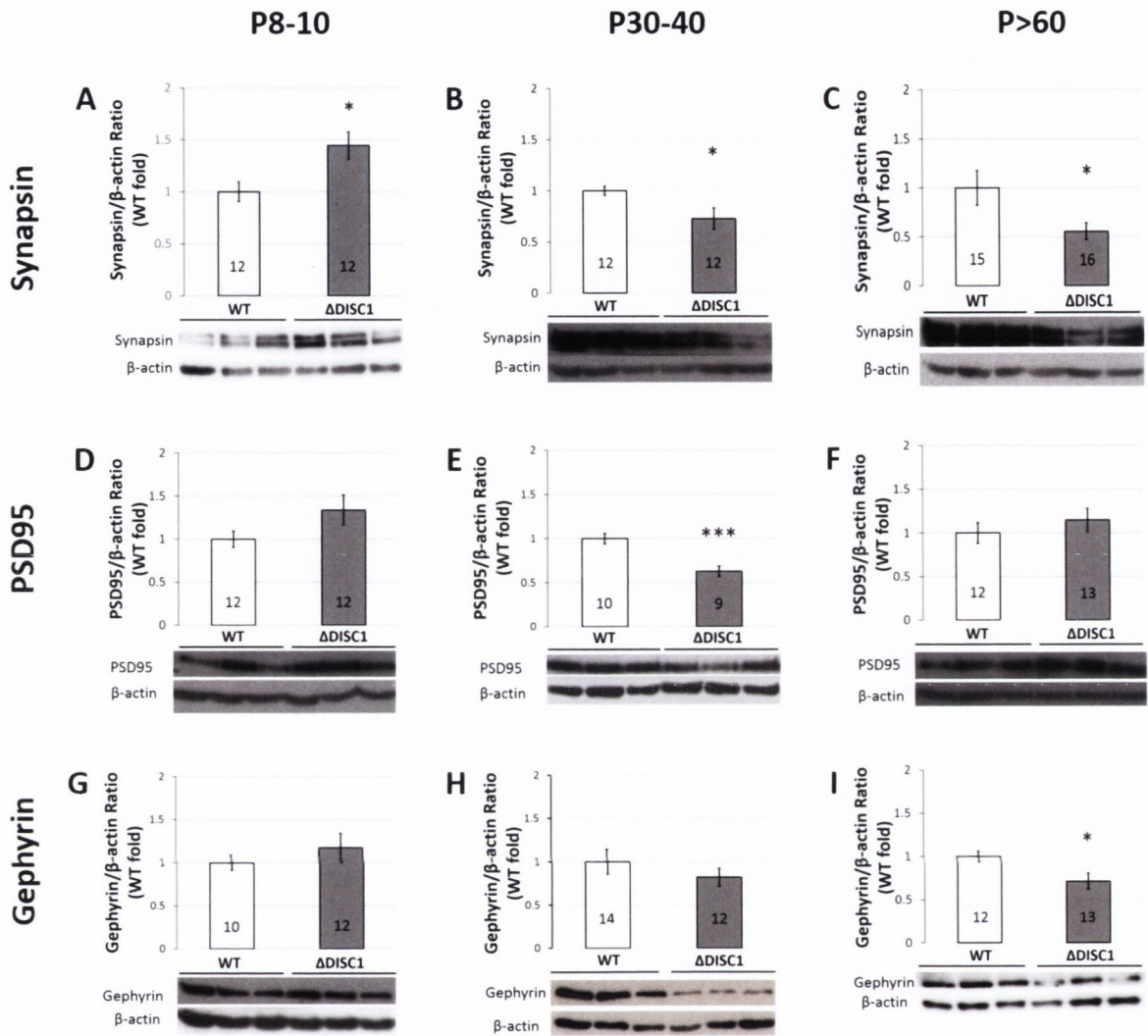


Figure 20. Levels of synaptic proteins are altered in *DiscI*-L100P mutant brains during postnatal development. Representative Western blots and quantitative analysis of the expression of synapsin, PSD95 and gephyrin in synaptoneurosomal protein fractions from wild type (WT) and *DiscI*-L100P mutant (Δ DISC1) brains, at three post-natal stages: P8-10, P30-40 and P>60. Synaptic proteins levels were normalized to the loading control β -actin, and fold changes are relative to WT. The number of animals is indicated on the graph column for each group. Relative protein levels are shown as average $\pm$ standard error (SE), difference between genotypes was assessed with Mann-Whitney test and considered statistically significant when P value was ≤ 0.05 . Synapsin: increased levels at P8-10 (A: $*P < 0.05$); decreased levels of expression at P30-40 and P>60 (B-C: $*P < 0.05$), in Δ DISC1 compared with WT. PSD95: comparable levels at P8-10 (D), followed by reduced levels at P30-40 (E: $***P < 0.005$) and comparable levels at P>60 (F), in Δ DISC1 compared with WT. Gephyrin: comparable levels at P8-10 and P30-40 (G and H respectively) followed by reduced levels at P>60 (I: $*P < 0.05$), in Δ DISC1 compared with WT.

4.1.2 Levels of glutamic acid decarboxylase (GAD) enzymes is decreased in *Disc1*-L100P mutant brains at P8-10 and P>60

In order to further investigate the inhibitory contribution of the synaptic dysregulation in *Disc1* mutant brains, we compared protein expression of GAD67 and GAD65 between cytoplasmic brain extracts from *Disc1* mutants and WT. Glutamic acid decarboxylases or GADs are enzymes responsible for the conversion of glutamate into the inhibitory neurotransmitter GABA which takes place into cytoplasm of inhibitory neurons.

At P8-10 we find comparable levels of GAD67 in *Disc1* mutants and WT but a substantial decrease (43%) of the levels of GAD65 in mutant brain extracts compared with WT (Table 7, Figure 21-A and 21-D). We do not find different protein levels of GADs between genotypes in cytoplasmic brain extracts at P30-40 (Table 7, Figure 21-B and 21-E). However, at P>60 protein levels of both enzymes in *Disc1* mutants are half of the ones in WT brains (Table 7, Figure 21-C and 21-F), which confirms the idea of a decreased number of inhibitory presynaptic terminals in adult mutant brains suggested by the results on synaptic proteins.

Post-natal Stage	Antigen	Number of Animals		Relative Protein Level ± Standard Error (SE)		P value
		WT	ΔDISC1	WT	ΔDISC1	
P 8-10	GAD67	11	11	1 ± 0.13	1.06 ± 0.2	NS
	GAD65	9	8	1 ± 0.15	0.6 ± 0.1	0.047
P 30-40	GAD67	11	10	1 ± 0.19	0.71 ± 0.15	NS
	GAD65	11	10	1 ± 0.14	0.87 ± 0.14	NS
P > 60	GAD67	12	13	1 ± 0.09	0.52 ± 0.09	0.003
	GAD65	12	13	1 ± 0.1	0.52 ± 0.08	0.002

Table 7. Details about Western blot for GAD65 and GAD67 on brain extracts from wild type (WT) and *Disc1* mutant (ΔDISC1) animals: post-natal stage, antigen, number of animals/genotype, relative protein level and standard error (protein of interest normalized to loading control and expressed as WT fold for each group) are listed. Statistical significance and P values were determined with Mann-Whitney test (NS = Not Significant).

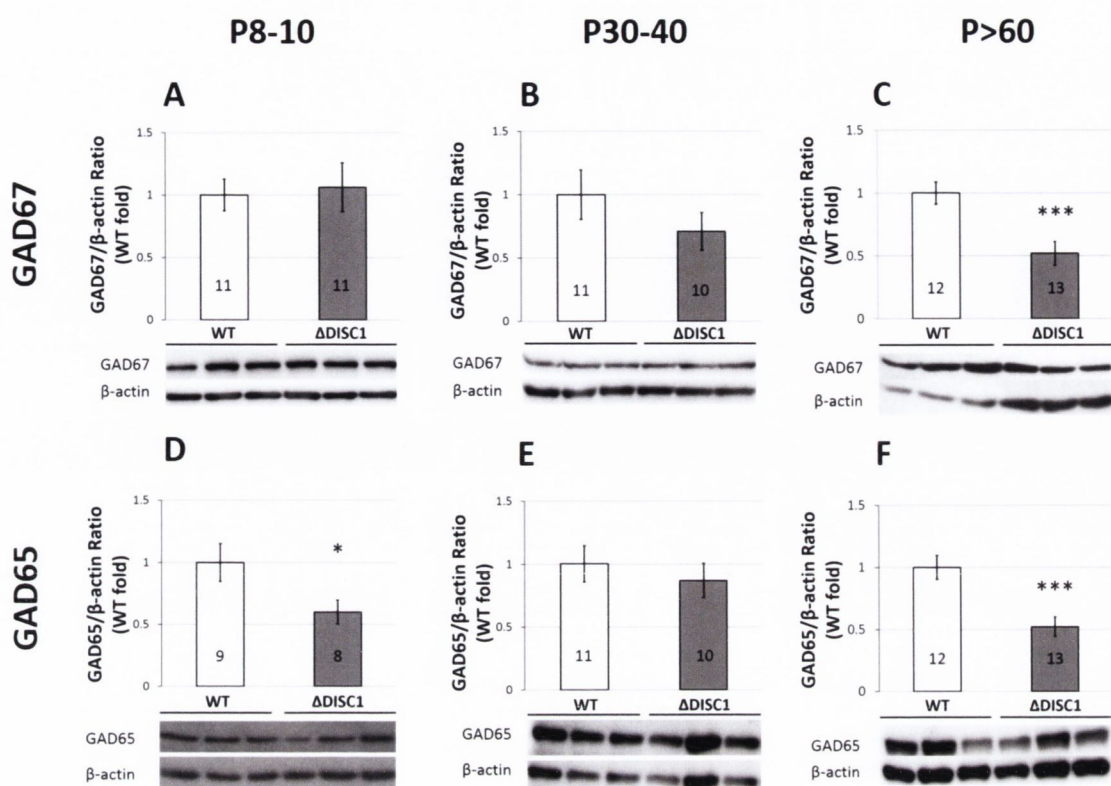


Figure 21. Expression of glutamic acid decarboxylase (GAD) enzymes is reduced during postnatal development in *Disc1*-L100P mutant brains. Representative Western blots and semi quantitative analysis of the expression of GAD67 and GAD65 enzymes in cytoplasmic protein fractions from wild type (WT) and *Disc1*-L100P mutant (Δ DISC1) brains, at three post-natal stages: P8-10, P30-40 and P>60. GADs levels were normalized to the loading control β -actin; levels are relative and expressed in WT fold. The number of animals is indicated on the graph column for each group. Relative protein levels are shown as average $\pm$ standard error (SE), difference between genotypes was assessed with Mann-Whitney test and considered statistically significant when P value was ≤ 0.05 .

GAD67: equivalent levels at P8-10 and P30-40 (A and B respectively), followed by decreased levels at P>60 (C: *** $P < 0.005$), in Δ DISC1 compared with WT. GAD65: decreased levels at P8-10 (D: * $P < 0.05$); comparable protein levels at P30-40 (E), and decreased levels at P>60 (F: *** $P < 0.005$), in Δ DISC1 compared with WT.

4.1.3 Levels of CREB and pCREB are not significantly changed in *Disc1*-L100P mutant brains during postnatal development

The L100P mutation leads to reduced brain interaction between DISC1 and the phosphodiesterase PDE4b (Clapcote et al. 2007). PDE4b is required for proper cAMP turnover and regulation of the phosphorylation/activation of the transcriptional factor CREB,

which in neurons is important for activity-dependent regulation of gene transcription. Therefore we tested the effect of the disrupted between DISC1 and PDE4b on CREB and pCREB levels. We measured levels of total CREB in cytoplasmic brain extracts and CREB activated isoform (pCREB) in nuclear extracts from *Disc1* mutant and WT brains. We find comparable levels of total CREB between WT and mutants at the age of synapses formation and pruning (Table 8, Figure 22-A and 22-B); there is a decrease of 40% in CREB levels in brains of adult mutant (Table 8, Figure 22-C), which however does not reach statistical significance. Contrary to our expectations, nuclear levels of pCREB, which is the active isoform of the transcriptional factor, are almost unchanged in L100P compared with WT during postnatal development (Table 8, Figure 22-D, 22-E and 22-F).

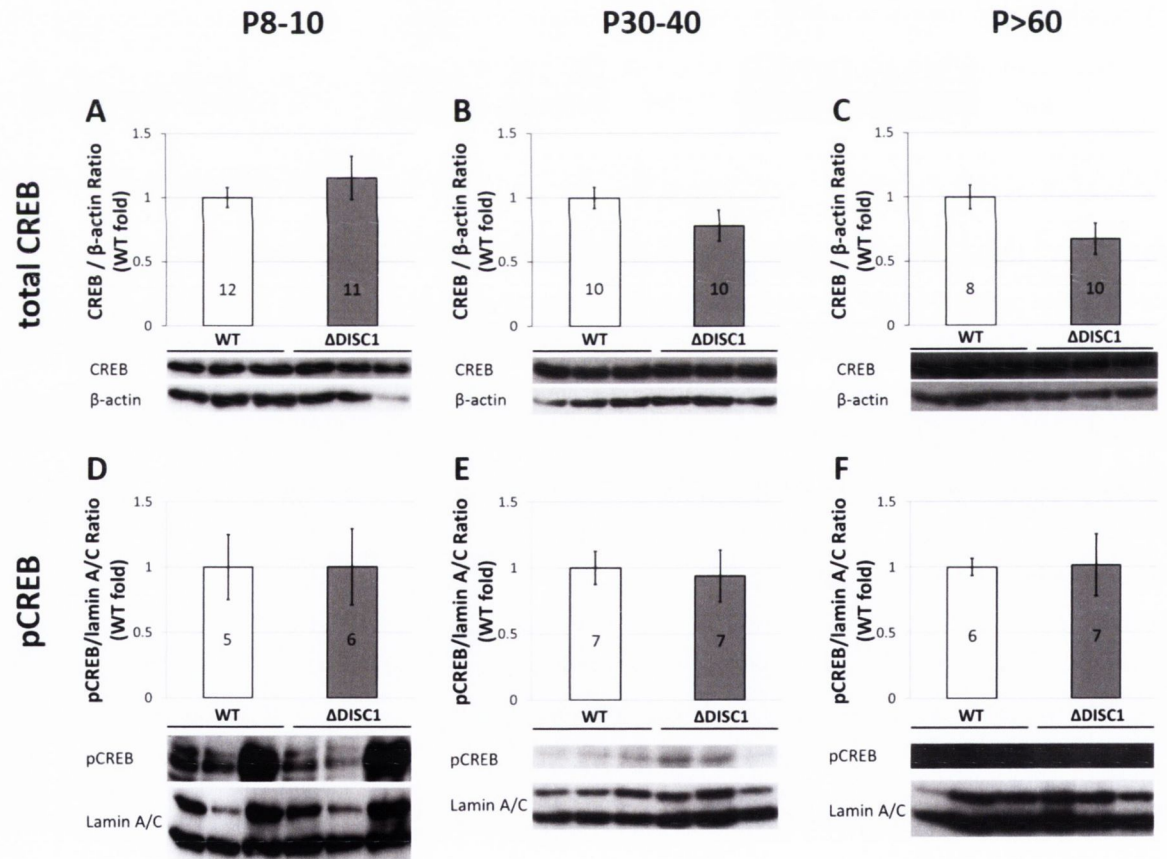


Figure 22. Levels of total CREB and pCREB are comparable during postnatal development in *Disc1*-L100P mutant and WT brains. Representative Western blots and semi quantitative analysis of protein levels of total CREB in cytoplasmic and pCREB in nuclear brain extracts from wild type (WT) and *Disc1*-L100P mutant

(Δ DISC1) brains, at three post-natal stages: P8-10, P30-40 and P>60. Cytoplasmic levels of total CREB in Δ DISC1 do not change significantly at P8-10 (A), P30-40 (B) and P>60 (C) compared with WT. Nuclear levels of pCREB are similar in Δ DISC1 and WT brains at P8-10 (D), P30-40 (E), and P>60 (F). CREB cytoplasmic levels were normalized to the loading control β -actin, while levels of activated CREB (pCREB) were normalized to the nuclear loading control lamin A/C; in all cases levels are relative and expressed in WT fold. The number of animals is indicated in the graph column for each group. Relative protein levels are shown as average $\pm$ standard error (SE), difference between genotypes was assessed with Mann-Whitney test.

4.1.4 Levels of pAKT, pGSK3 β , pMAPK (pERK1/2) and pCaMKIV, are altered in *Disc1*-L100P mutant brain during postnatal development

In order to identify possible cellular pathways affected by the L100P mutation, we measured by Western blot phosphorylated levels of AKT, GSK3 β , MAPKs (ERK1/2) and CaMKIV in cytoplasmic extracts from *Disc1* mutant and WT brains. At P8-10 the levels of pAKT are on average increased by 60% in *Disc1* mutant brains; however this increase fails to reach statistical significance due to the high standard error associated to the mutant dataset (Table 8, Fig. 23-A). At P30-40, *Disc1* mutants and WT show equal expression levels of pAKT (Table 8, Fig. 23-B), while at P>60 *Disc1* mutants show a sharp (93%) decrease of pAKT levels (Table 8, Fig. 23-C), together with a 80% decreased of the levels of pGSK3 β (Table 8, Figure 24). Levels of pERK1/2 are comparable at all the three postnatal stages, although at P30-40 *Disc1* mutants show more than twofold average increase compared with WT, which is not statistically significant (Table 8, Fig 23-D, 22-E and 23-F). pCaMKIV levels are reduced in P8-10 mutants compared with the WT (Table 8, Fig 23-G), while no significantly different levels are observed at later stages of development (Table 8, Fig 23-H and 23-I). The observed changes in changes pAKT, pGSK3 β , pERK1/2 and pCaMKIV might be due to increased availability of un-phosphorylated substrate or increased phosphorylation rate following changes in the activity of upstream signaling pathways. Our current data does not allow us to discern these two possible mechanisms, although we would like to pursue this question in the near future. Measuring the levels of the phosphorylated isoforms only allow us to monitor the factors directly regulating CREB activation. A measure of the total protein would be valuable because it would allow us to clarify the cellular mechanisms responsible for the observed changes. In summary, the analysis of molecular pathways converging on CREB shows dysregulated levels of activated kinases in *Disc1*-L100P mutants during postnatal development.

Post-natal Stage	Antigen	Number of Animals		Relative Protein Level ± Standard Error (SE)		P value
		WT	ΔDISC1	WT	ΔDISC1	
P 8-10	pAKT	11	12	1 ± 0.11	1.57 ± 0.42	NS
	pMAPKs (ERK1/2)	9	9	1 ± 0.09	1.31 ± 0.24	NS
	pCaMKIV	9	8	1 ± 0.06	0.66 ± 0.07	0.01
	CREB	12	11	1 ± 0.08	1.15 ± 0.17	NS
	pCREB	5	6	1 ± 0.25	1 ± 0.29	NS
P 30-40	pAKT	11	10	1 ± 0.08	1 ± 0.15	NS
	pMAPKs (ERK1/2)	10	10	1 ± 0.15	2.26 ± 0.64	NS
	pCaMKIV	10	10	1 ± 0.11	1.02 ± 0.10	NS
	CREB	10	10	1 ± 0.08	0.78 ± 0.16	NS
	pCREB	7	7	1 ± 0.13	0.94 ± 0.20	NS
P > 60	pAKT	5	6	1 ± 0.23	0.07 ± 0.04	0.02
	pGSK3β	6	6	1 ± 0.20	0.20 ± 0.05	0.01
	pMAPKs (ERK1/2)	9	10	1 ± 0.18	1.06 ± 0.10	NS
	pCaMKIV	9	9	1 ± 0.05	0.88 ± 0.05	NS
	CREB	11	13	1 ± 0.07	0.76 ± 0.11	NS
	pCREB	6	7	1 ± 0.07	1.02 ± 0.24	NS

Table 8. Details about Western blot results for pAKT, pGSK3β, pMAPKs (ERK1/2), pCaMKIV, CREB and pCREB on brain extracts from wild type (WT) and *Disc1* mutant (ΔDISC1) animals: post-natal stage, antigen, number of animals/genotype, relative protein level and standard error (protein of interest normalized to loading control and expressed as WT fold for each group) are listed. Statistical significance and P values were determined with Mann-Whitney test (NS = Not Significant).

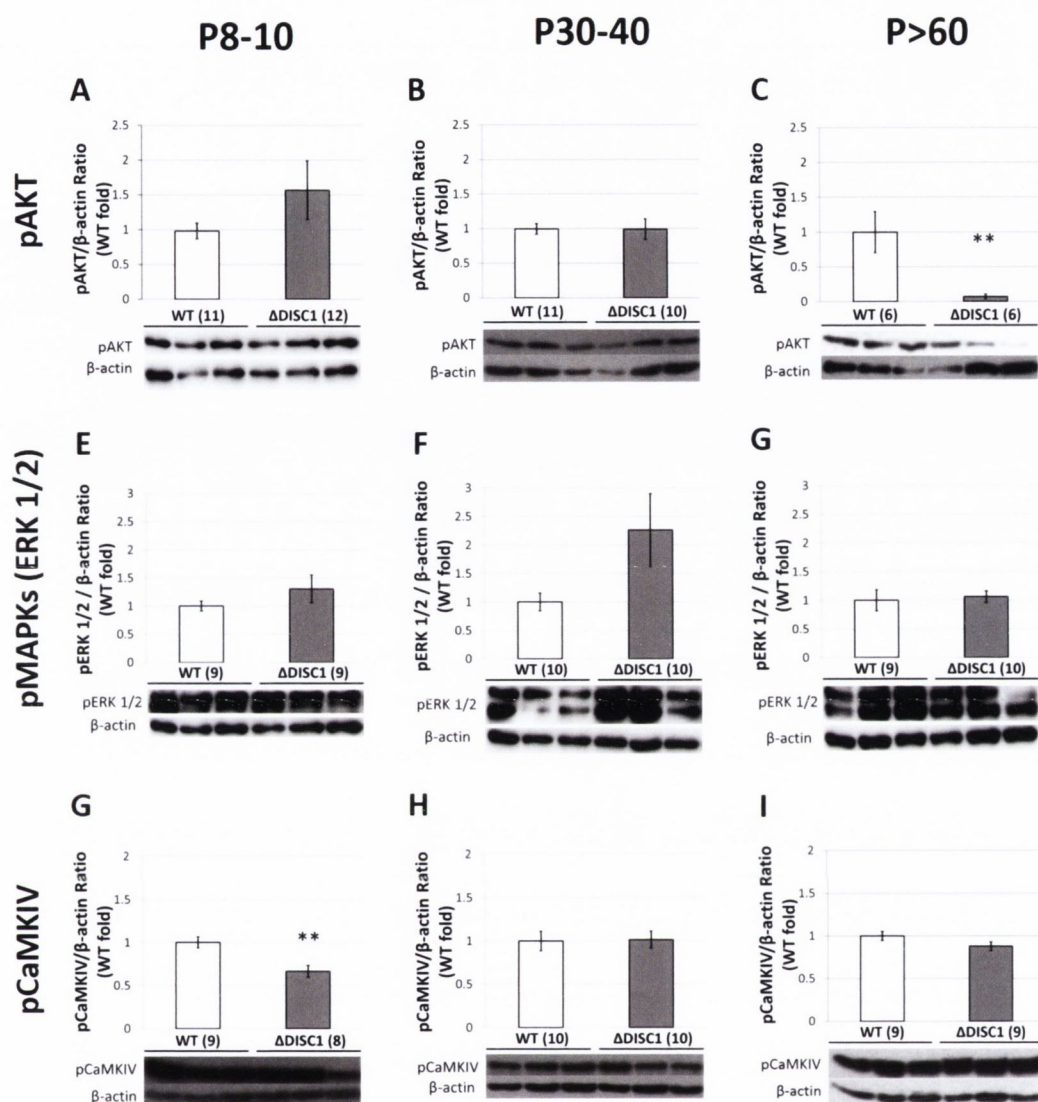


Figure 23. Levels of pAKT, pMAPKs (ERK1/2) and pCaMKIV are altered during postnatal development in *Disc1*-L100P mutant brains. Representative Western blots and semi quantitative analysis of the expression of pAKT, pMAPKs (ERK1/2) and pCaMKIV in cytoplasmic protein extracts from wild type (WT) and *Disc1*-L100P mutant (Δ DISC1) brains, at three post-natal stages: P8-10, P30-40 and P>60. Active kinase protein levels were normalized to the loading control β -actin, expression levels are relative to WT. Number of animals is indicated in brackets for each group. Relative protein levels are shown as average $\pm$ standard error (SE), difference between genotypes was assessed with Mann-Whitney test and considered statistically significant when P value was ≤ 0.05 . pAKT: equivalent levels at P8-10 and P30-40 (A and B respectively) and decreased levels at P>60 (C: ** $P < 0.01$), in Δ DISC1 compared with WT. pMAPKs (ERK1/2): equivalent expression levels at P8-10, P30-40 and P>60 (D, E and F respectively), in Δ DISC1 compared with WT. pCaMKIV: reduced levels at P8-10 (G: ** $P < 0.01$), followed by equivalent levels in P30-40 and P>60 (H and I respectively), in Δ DISC1 compared with WT.

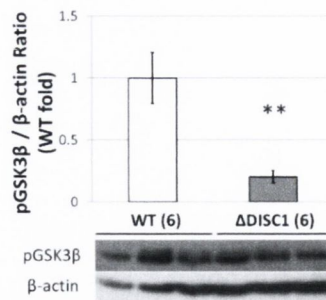


Figure 24. Levels of pGSK3 β are altered in P>60 *Disc1*-L100P mutant brains. Representative Western blots and semi quantitative analysis of the expression cytoplasmic protein extracts from P>60 wild type (WT) and *Disc1*-L100P mutant (Δ DISC1) brains. Active kinase protein levels were normalized to the loading control β -actin, expression levels are relative to WT. Number of animals is indicated in brackets for each group. Relative protein levels are shown as average $\pm$ standard error (SE). **P=0.01 (calculated with Mann-Whitney test).

4.1.5 Levels of pAKT, pGSK3 β and pMAPKs (pERK1/2) are altered in *Disc1*-L100P mutant cerebella compared with WT during postnatal development

To test the hypothesis of spatial-temporal dependent changes in the expression of cellular pathways converging on CREB activation, we also measured levels of pAKT, pGSK3 β and pERK1/2 in cytoplasmic extracts from WT and *Disc1* mutant cerebellum (CRB) tissue. For experiments on CRB protein extracts, the antibodies described in paragraph 3.4.1 were used, which presented the same band pattern described for brain blots.

At P8-10 we find similar levels of pAKT and pERK1/2 in *Disc1* mutant and WT cerebella (Table 9, Fig 25-A and 25-D). However, levels of both pAKT and pGSK3 β are reduced of about 40% in mutant CRB compared with WT at P30-40 (Table 9, Fig 25-B and Fig 26 respectively), and comparable levels of pAKT between genotypes in CRB of adult animals (Table 9, Fig 25-C). Moreover, pERK1/2 levels are significantly increased in mutant CRB compared with WT at P30-40 and P>60 (Table 9, Figure 25-E and 25-F). These results show that pAKT/pGSK3 β and pERK1/2 pathways are altered in *Disc1* mutant cerebella during postnatal development with different trends compared to what observed for brain.

Post-natal Stage	Antigen/ Cellular Fraction	Number of Animals		Relative Protein Level ± Standard Error (SE)		P value
		WT	ΔDISC1	WT	ΔDISC1	
P8-10	pAKT	6	6	1 ± 0.09	1.14 ± 0.1	NS
	pERK1/2	6	6	1 ± 0.05	1.04 ± 0.17	NS
P30-40	pAKT	6	6	1 ± 0.05	0.67 ± 0.12	0.04
	pGSK3β	6	6	1 ± 0.02	0.65 ± 0.13	0.04
	pERK1/2	6	6	1 ± 0.14	1.55 ± 0.11	0.01
P>60	pAKT	6	6	1 ± 0.11	1 ± 0.23	NS
	pERK1/2	6	6	1 ± 0.11	1.43 ± 0.10	0.01

Table 9. Details about Western blot results for pAKT, pGSK3β and pMAPKs (ERK1/2) on cerebellum (CRB) extracts from wild type (WT) and *Disc1* mutant (ΔDISC1) animals: post-natal stage, antigen, number of animals/genotype, relative protein level and standard error (protein of interest normalized to loading control and expressed as WT fold for each group) are listed. Statistical significance and *P* values were determined with Mann-Whitney test (NS = Not Significant).

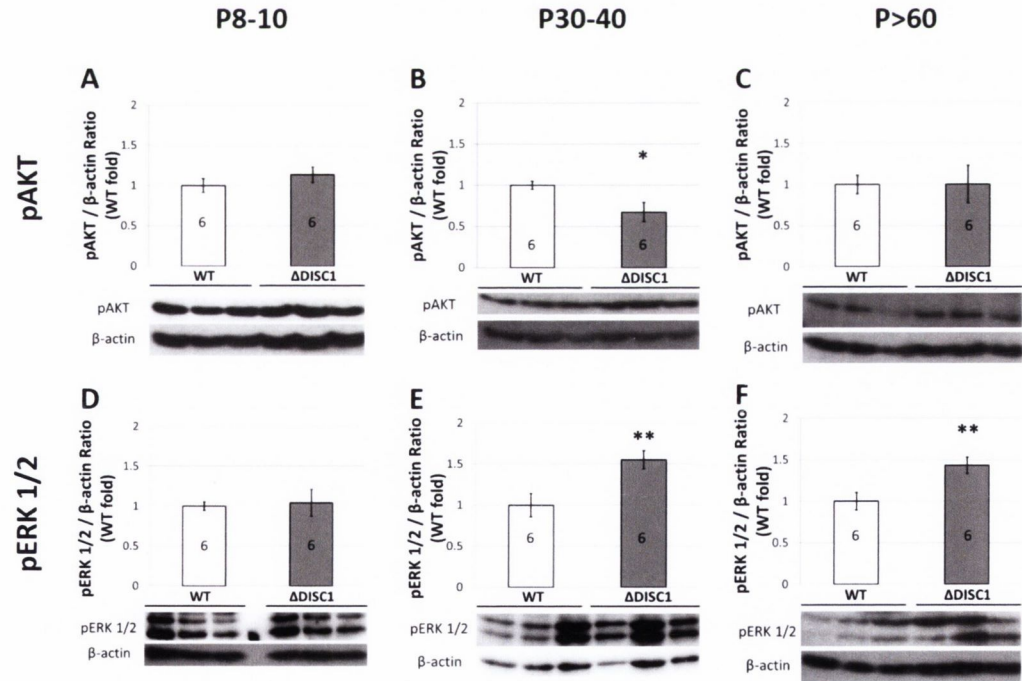


Figure 25. Levels of pAKT and pMAPKs (ERK1/2) are altered during postnatal development in *Disc1*-L100P mutant cerebellum (CRB). Representative Western blots and semi quantitative analysis of the expression of pAKT and pMAPKs (ERK1/2) in cytoplasmic protein extracts from wild type (WT) and *Disc1*-L100P mutant

(Δ DISC1) CRB, at three post-natal stages: P8-10, P30-40 and P>60. Active kinase protein levels were normalized to the loading control β -actin, levels are relative and expressed in WT fold. The number of animals is indicated in graph bars for each group. Relative protein levels are shown as average $\pm$ standard error (SE), difference between genotypes was assessed with Mann-Whitney test and considered statistically significant when P value was ≤ 0.05 . pAKT: equivalent levels at P8-10 and P>60 (A and C respectively) and decreased levels at P30-40 (B: $*P < 0.05$), in Δ DISC1 compared with WT. pMAPKs (ERK1/2): equivalent expression levels at P8-10 (E), increased levels at P30-40 and P>60 (F and G respectively, $**P = 0.01$), in Δ DISC1 compared with WT.

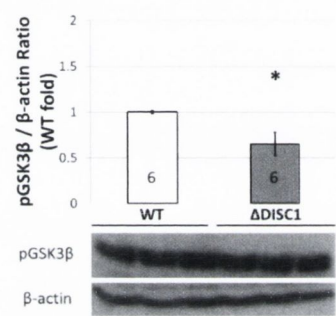


Figure 26. Levels of pGSK3 β are altered in P30-40 *Disc1*-L100P mutant cerebella (CRB). Representative Western blots and semi quantitative analysis of the cytoplasmic expression in P30-40 wild type (WT) and *Disc1*-L100P mutant (Δ DISC1) CRB. Levels were normalized to β -actin and expressed as WT fold. The number of animals is indicated in graph bars for each group. Relative protein levels are shown as average $\pm$ standard error (SE); difference between genotypes was assessed with Mann-Whitney test ($*P < 0.05$).

4.1.6 Levels of PAK7, but not nNOS, are decreased in *Disc1*-L100P mutant brains during postnatal development

Genome-wide association analyses demonstrated that a number of SCZ risk factors cluster in functionally related groups. Interestingly some risk genes encodes for proteins that play a role at the level of the postsynaptic density. In order to test hypothesis of a pathophysiological interaction between different SCZ risk factors, we measured levels of the SCZ risk factors nNOS and PAK7 in *Disc1*-L100P and WT brain extracts. Neuronal nitric oxide synthase (nNOS) is key enzyme involved in axon formation, synaptogenesis and spine remodeling. nNOS exerts its functions in an activity-dependent manner through the interaction with the NMDA-PSD95 complex in spines (Nikonenko et al. 2008). Recently, nNOS was demonstrated to interact with DISC1 (Zoubovsky et al. 2011). p-21 activated kinase 7

(PAK7) is a brain-restricted kinase with uncertain functions and it was recently identified as a penetrant risk factor for SCZ (Morris et al. 2014). Other members of the p-21 activated kinases family were reported to be involved in spine dynamics in a DISC1/PSD95-dependent manner; therefore, we tested PAK7 for a similar connection. We show that cytoplasmic levels of nNOS are mostly unchanged in *Disc1* mutant brains when compared with WT littermates during postnatal life (Table 10, Fig 27-A, 27-B, and 27-C). On the contrary, levels of PAK7 are decreased in *Disc1* mutants in specific cellular compartment at selected postnatal stages: PAK7 levels are reduced in mutants of about 40% at P8-10 when measured in synaptoneurosomal extracts and not altered compared with WT at P30-40 and P>60 (Table 10, Fig 27-D, 27-E, and 27-F); cytoplasmic levels of PAK7 are not significantly different in mutants and WT at P8-10 and P>60 (Table 10, Fig 27-G and 27-I respectively) but reduced more than 60% in *Disc1* mutant brains compared with WT at the age of pruning (Table 10, Fig 27-H). These results suggest that the L100P mutation in *Disc1* gene affect protein levels of PAK7 in a spatial-temporal regulated manner.

Post-natal Stage	Antigen/ Cellular Fraction	Number of Animals		Relative Protein Level ± Standard Error (SE)		P value
		WT	ΔDISC1	WT	ΔDISC1	
P 8-10	nNOS	12	12	1 ± 0.16	1.59 ± 0.38	NS
	PAK7 / Synaptoneurosomal	7	9	1 ± 0.14	0.62 ± 0.11	0.05
	PAK7 / Cytoplasmic	9	9	1 ± 0.07	0.99 ± 0.08	NS
P 30-40	nNOS	8	7	1 ± 0.23	1.35 ± 0.48	NS
	PAK7 / Synaptoneurosomal	9	9	1 ± 0.09	1.39 ± 0.18	NS
	PAK7 / Cytoplasmic	14	12	1 ± 0.12	0.42 ± 0.07	0.0004
P >60	nNOS	12	13	1 ± 0.09	1 ± 0.1	NS
	PAK7 / Synaptoneurosomal	5	5	1 ± 0.11	1.33 ± 0.16	NS
	PAK7 / Cytoplasmic	6	6	1 ± 0.13	1.01 ± 0.14	NS

Table 10. Details about Western blot results for nNOS and PAK7 on brain extracts from wild type (WT) and *Disc1* mutant (ΔDISC1) animals: post-natal stage, antigen/cellular fraction, number of animals/genotype,

relative protein level and standard error (protein of interest normalized to loading control and expressed as WT fold for each group) are listed. Statistical significance and *P* values were determined with Mann-Whitney test (NS = Not Significant).

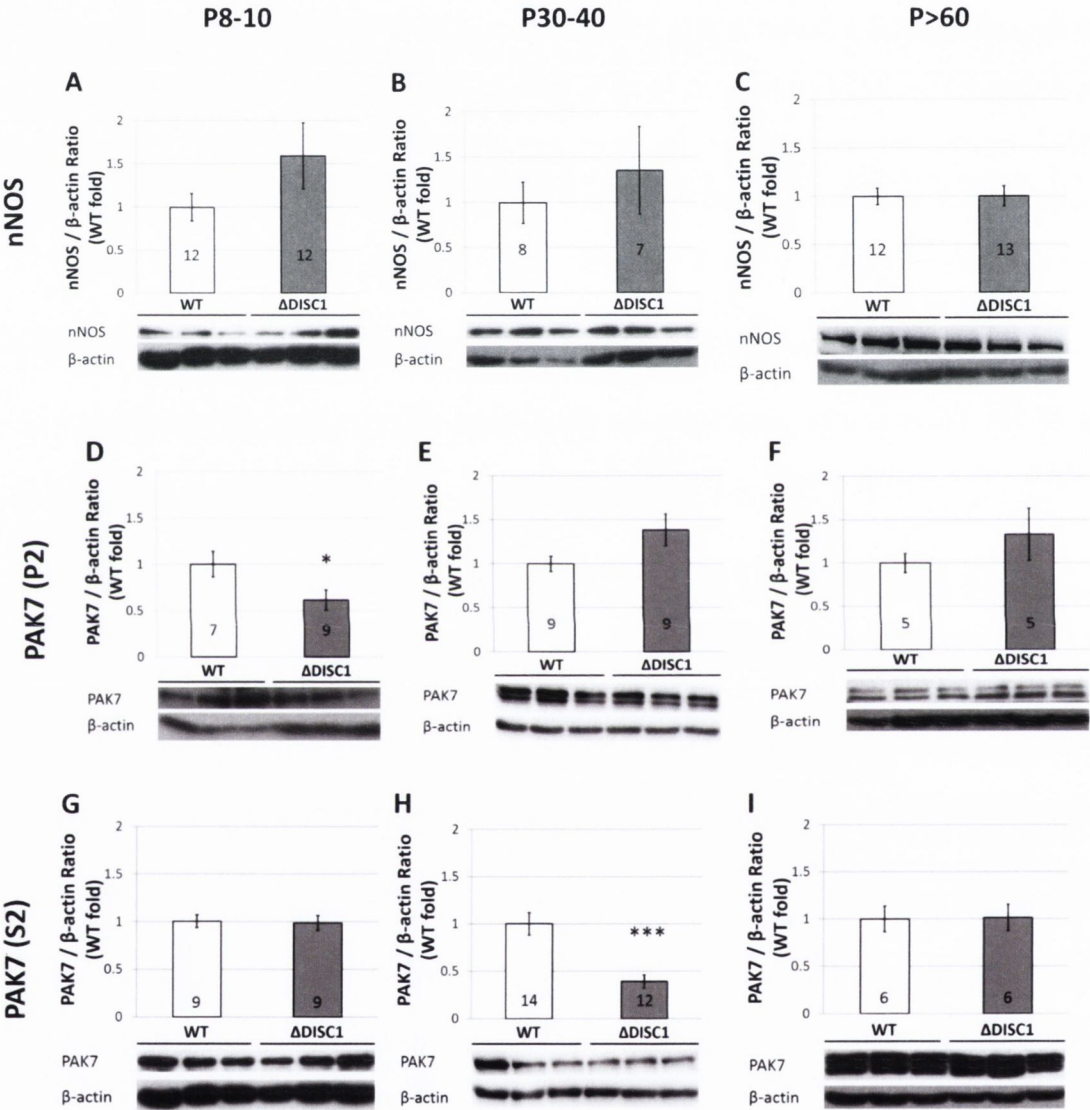


Figure 27. Levels of PAK7, but not nNOS, are reduced in specific neuronal compartments during postnatal life in *Disc1*-L100P mutant brains. Representative Western blots and quantitative analysis of the expression of nNOS and PAK5 in synaptic or cytoplasmic protein fractions from wild type (WT) and *Disc1*-L100P mutant (Δ DISC1) brains, at three post-natal stages: P8-10, P30-40 and P>60. Protein levels were normalized to the loading control β -actin, and fold changes are relative to WT. The number of animals is indicated in the graph column for each group. Relative protein levels are shown as average $\pm$ standard error (SE), difference between

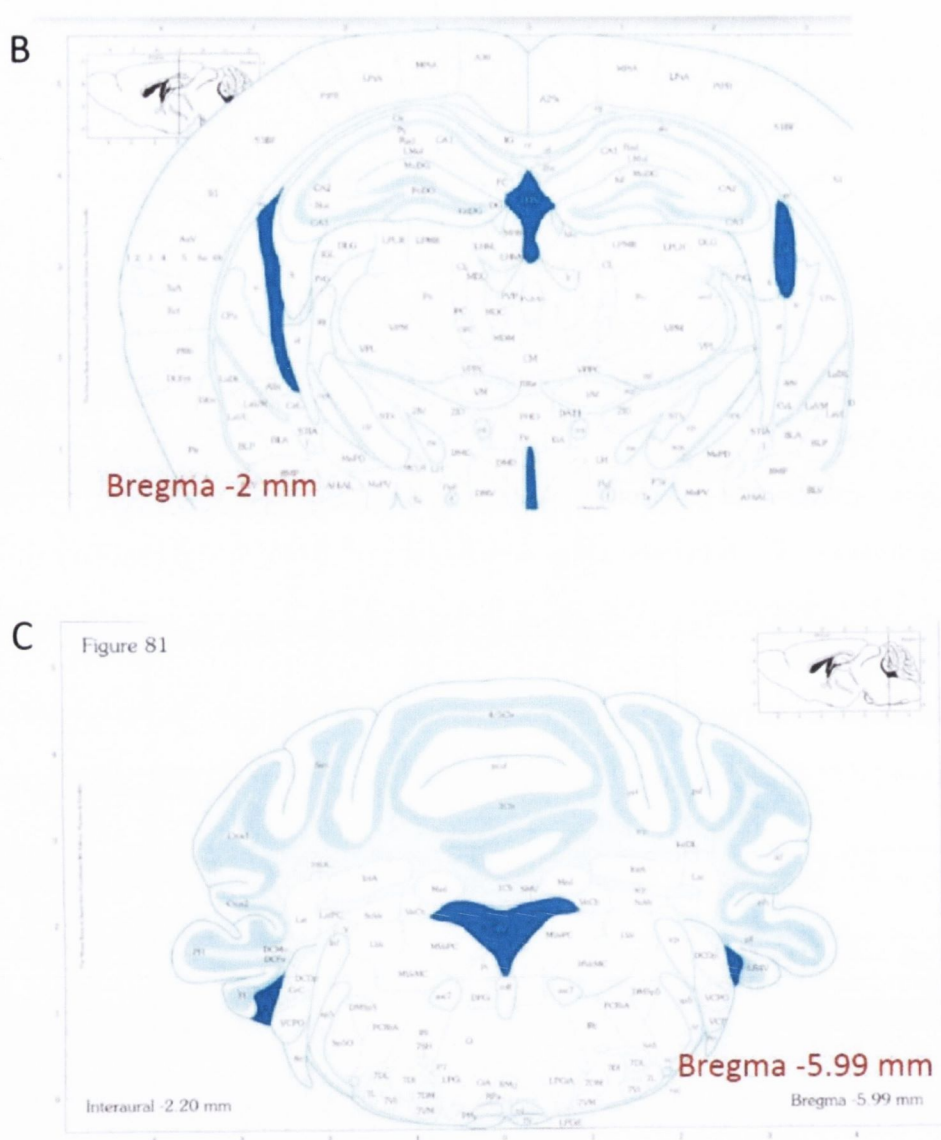


Figure 28. Schematic representation of coronal section plans selected for immunostaining of PFC (A), HP (B) and CRB (C) within established bregma intervals. Bregma values of each section plan is reported in red (bottom right of each panel), and its position is indicated as a vertical line in the sagittal view of the brain on top right of each panel.

We measured levels of synapsin I/II, PSD95, GAD65, total CREB, pCREB, PDE4b and cAMP, whose roles in neurons have been described above; we subsequently compared the intensity of the immunostaining for each of them between genotypes in individual brain regions. These antigens show different subcellular distribution within the neuron, and immunolabeling for each of them produced different patterns of distribution of the

fluorescent signal. Accordingly, different criteria were used to quantify intensity of the obtained IS (immunostaining) for each of them as described below. IS measurement of all antigens was performed on individual Z stacks as described above (Paragraph 3.6, Chapter 3). Synaptic proteins such as synapsin and PSD95 are exclusively located at synaptic terminals, but not at the level of cell body and nucleus of the neuron. This distribution results in a punctate staining that is typical of synaptic proteins; in tissue this pattern is often not easily recognizable due to the density of synaptic connections throughout the thickness of the tissue. Therefore, we performed confocal imaging of subsequent 5 μ m Z stacks for each considered field of view and we used a 63X oil-immersion objective (numerical aperture = 1.4) in order to better distinguish individual puncta from background staining. A lower amount of these synaptic proteins can be detected in the cytoplasmic compartment and does represent a portion of protein that is being translocated to its final subcellular compartment. Synaptic proteins punctate staining results in the absence of signal at the level of the cell body and nucleus: this produces the “ghosts”, which are not stained cell body/nuclear-spots. The average IS of the ghosts was measured for each field analyzed in the synaptic proteins images, and subtracted from the signal because considered background staining.

Immunostaining of both GAD65 and PDE4b in tissue sections produces a diffuse cytoplasmic and dendritic signal visible throughout the images except in cellular nuclei, which should appear black: IS that was present at the nuclear level was then considered background staining and subtracted from the cytoplasmic staining for quantification.

cAMP is visible at the cytoplasmic level but not in dendrites and nuclear compartment; in order to make measurement for this molecule only the cytoplasmic signal was considered specific, while the IS in nuclei and outside cytoplasm was measured and subtracted as background. CREB is a transcriptional factor that is located at the cytoplasmic level; then, it migrated into the nucleus where it gets activated: CREB measurements in both cellular compartments were combined in our analysis. pCREB is the nuclear activated isoform of CREB located uniquely in the cellular compartment; immunostaining was measured in nuclei and extra-nuclear signal was subtracted. As a general criterion, images in which the explained patterns of expression of the specific antigens were not recognized were excluded from quantification. Antigens like cAMP, PDE4b, CREB and pCREB are present in both glial cells and neurons. The pattern of staining of cAMP and pCREB, together with the

comparison with the DAPI staining, make it possible to distinguish the signal between one cell type and the other. However, total brain levels of all of these antigens were measured in our analysis. In our experiments, each brain section tested was stained for multiple antigens, using primary antibodies raised in three different animals and therefore detectable using secondary antibodies conjugated to three different dyes (DL488, Cy3 and DL649). A no primary/secondary antibodies only control was performed for each immunofluorescence experiment and involved the use of all secondary antibodies used in individual experiments and the omission of the primary antibodies incubation step (described above); these control experiments confirmed low levels of background staining resulting from secondary antibodies-aspecific binding (Figure 29).

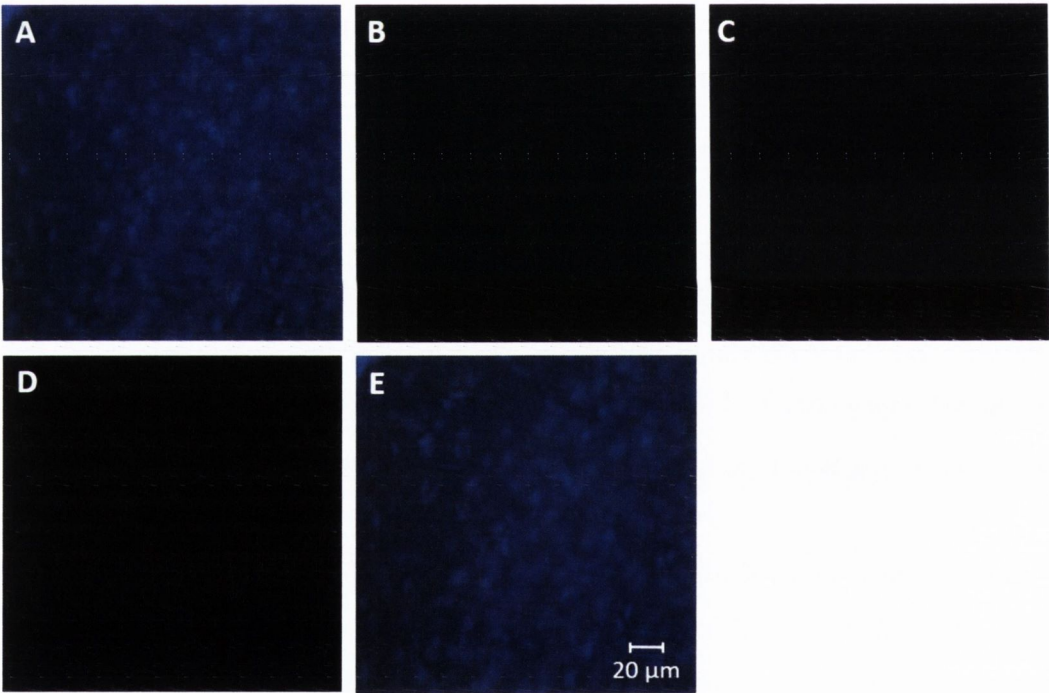
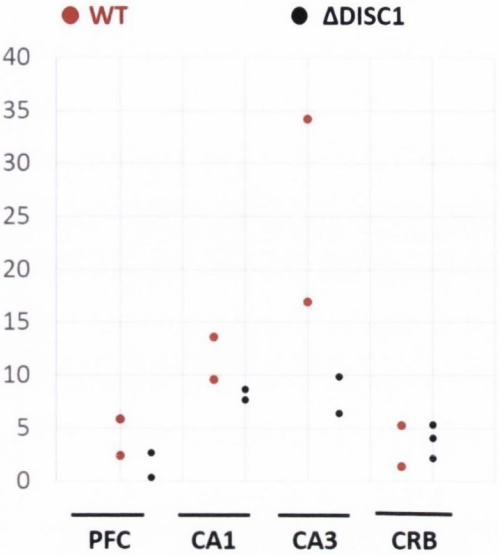


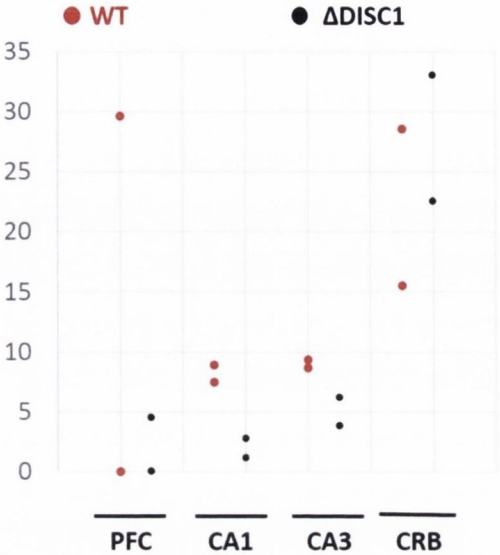
Figure 29. No primary/secondary antibodies only controls for immunofluorescence staining of tissue sections. This figure provide an example of secondary antibodies control, in which the use of the following secondary antibodies was combined in immunofluorescence experiments on brain tissue sections, and primary antibodies were omitted. A: DAPI nuclear staining; B: acquisition from green channel that shows anti-rabbit/DL488-conjugated staining; C: acquisition from the red channel that shows anti-guinea pig/Cy3-conjugated staining; D: acquisition from far red channel that shows and anti-mouse/DL649-conjugated staining; E: merged signal from blue, green, red and far red channels.

In order to identify differences in the expression of the proteins in PFC, HP and CRB between WT and *Disc1* mutant brains we performed separate immunofluorescence experiments using two different animals for each genotype. In general, two animals of each genotype and two sections from PFC, HP and CRB from each animal were used for each antigen to be tested. In some experiments a single section of wild type CRB was included (GAD65, cAMP, PDE4b), and three sections of CRB were used from the WT brain in one of the experiments (pCREB). In one experiment only (totCREB_experiment1) CRB sections were not included, resulting in the comparison of single animals for this antigen in this brain area. Further experiments are required in the future to complete this analysis. Imaging of stained sections was performed using a confocal microscope as described; a single field from each section was selected within the area of interest and a variable number of Z stacks (4 on average) were imaged, with a distance of 5µm between two consecutive stacks. Intensity of fluorescence was measured from each single Z stack, and an average value was calculated for each field, so that a single IS value was obtained for each biological replicate (section). As reasonably expected, the CV (coefficient of variability) - calculated for the Z stacks averages from each section of the same brain area - was lower than the CV calculated for the same before averaging Z stacks. Only Z stacks averages were considered, and they represented the single data points used for subsequent analysis. In each of the two experiments and within the same brain area (PFC, CA1 and/or CA3 of the HP, or CRB) the IS intensity of section fields were normalized to the average of the WT fields. The normalized values from the two individual experiments were averaged and incorporated into the Mann-Whitney test in order to assess differences in the expression of each protein, in each brain area, between WT and mutants. Differenced in the expression of the markers among brain areas were not assessed. Figure 30 reports plotted values of Z stacks averages of each analyzed field before normalization to the WT IS: each graph reports single data points used for analysis for each genotype and brain area.

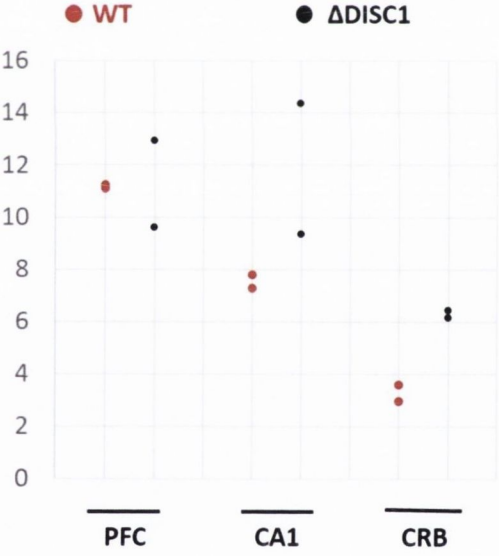
synapsin EXPERIMENT 1



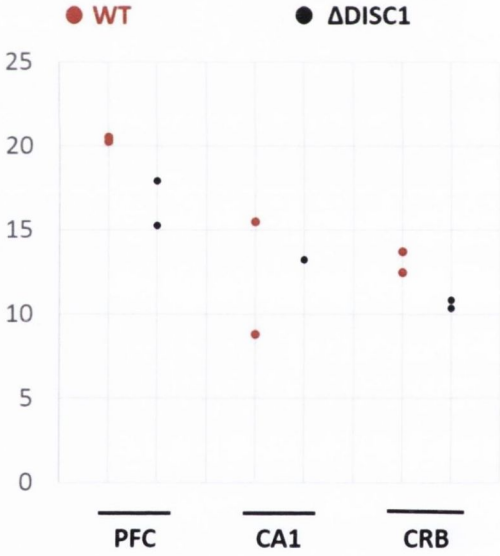
synapsin EXPERIMENT 2



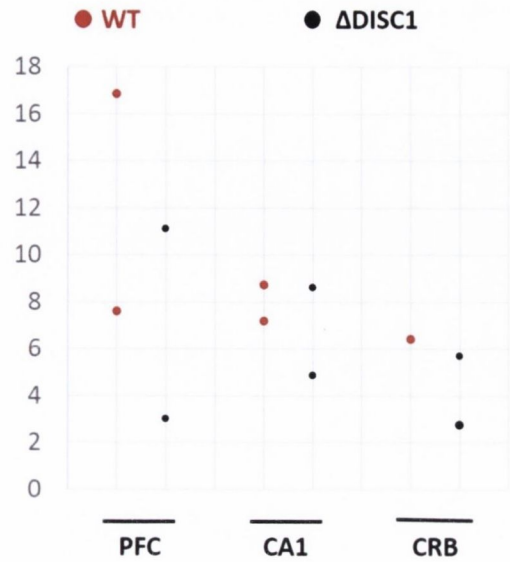
PSD95 EXPERIMENT 1



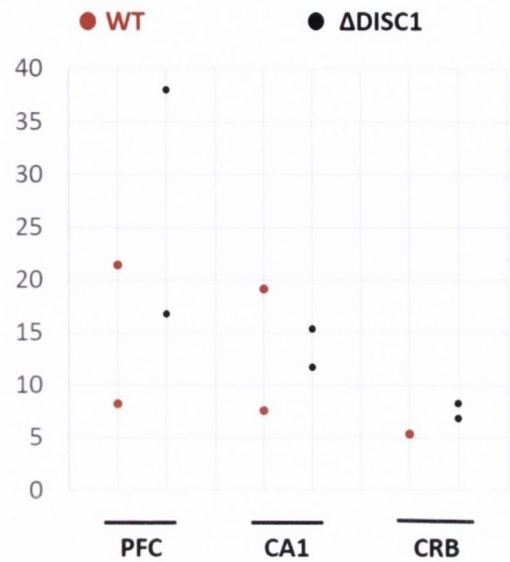
PSD95 EXPERIMENT 2



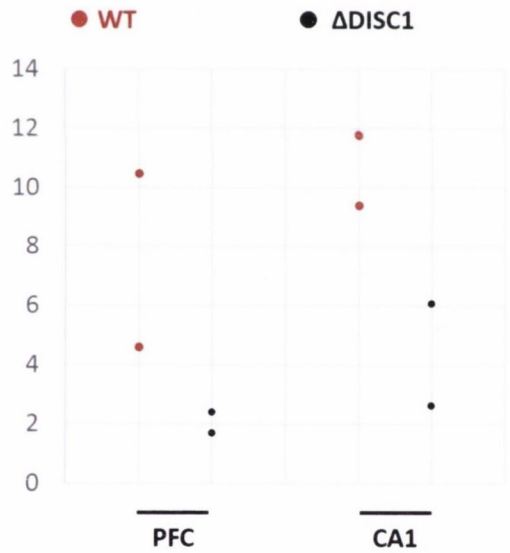
GAD65_EXPERIMENT 1



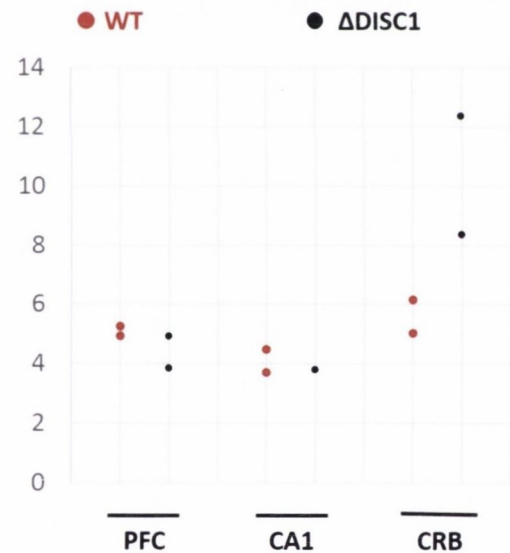
GAD65_EXPERIMENT 2

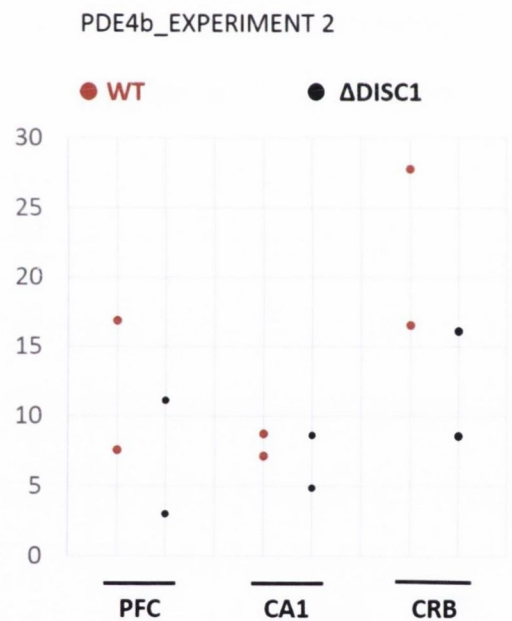
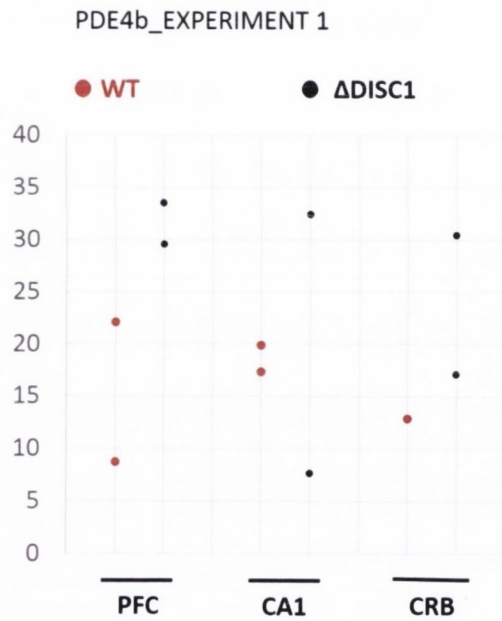
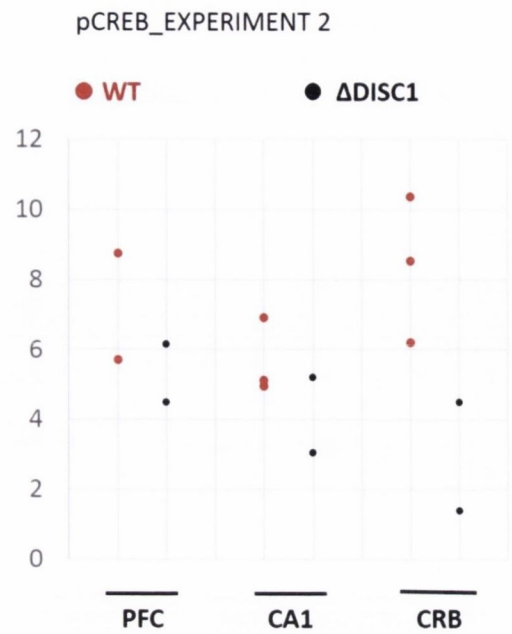
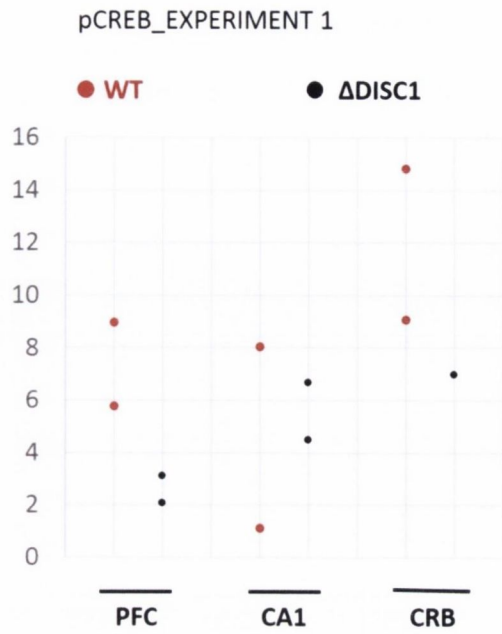


totCREB_EXPERIMENT 1



totCREB_EXPERIMENT 2





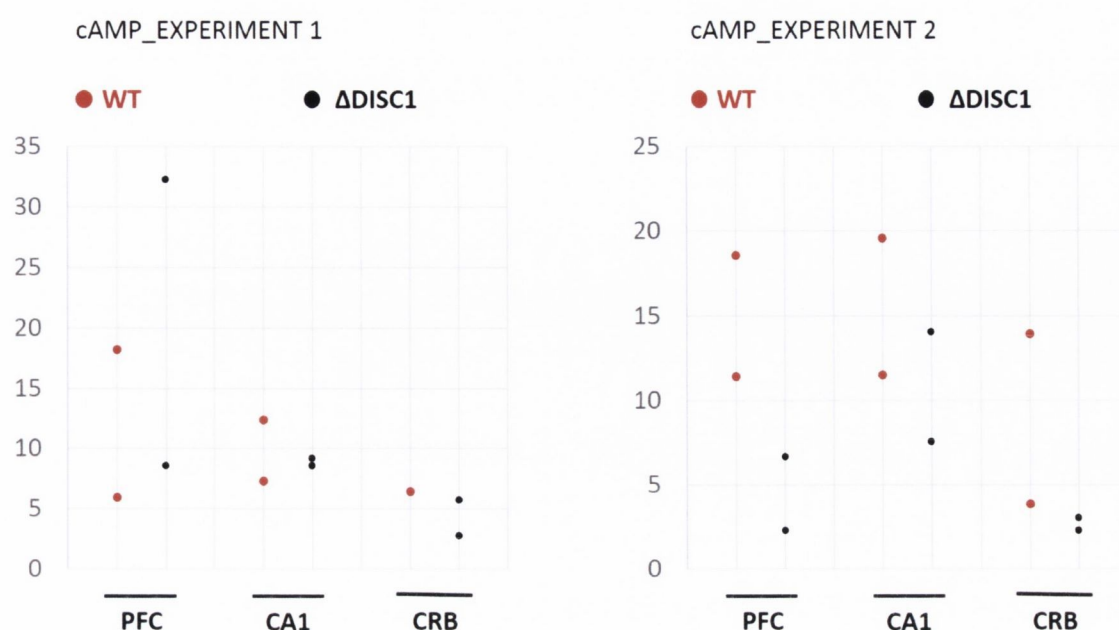


Figure 30. Plotted values of individual IS data points for WT (red dots) and *Disc1* mutant (black dots) sections before normalization to the WT IS. Each graph visualize distribution of the IS values measured from the experiments performed. Two experiments were performed for each antigen to be analyzed; an average of two sections/brain area (PFC, HP and CRB) were included in each experiment. Individual points represent the average of IS measured from each Z stack from individual sections of each brain area.

4.2.1 Levels of synapsin and pCREB are significantly decreased in selected brain areas of adult *Disc1*-L100P mutant mice

Following the same trend of what we observe from WB measurements, we find a decrease of more than 50% of synapsin levels specifically in hippocampus (CA1 and CA3), but not in CRB, in sections from *Disc1* mutants compared to WT; an average reduction of synapsin levels is found in PFC of mutants; this difference is not statistically significant due to the small number of section used here. Additional experiments are required to clarify this pattern. (Table 11, Fig 31). Levels of PSD95 and GAD65 measured in PFC, HP (CA1) and CRB are instead comparable between *Disc1* mutants and wild types during adulthood (Table 11, Fig 32 and 33 respectively). Additionally, to assess a possible brain area-specific alteration of the activation of the transcriptional factor CREB, we measured IS intensity of both total CREB and the active form pCREB in the three brain areas. We show a decrease of 45% and 57% of the levels of pCREB in mutants which occurs in PFC and CRB respectively (Table

11, Figure 34); levels of total CREB are increased in CRB of mutants, but not significantly different in mutants compared with WT in all areas (Table 11, Figure 35).

In order to test a possible cAMP dependent decrease of pCREB in the brain of adult mutants, we measured immunostaining of the phosphodiesterase PDE4b and the second messenger cAMP. We show indistinguishable levels of PDE4b and cAMP in PFC, hippocampus (CA1) and CRB (Table 11, Figure 36 and 37 respectively).

These results from histological immunostaining suggest that changes in the levels of synaptic and neuronal markers in adult *Disc1* mutant brains are brain area specific: decreased levels of synapsin are observed in hippocampus and levels of pCREB are impaired specifically in PFC and CRB of mutants; measurements of PDE4b and cAMP suggest that the reduction of pCREB in these areas is not cAMP-dependent.

Postnatal Stage	Antigen	Brain Area		Relative Immunostaining (IS)		P value
				WT	Δ DISC1	
P>60	Synapsin	PFC		1 ± 0.34	0.22 ± 0.12	NS
		HP	CA1	1 ± 0.09	0.48 ± 0.09	0.03
			CA3	1 ± 0.15	0.45 ± 0.09	0.03
		CRB		1 ± 0.25	0.96 ± 0.13	NS
	PSD95	PFC		1 ± 0.004	0.91 ± 0.09	NS
		HP (CA1)		1 ± 0.11	1.41 ± 0.25	NS
		CRB		1 ± 0.02	0.91 ± 0.08	NS
	GAD65	PFC		1 ± 0.23	1.21 ± 0.49	NS
		HP (CA1)		1 ± 0.18	0.93 ± 0.12	NS
		CRB		1 ± 0.07	1 ± 0.27	NS
	PDE4b	PFC		1 ± 0.22	1.29 ± 0.47	NS
		HP (CA1)		1 ± 0.18	1.15 ± 0.37	NS
		CRB		1 ± 0.16	1.21 ± 0.43	NS
	cAMP	PFC		1 ± 0.23	0.99 ± 0.57	NS
		HP (CA1)		1 ± 0.15	0.80 ± 0.10	NS
		CRB		1 ± 0.47	0.46 ± 0.15	NS
	CREB	PFC		1 ± 0.16	0.57 ± 0.18	NS
		HP (CA1)		1 ± 0.05	0.68 ± 0.25	NS
		CRB		1 ± 0.10	1.85 ± 0.36	-
	pCREB	PFC		1 ± 0.12	0.55 ± 0.13	0.04
		HP (CA3)		1 ± 0.25	0.96 ± 0.19	NS
		CRB		1 ± 0.11	0.43 ± 0.13	0.02

Table 11. Details about immunofluorescence on PFC, HP and CRB sections obtained from wild type (WT) and *Disc1* mutant (Δ DISC1) adult (P>60) animals. Relative immunostaining (IS) intensity $\pm$ standard error (SE) and *P* value are reported for each marker and area. IS intensity is expressed in WT fold and differences between genotypes were assessed using Mann-Withney test.

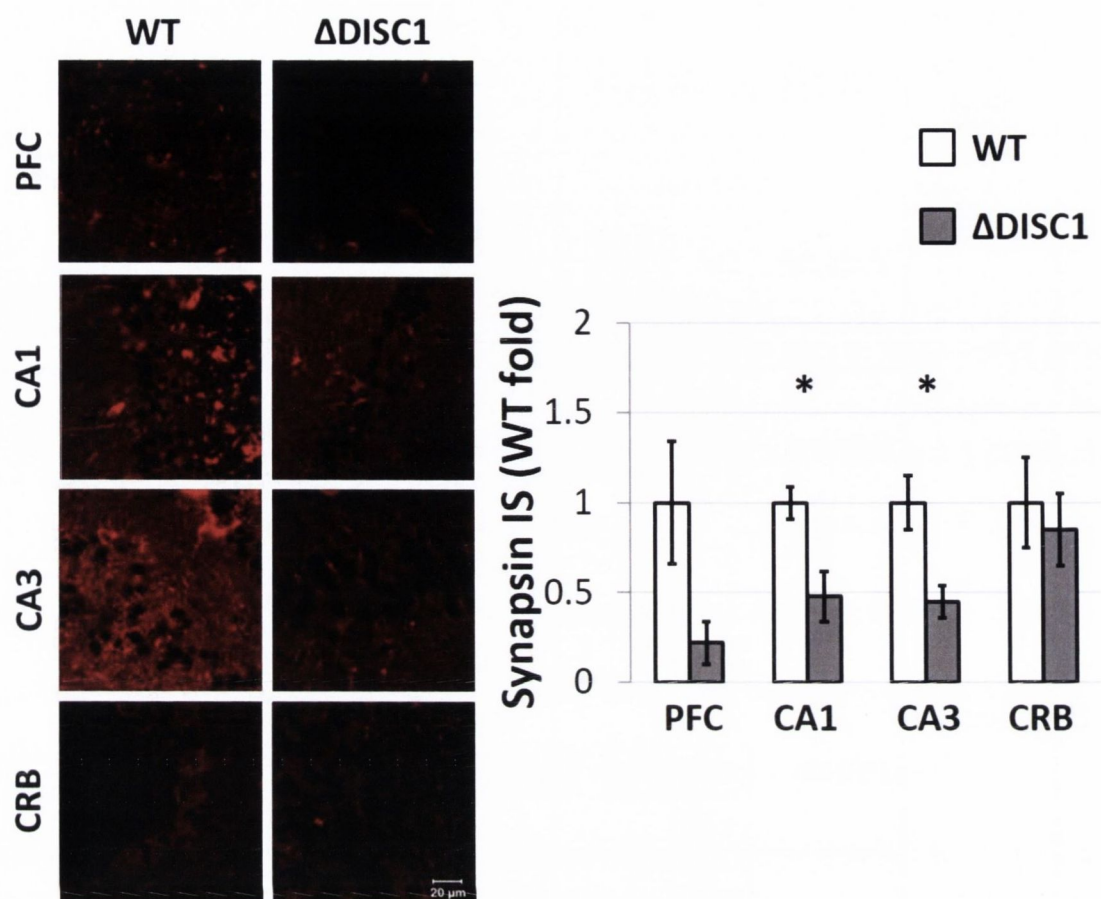


Figure 31. Levels of synapsin are reduced in histological sections from HP (both CA1 and CA3), but not PFC or CRB, in *Disc1*-L100P mutants (Δ DISC1) at P>60 (* $P=0.03$).

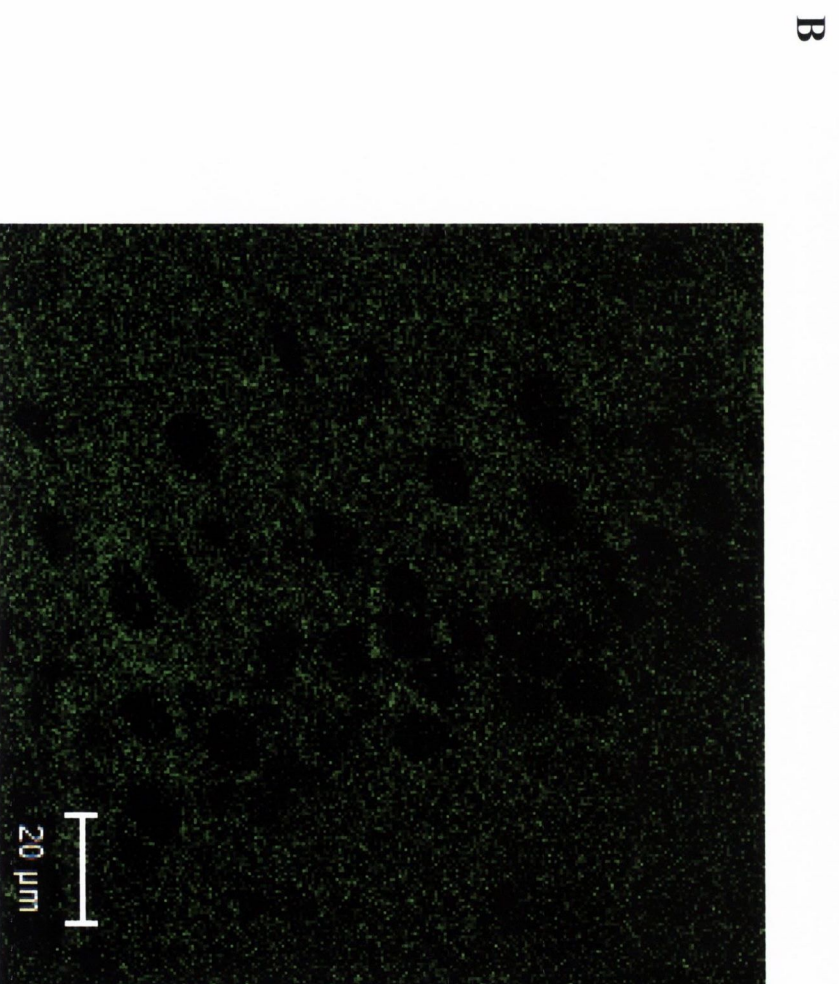
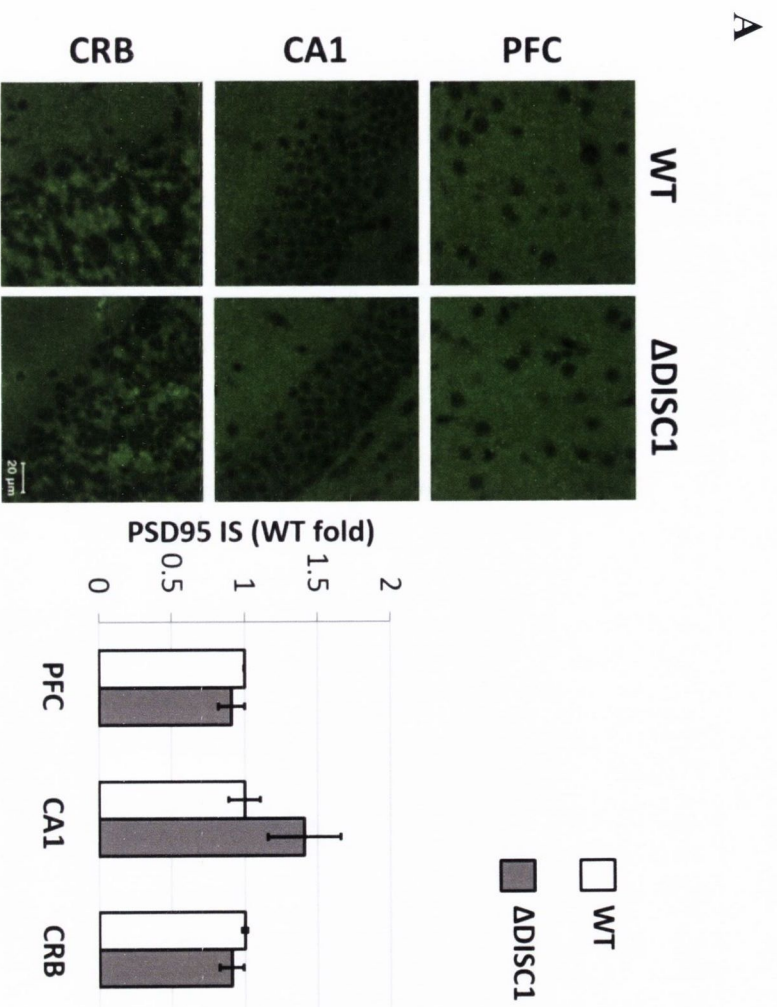


Figure 32. A: Levels of PSD95 are comparable in histological sections from PFC, hippocampus (CA1) and CRB, in WT and *Disc1*-L100P mutants (Δ DISC1).
 B: High magnification of an individual Z focal plane obtained from scan imaging of one of the WT hippocampal (CA1) sections included in our analysis: the image show the punctate pattern typical of PSD95 staining, which is not clearly recognizable in low magnification projection images (A).

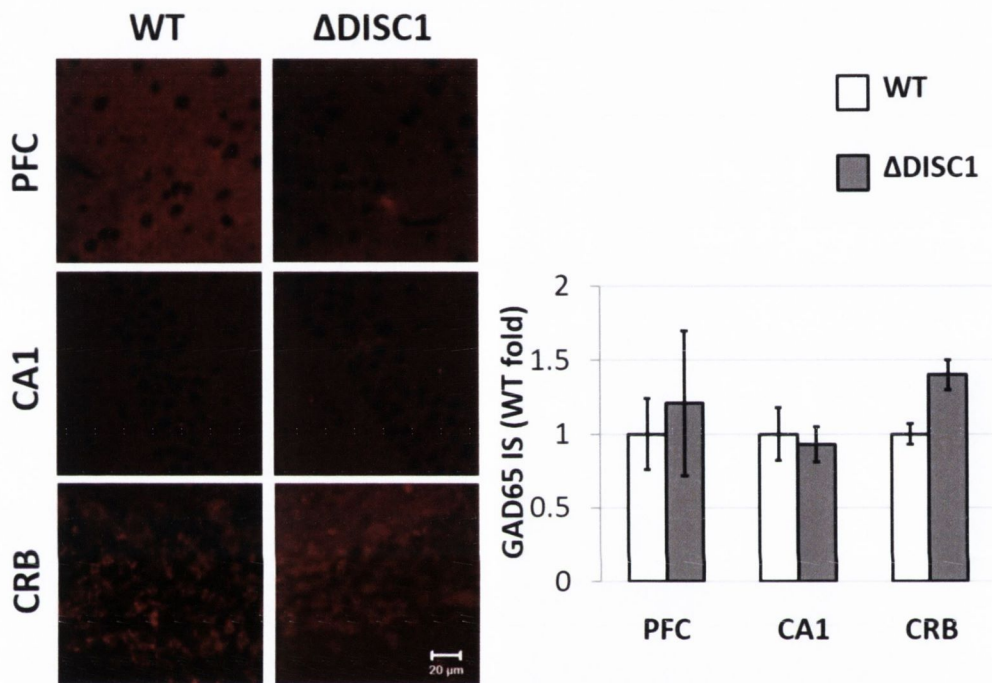


Figure 33. Levels of GAD65 are comparable in histological sections from PFC, hippocampus (CA1) and CRB, in WT and *Disc1*-L100P mutants (Δ DISC1).

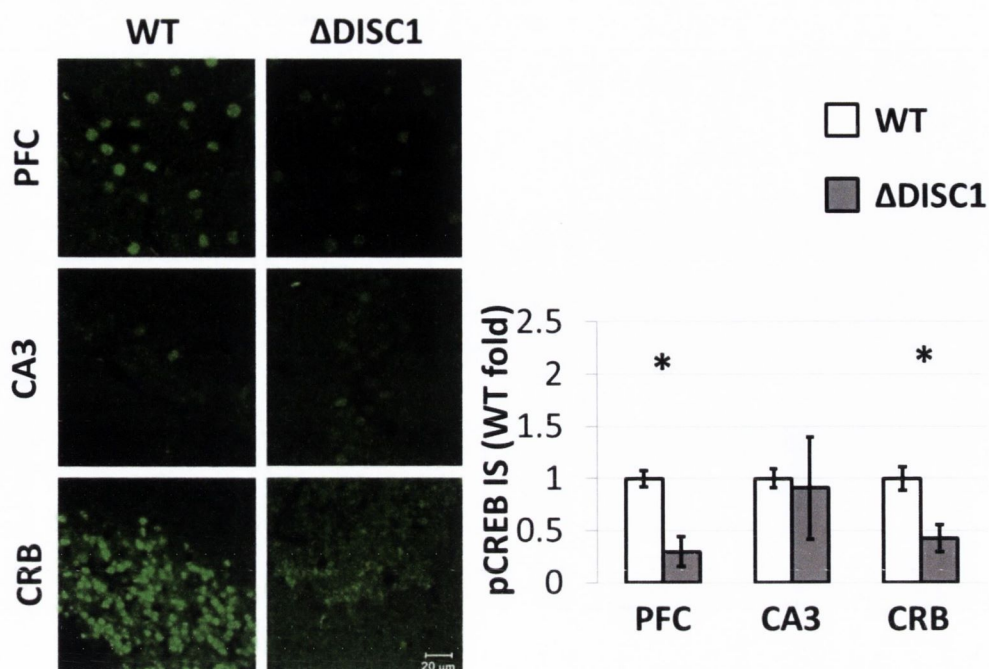


Figure 34. Levels of pCREB are reduced in histological sections from PFC and CRB, but not hippocampus (CA1), in *Disc1*-L100P mutants (Δ DISC1) at P>60 (* $P < 0.05$).

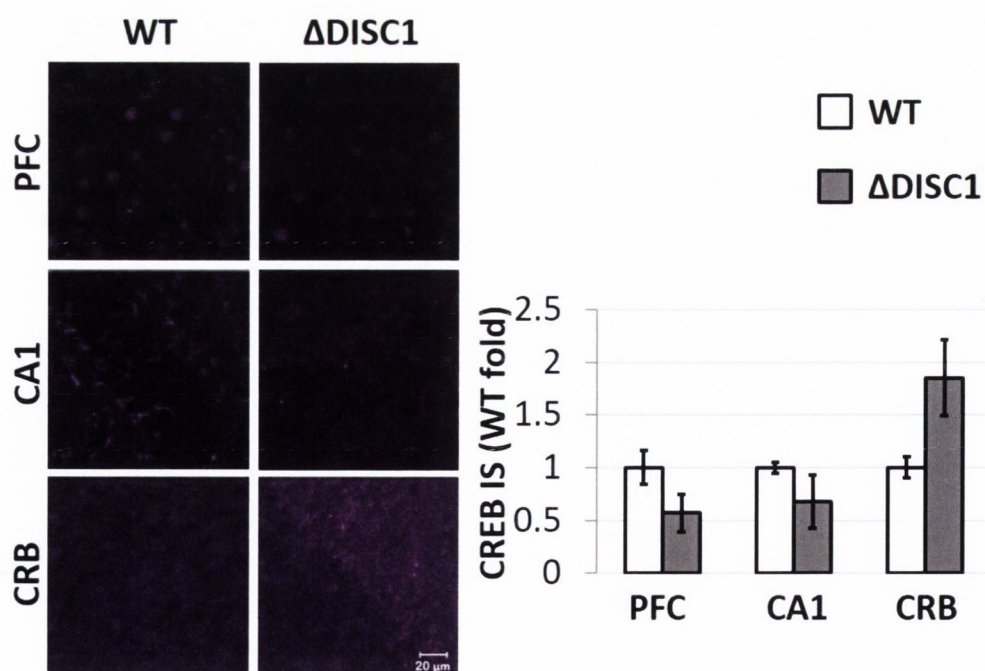


Figure 35. Levels of CREB are not significantly different in histological sections from PFC, hippocampus (CA1) and CRB, in WT and *Disc1*-L100P mutants (Δ DISC1).

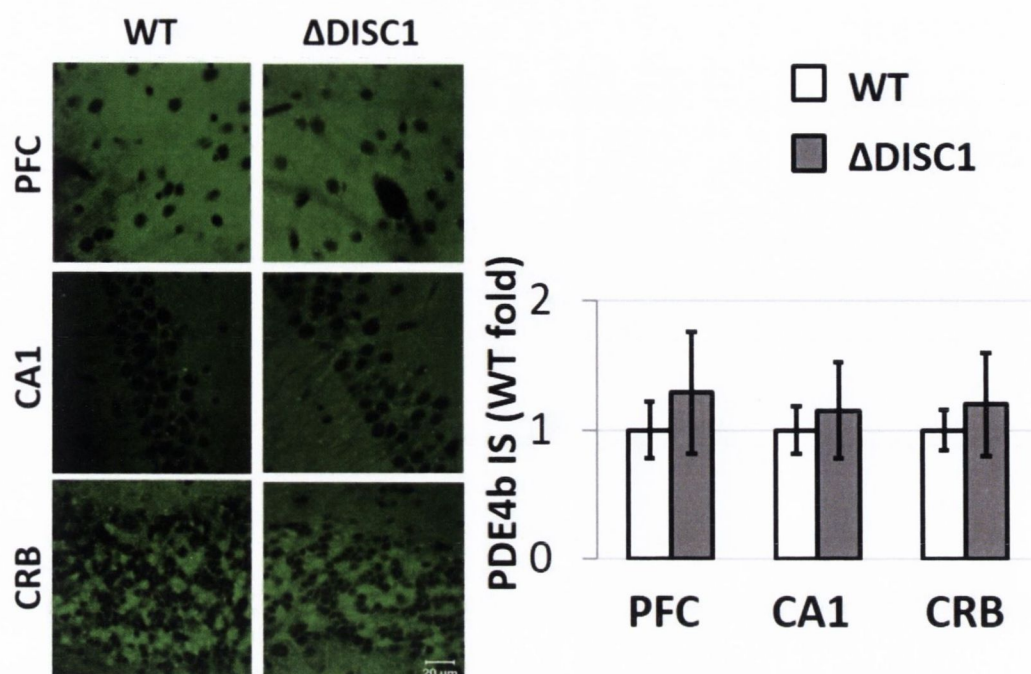


Figure 36. Levels of PED4b are comparable in histological sections from PFC, hippocampus (CA1) and CRB, in WT and *Disc1*-L100P mutants (Δ DISC1).

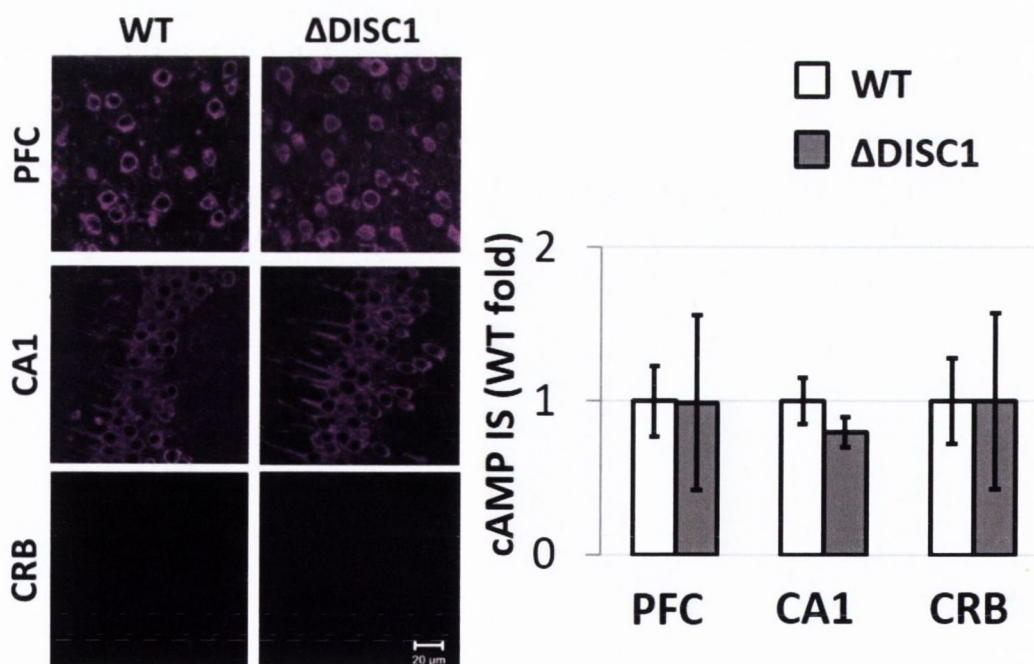


Figure 37. Levels of cAMP are comparable in histological sections from PFC, hippocampus (CA1) and CRB, in WT and *Disc1*-L100P mutants (Δ DISC1).

4.3 Functional characterization of PAK7

PAK7 is a brain-specific isoform of PAK (p21-activated kinase), which can be activated by the small GTPases Rac and Cdc42. Although in vitro evidences suggested implication of PAK7 in neurite outgrowth and synaptic vesicles trafficking (Dan et al. 2002b; Strohlic et al. 2012), the role of the kinase in neuronal physiology remains largely unknown. Mice knock-out (KO) for *Pak7* are viable and do not show gross brain anatomical alterations; however, they have deficits in motor coordination and are less aggressive than wild type littermates (Nekrasova et al. 2008; Furnari et al. 2013). Double *Pak6/Pak7* KO exhibit behavioral and learning aberrations, with possible functional redundancy for the two isoforms (Nekrasova et al. 2008). Other members of the PAK family have been demonstrated to participate to the NMDA-dependent spine dynamic processes as a downstream effector of the DISC1/kalirin7/PSD95 protein complex (Hayashi-takagi et al. n.d.). Our results show that PAK7 protein levels are altered during postnatal development in *Disc1*-L100P mutant brains (Figure 27, Page 78); therefore, we sought to assess a possible co-localization and interaction between DISC1 and PAK7 at different postnatal stages in the mouse brain. Additionally, in order to test a possible activity-dependent regulation of PAK7 expression, we tested the effect of different forms of neuronal stimulation on the levels of the PAK7 protein in primary neuronal cultures.

4.3.1 DISC1/PAK7 colocalization and interaction studies

In this study we investigated the putative colocalization and interaction between the two risk factors for SCZ DISC1 and PAK7 at the protein level. In our experiments, we used two commercially available antibodies directed anti-DISC1 (Invitrogen 40-6900 made in rabbit for western blot, and Santa Cruz sc-47990 made in goat for immunoprecipitation) and two directed anti-PAK7 (Sigma SAB-3500335 made in rabbit for western blot, and Santa Cruz sc-22155 for immunoprecipitation). We tested the specificity of both antibodies anti-PAK7 in preliminary WB experiments in which a band at the expected molecular weight of 81 kDa was detected in brain extracts from WT animals, but not in extracts from *Pak7* KO mice that only retain regulatory regions of the gene (Figure 52, Page 118). Multiple isoforms of DISC1 have been described, and the commercially available antibodies directed against DISC1 recognize multiple bands at different molecular weight, which represent distinct isoforms of

the protein and possible polymeric structures given by association of single monomeric isoforms. One study reported in literature (Kuroda et al. 2011) describes two homemade antibodies (raised against either C- and N-termini of DISC1); the antibodies were tested for specificity using a mouse lacking of most of exon 2 and exon 3 of the *Disc1* gene. The authors provide evidence that a single band of 100 kDa is recognized by the two antibodies, which is not present in brain extracts from mutant mice lacking exon 2 and 3. As the authors report, these antibodies do not detect the presence of any of the additional isoforms reported in the literature (for revision see Chubb, Bradshaw, Soares, Porteous, & Millar, 2008). Additionally, they show the presence of transcripts from exons 7 to 10 of *Disc1*, which suggests the presence of alternative *Disc1* transcripts - other than the 100 kDa one - in their model. This work emphasizes one more time on the difficulties in using DISC1 antibodies; however the presented evidences do not exclude the presence of DISC1 isoforms that can be recognized by commercially available antibodies.

4.3.1.1 DISC1 and PAK7 colocalize in brain cortex and hippocampus of wild type mice during postnatal development

We investigated the distribution of DISC1 and PAK7 in histological sections over postnatal development. We performed a double immunostaining for DISC1 and PAK7 on brain sections obtained at the level of somatosensory cortex (SSC) and hippocampus (DG or dentate gyrus) from WT brains at P8-10 and P>60. In order to increase reliability of this experiment, we used secondary antibodies conjugated to DL488 to detect PAK7 and to DL-649 to detect DISC1 as described in the material and methods chapter. The two fluorophores emit fluorescence in the range of green and far red (purple) respectively and their emission spectra do not overlap; this choice eliminated the chance of bleed-through effect, which represents a possibility when green dyes are used with red ones for co-immunostaining experiments. After imaging, we converted purple images into red images for graphic purposes. We show that there is overlapping immunostaining for DISC1 (red) and PAK7 (green) in both SSC and hippocampus at all postnatal stages; co-localization is visible as a punctate yellow staining that presents a perisomal distribution (Figure 38). Two individual single primary/double secondary control immunofluorescence experiments were performed on P>60 SSC sections, using primary antibody goat anti-PAK7 (Figure 39) or rabbit anti-

DISC1 (Figure 40) followed by addition of both DL488-conjugated anti –goat and DL649-conjugated anti –rabbit secondary antibodies: merged-channels images resulting from this two experiments did not show overlap between DL488 and DL649 signal. These controls confirmed that the overlapping staining was detected as a result of colocalization of DISC1 and PAK7, rather than random merge of background given by aspecific secondary antibody binding. Double immunolabeling and control experiments were imaged using a 63X magnification and oil-immersion objective with NA=1.4. Additionally, we performed three-dimensional (3D) imaging on SSC and hippocampus sections from a P30-40 WT brain; we observe overlapping immunostaining for DISC1 and PAK7 throughout the entire thickness of the tissue at this age (3D imaging not shown here). These results show colocalization of DISC1 and PAK7 during postnatal development and suggest that, similarly to what happens for other member of the PAK family, the two proteins might interact in neurons.

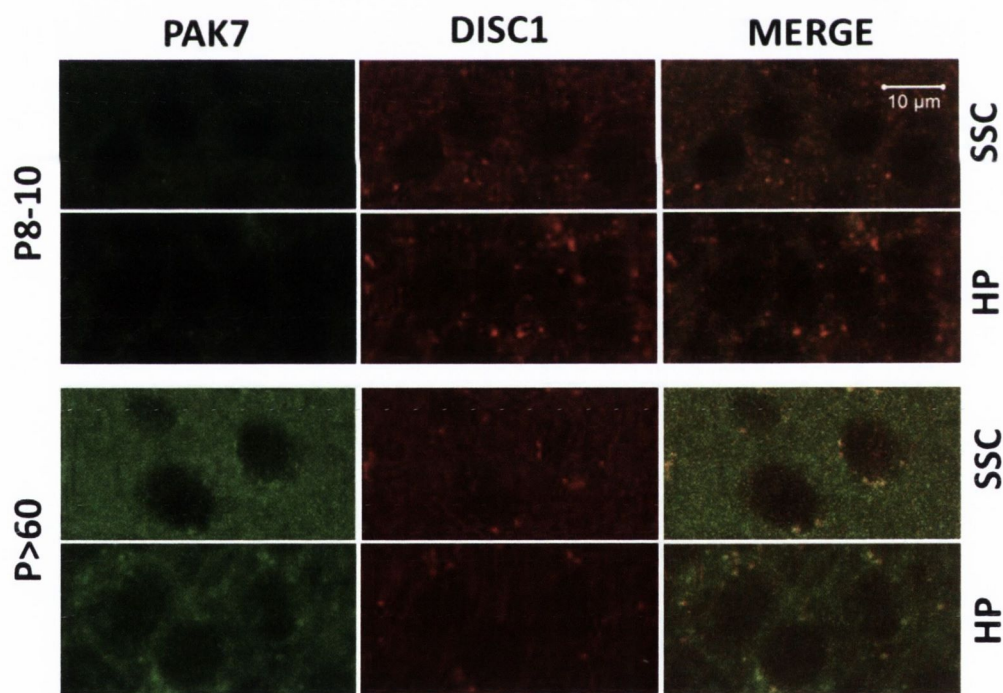


Figure 38. Colocalization of DISC1 and PAK7 in somatosensory cortex (SSC) and hippocampus of WT mice during postnatal development. Immunostaining of DISC1 (red) and PAK7 (green) histological sections of SSC and HP obtained from P8-10 and P>60 WT brains. Overlapping staining (yellow) indicates colocalization of DISC1 and PAK7.

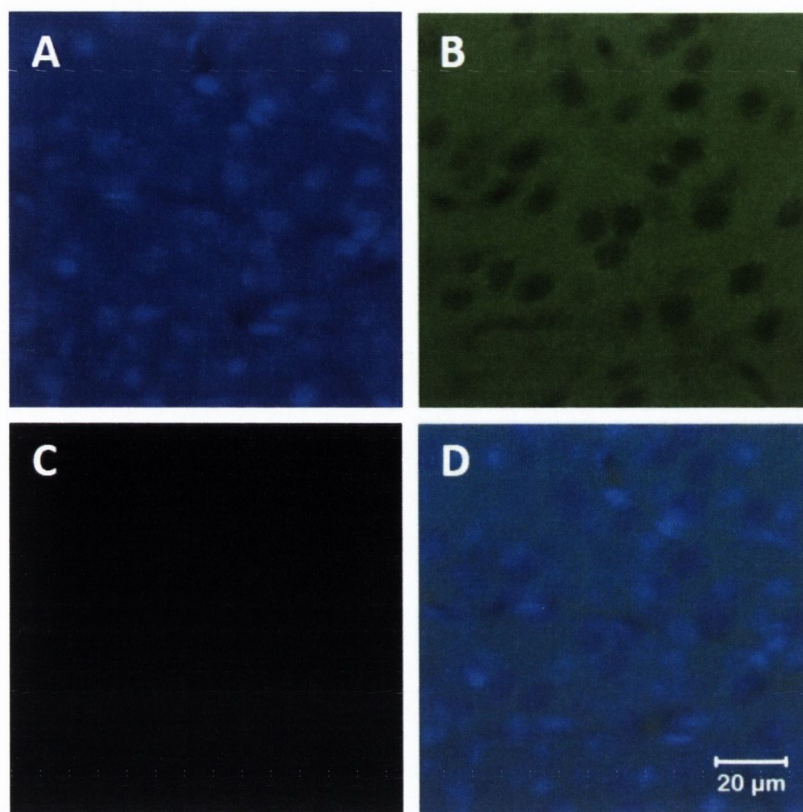


Figure 39. Single primary (anti-PAK7)/double secondary antibodies control for colocalization experiment. P>60 SSC sections were stained using primary antibody anti-PAK7 (Santa Cruz sc-22155, made in goat), followed by incubation with DL488-conjugated anti-goat and DL649-conjugated anti-rabbit immunoglobulins. A: DAPI nuclear staining (blue channel); B: PAK7 (green channel); C: far red (purple) channel acquisition, showing undetectable signal; D: merged signals from blue, green and far red channel.

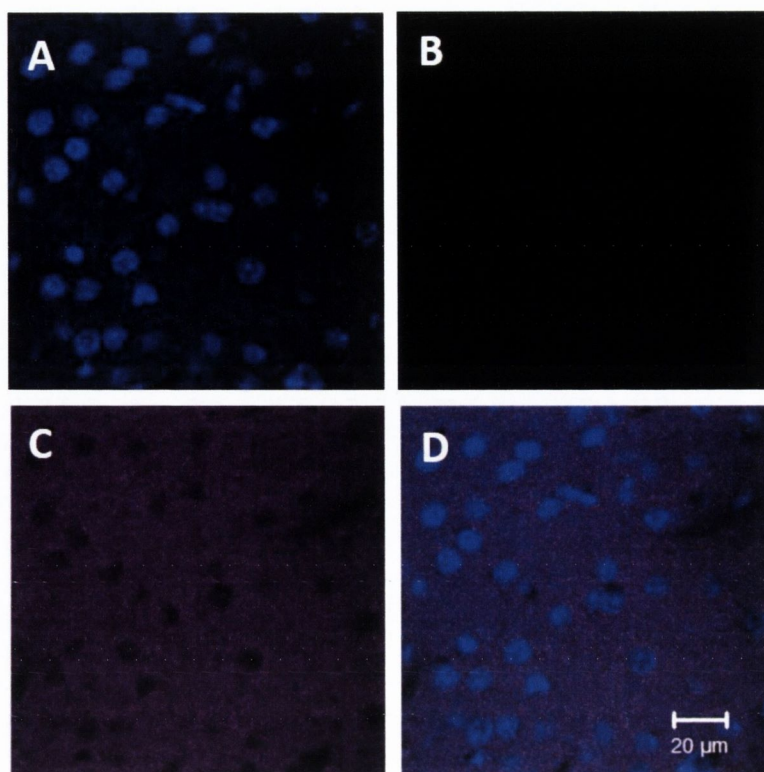


Figure 40. Single primary (anti-DISC1)/double secondary antibodies control for colocalization experiment. P>60 SSC sections were stained using primary antibody anti-DISC1 (Invitrogen 40-6900, made in rabbit), followed by incubation with DL488-conjugated anti-goat (green) and DL649-conjugated anti-rabbit (purple) immunoglobulins. A: DAPI nuclear staining (blue channel); B: green channel acquisition, showing undetectable signal; C: DISC1 (far red/purple channel); D: merged signals from blue, green and far red channel.

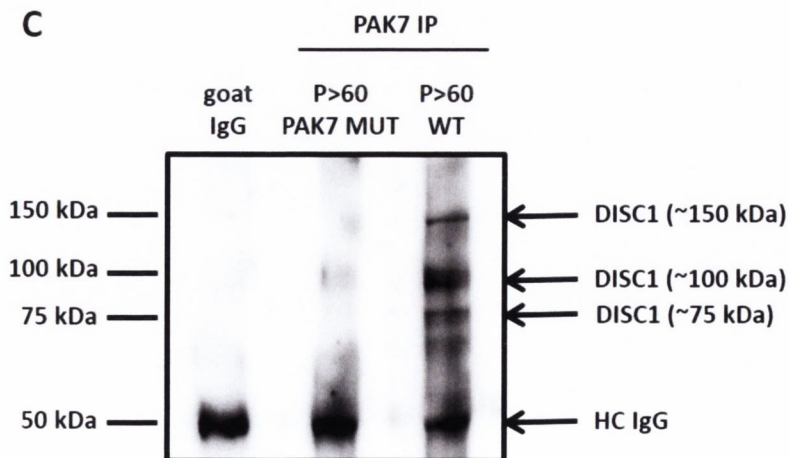
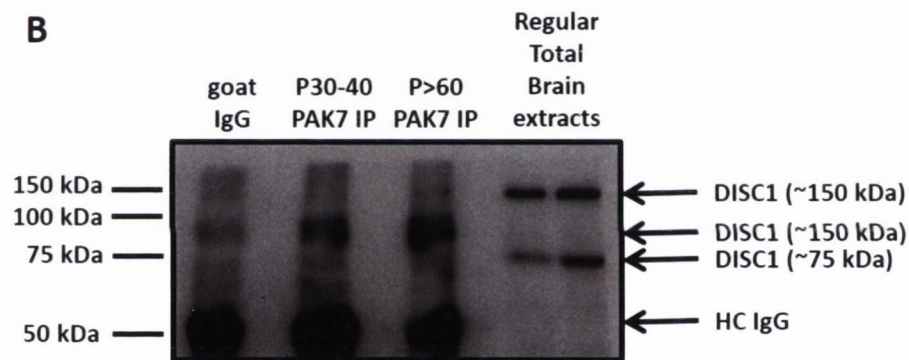
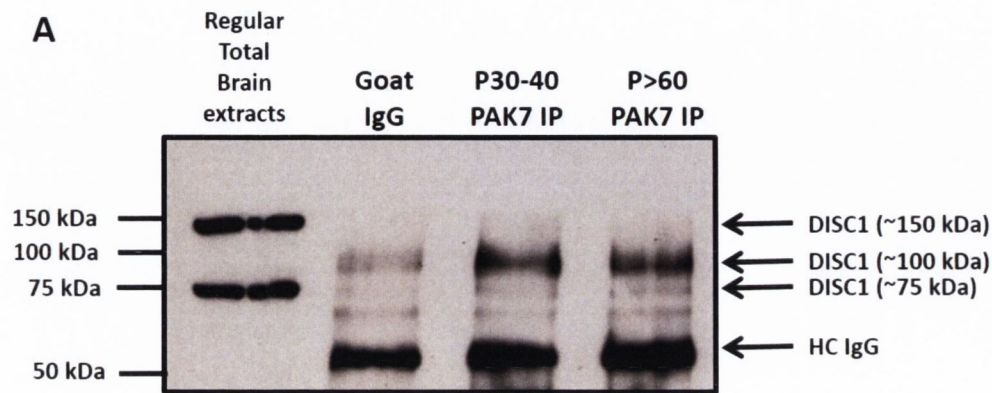
4.3.1.2 DISC1 and PAK7 interact in juvenile and adult wild type brains

In order to check for protein-protein interaction between DISC1 and PAK7 we performed co-IP (co-immunoprecipitation) experiments using total protein extracts obtained from juvenile and adult WT mice. Our experiments show that when PAK7 was immunoprecipitated in total brain extracts, a DISC1 isoform was detected among co-immunoprecipitated proteins at both ages (Figure 41-A). These results suggest direct or indirect physiological interaction between the two proteins during postnatal development. Since other members of the PAK family have been involved in DISC1-dependent spine dynamic mechanisms, putative DISC1/PAK7 interaction at the synaptic level was also assessed: we carried out co-IP experiments using synaptoneurosomal protein extracts obtained from WT P30-40 and P>60 brains. Synaptoneurosomal extracts were also prepared

from P>60 *Pak7* KO mice and used as a negative control. We show that DISC1 is co-immunoprecipitated with PAK7 in WT at P30-40 and P>60 (Figure 41-B), but not in P>60 brains of mice KO for *Pak7* (Figure 41-C). In addition, PAK7 is co-immunoprecipitated with DISC1 in P>60 WT extracts (Figure 41-D), which confirm an interaction for the two proteins at the synaptic level in the mouse brain; an image including regular total brain extracts lanes is not available for this experiments, however, PAK7 band was detected at 81 kDa as expected. We performed at least three independent experiments - using brain extracts made from different WT mice - that employed a DISC1 antibody (rabbit antibody) in western blot after immunoprecipitation of PAK7 (goat antibody). Our results strongly confirm the physiological interaction between DISC1 and PAK7 in the mouse brain. The antibody against DISC1 that we used following IP of PAK7 did not recognize the major isoform of DISC1 detectable at 100 kDa in regular brain extracts. However, in all replicates, and in both total brain and synaptoneurosomal extracts co-immunoprecipitated within the same experiment a band of approximately 100 kDa was identified, correspondent to the band of approximately 75 kDa observed in regular total brain extracts run alongside.

The presence of two controls validate our findings: the reverse co-IP experiment detected a single band correspondent to the molecular weight of PAK7 (81 kDa); additional support is provided by the lack of bands in the experiment that employed a *Pak7* KO brain extract. In one experiment only (Figure 41-C) we detected two extra bands following immunoprecipitation of PAK7 and WB immunodetection of DISC1: one of molecular weight included between 100 and 150 kDa and the other of approximately 75 kDa. However, even in this experiment the most intense band detected corresponded to a MW of approximately 100 kDa in co-IP samples. Therefore, we believe that the DISC1 isoform identified to interact with PAK7 in our conditions is the one that shows a MW of approximately 100 kDa in co-IP samples and approximately 75 kDa in regular total brain extracts. Slight differences of observed molecular weight of bands between co-IPed (coimmunoprecipitated) samples and regular total protein extracts are due to different nature of the individual samples: in fact, the total brain extracts not subjected to immunoprecipitation procedure were obtained using a regular protocol of total protein extraction. Total brain and synaptoneurosomal extracts to be used for IP were conversely made using a low ionic strength extraction buffer and they did not underwent sonication. Differently from what happened for non-IPed samples, the

samples made for IP using the mild condition described above, were likely to have maintained post-translational modifications.



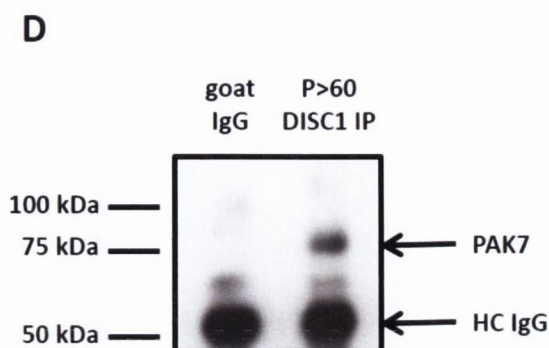


Figure 41. DISC1 and PAK7 interact in juvenile (P30-40) and adult (P>60) WT brains. A: immunostaining for DISC1 on total protein extracts from P30-40 and P>60 WT where PAK7 was immunoprecipitated (IP), DISC1 is detected at ~ 100 kDa in co-IP samples; B: immunostaining for DISC1 on synaptoneurosomal extracts from P30-40 and P>60 WT where PAK7 was IP, DISC1 is detected at 100 kDa in IP samples; C: immunostaining for DISC1 on synaptoneurosomal extracts from either *Pak7* KO or WT mice at P>60, three different isoforms of DISC1 are detected at 75 kDa, ~100 kDa and ~150 kDa. D: immunostaining for PAK7 on synaptoneurosomal extracts from P>60 WT where DISC1 was IP, PAK7 is detected at 81 kDa; Bands of approximately 150 kDa and 75 kDa were identified in regular total brain extracts probed for DISC1 (A-B). Normal goat IgG was used in all experiments as a negative control (55 kDa).

4.3.2 Protein levels of PAK7 are regulated by neuronal stimulation in primary cultures from CD1 wild type mice

To further characterize the novel SCZ risk factor PAK7 at the protein level, we investigate the effect of neuronal stimulation on the expression of PAK7 in vitro. We prepared primary neuronal cultures from prefrontal cortex (PFC) or visual cortex (VC) of P0-1 CD1 wild type pups as described in section 3.7, and we treated them with different protocol of stimulations that induce either Hebbian plasticity (LTP, LTD or rapid potassium depolarization) or homeostatic plasticity (TTX and two days KCl) with different stimulation protocols as described in section 3.8. We performed immunofluorescence on treated cultures using NeuN as a neuronal marker, and we measured neuronal levels of cytoplasmic and nuclear PAK7 in response to different treatments. A no primary/secondary antibodies only-control was performed for all secondary antibodies used for immunostaining experiments in order to confirm IS specificity (Figure 42).

We find that in PFC cultures cytoplasmic neuronal levels of PAK7 are not affected by any applied form of neuronal stimulation (Figure 43, 44, and 47-A), while nuclear levels of PAK7

are slightly but consistently increased (15%) in response to long term treatment with KCl (Figure 43, 44, 47-B). Surprisingly, in VC cultures all short and long term stimulations elicit a decrease of PAK7 cytoplasmic levels (Figure 47-C): TTX 15%, KCl 22%, LTP 25%, LTD 21% and K^+ 43%. In VC a similar downward expression trend is found for nuclear PAK7 (Figure 47-D): TTX 16%, KCl 8%, LTP 23%, LTD 21% and K^+ 47%; however, the decrease of expression of PAK7 in the nuclear compartment in response to 2 days treatment with KCl is not statistically significant. Effect of long and fast protocols of stimulation on VC cultures is shown by Figure 45 and Figure 46 respectively. These results show that cytoplasmic and nuclear PAK7 levels are altered by plasticity stimulation in primary neurons from PFC and VC. Visual cortex cultures are more sensitive to stimulation; in these neurons the maximal effect on PAK7 protein levels is elicited by short term stimulations. It is common practice in cellular and molecular studies using primary neuronal cultures to use single entities (cells or dendrites) as single data points for the statistical analysis of data. However, we performed additional analysis using the mean values of each biological replicates and each treatments: Kruskal-Wallis analysis of variance among groups did not found differences in this analysis (Figure 52).

LTP and LTD induction protocols have long been demonstrated to induce morphological remodeling of spines and to regulate synaptogenesis in vitro: many studies provide evidence that LTP stimulation produces expansion of the size and number of synaptic spines, while the opposite effect is elicited by LTD stimulation (reviewed by De Roo, Klauser, Garcia, Poglia, & Muller, 2008). PSD95 (postsynaptic density 95) is a major component of the postsynaptic density: expression levels of PSD95 are well known to be directly proportional to the size and number of spines in neurons (El-Husseini et al. 2000). In order to support our results from the stimulation protocols, we measured the expression of synaptic proteins in control and treated primary neuronal culture (data not shown); we confirmed significantly increased expression of PSD95 in LTP-stimulated cultures (Tropea 2011). Although our results for short term stimulation protocols align with previous literature, the effect on the expression of comparable proteins have not been studied in prolonged stimulation protocols. Additional experiments are required to establish appropriate control protein to test the effect of long term stimulation in vitro.

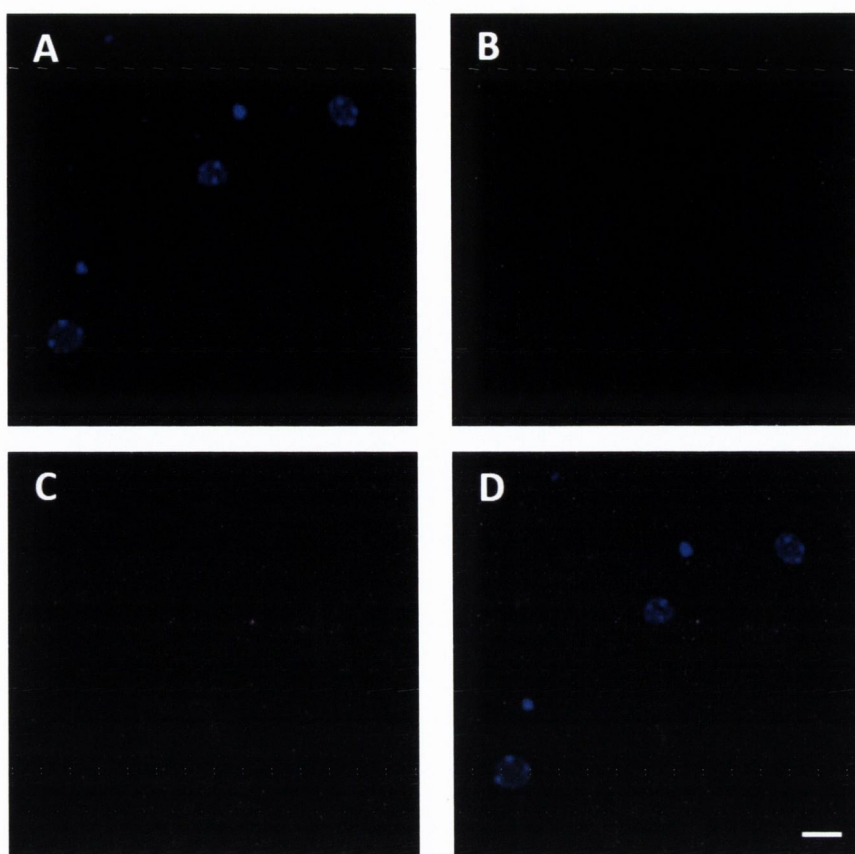


Figure 42. No primary/secondary antibodies only control in primary neuronal cultures. The figure reports an example of a control immunostaining for secondary antibodies specificity. Primary neuronal cultures were subjected to regular immunostaining protocol in which incubation with primary antibodies against PAK7 and NeuN were omitted. Conversely, both secondary antibodies DL488-conjugated anti-rabbit and DL649-conjugated anti-mouse were used. A: acquisition from blue channel (DAPI nuclear staining); B: acquisition from the green channel (488 nm wavelength); C: acquisition from the far red channel (649 nm wavelength); D: merged signal of blue, green and far red channels. Scale: 20 μ m.

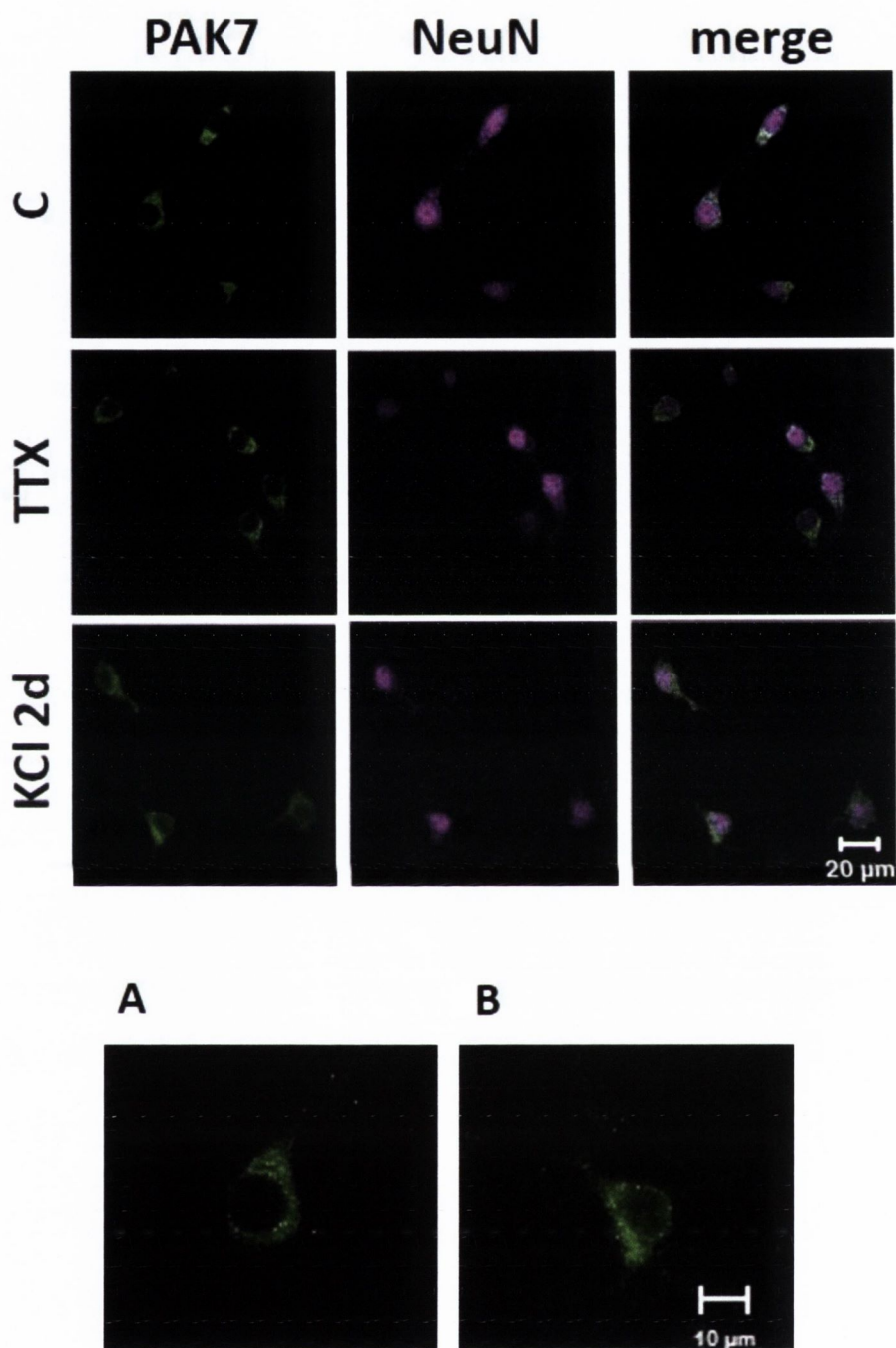


Figure 43. Immunofluorescence staining of primary neuronal cultures obtained from PFC and treated with stimulation protocols that induce long-term forms of synaptic plasticity (TTX or KCl 2d). Cultures were stained for PAK7 (green) and NeuN (purple). PAK7 show both a cytoplasmic and a neuronal subcellular localization. Panel A: close-up view of a representative neuron stained for PAK7 in control cultures. Panel B: close-up view of a representative neuron stained for PAK7 in cultures treated with KCl for 2 days. Panels A and B show increased nuclear staining for PAK7 in cultures treated with KCl for 2 days compared with control neurons.

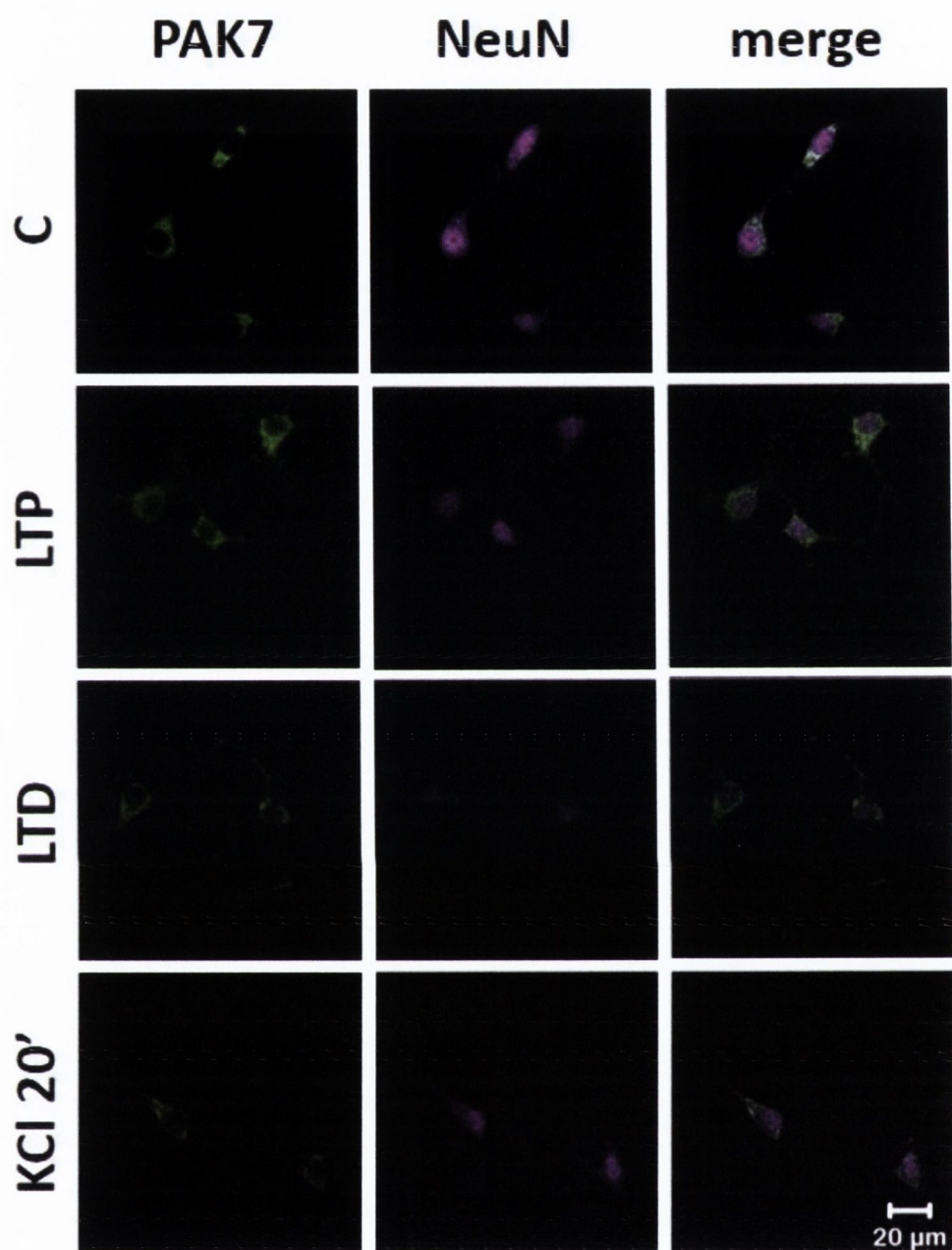


Figure 44. Immunofluorescence staining of primary neuronal cultures obtained from PFC and treated with stimulation protocols that induce short-term forms of synaptic plasticity (LTP, LTD or KCl 20'). Cultures were stained for PAK7 (green) and NeuN (purple). PAK7 show both a cytoplasmic and a neuronal subcellular localization.

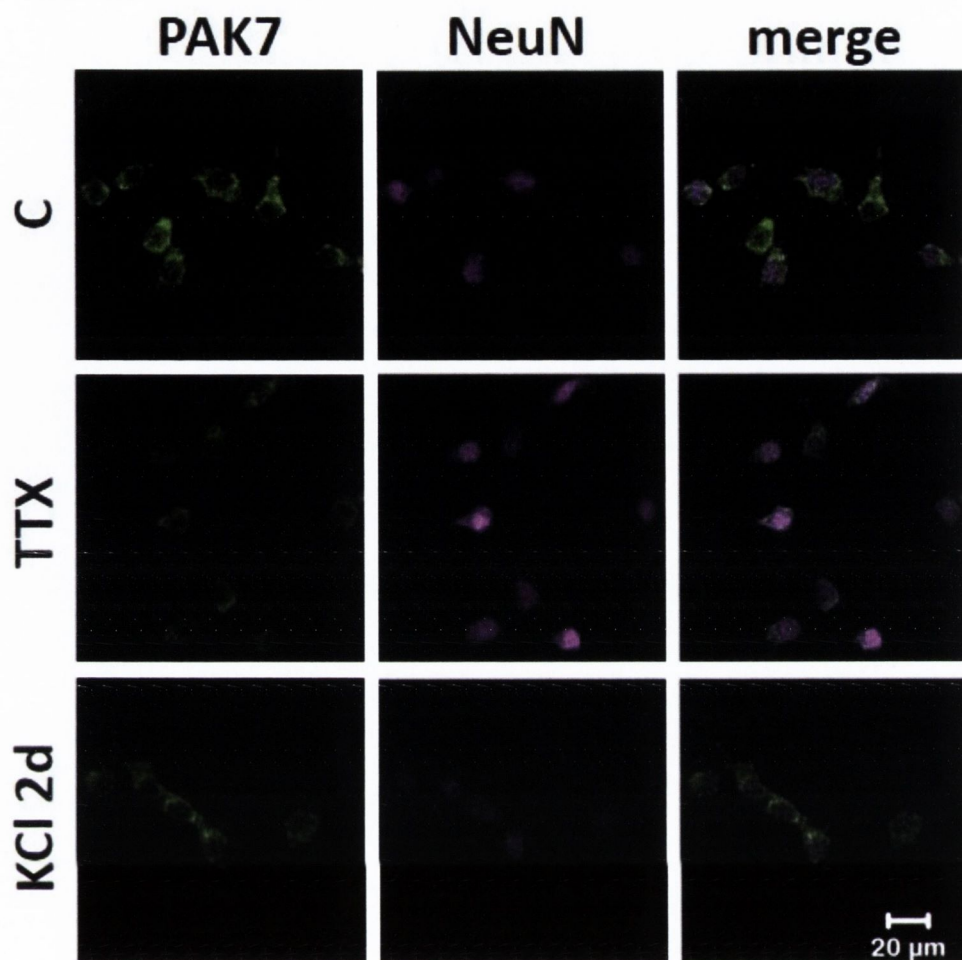


Figure 45. Immunofluorescence staining of primary neuronal cultures obtained from VC and treated with stimulation protocols that induce long-term forms of synaptic plasticity (TTX or KCl 2d). Cultures were stained for PAK7 (green) and NeuN (purple). PAK7 show both a cytoplasmic and a neuronal subcellular localization. Cytoplasmic staining for PAK7 is decreased in response to both treatments when compared to control stimulation; while nuclear staining for the protein is reduced by treatment with TTX, but not KCl 2 days.

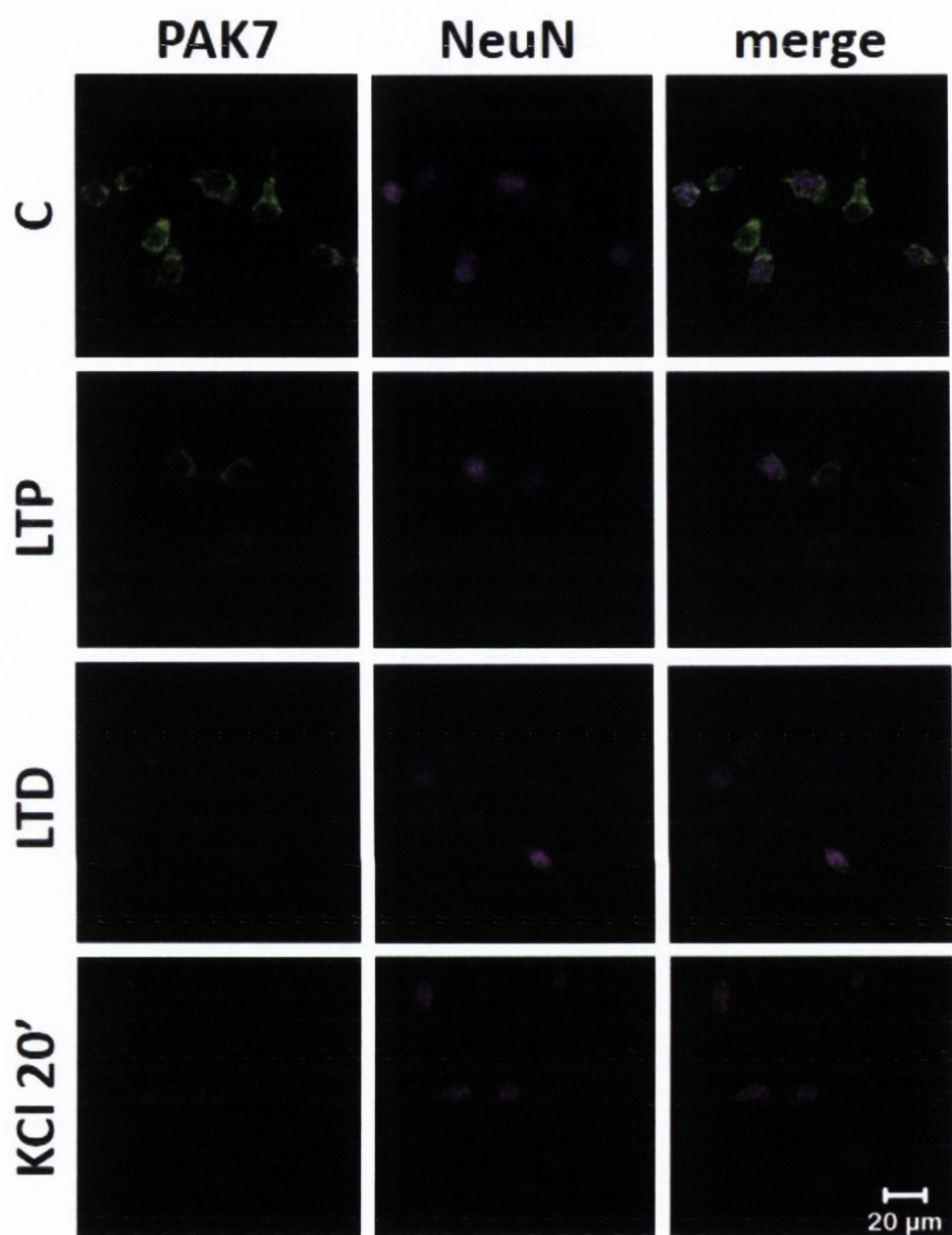


Figure 46. Immunofluorescence staining of primary neuronal cultures obtained from VC and treated with stimulation protocols that induce short-term forms of synaptic plasticity (LTP, LTD or KCl 20'). Cultures were stained for PAK7 (green) and NeuN (purple). PAK7 show both a cytoplasmic and a neuronal subcellular localization: PAK7 IS intensity is decreased in both cytoplasmic and nuclear compartments.

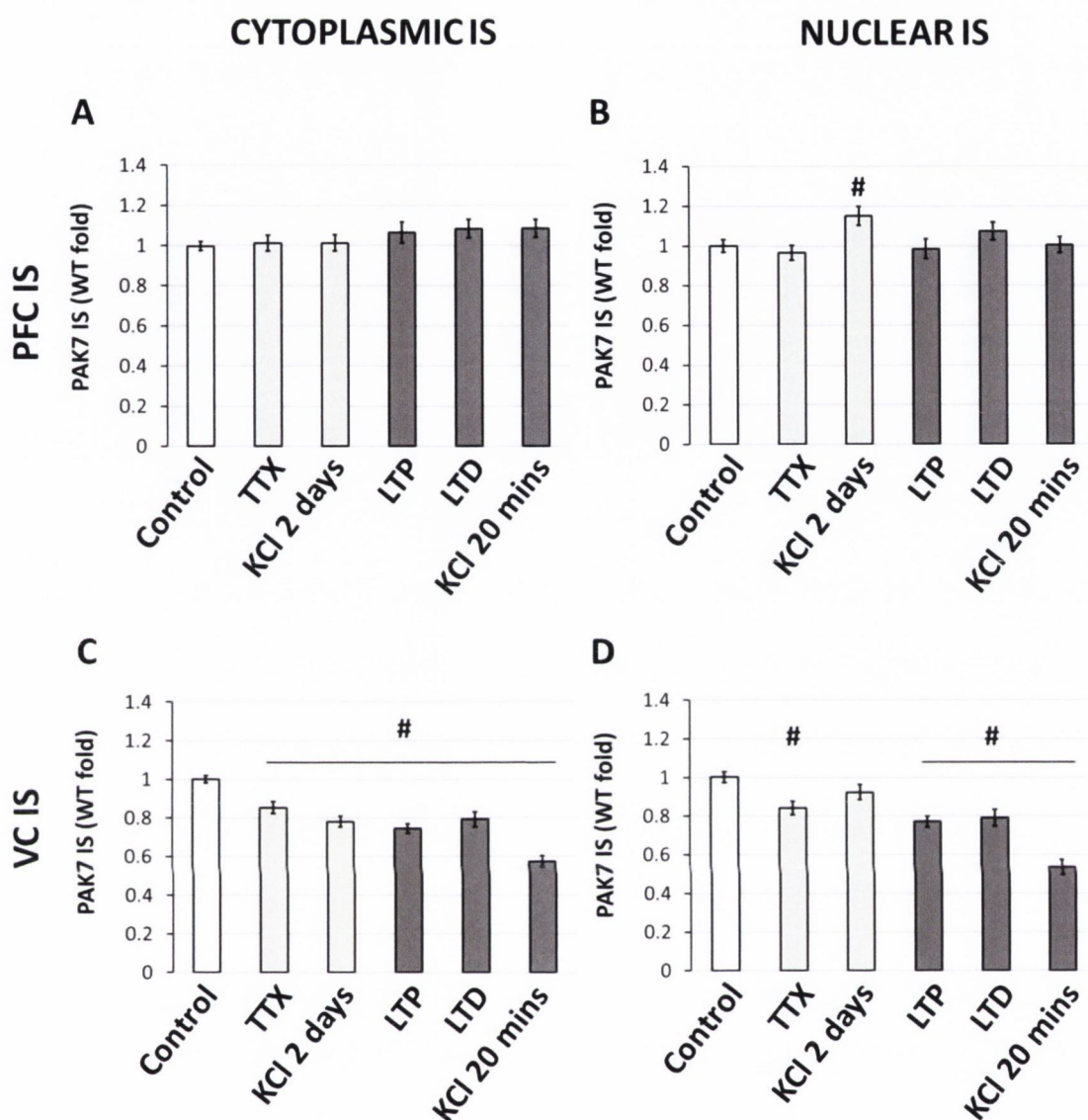
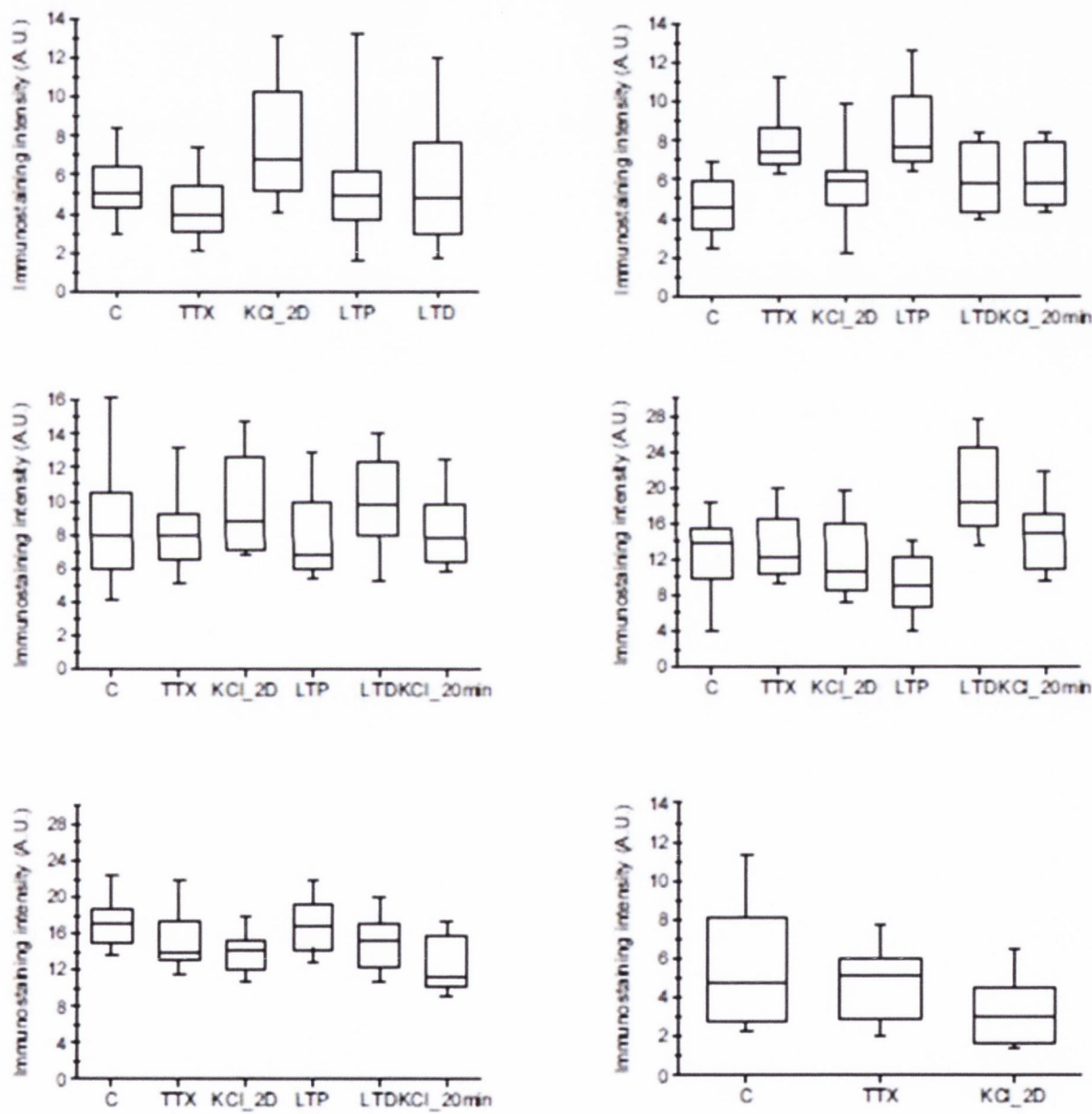


Figure 47. Levels of PAK7 are regulated by neuronal stimulation in primary neuronal cultures from WT mice. Semi-quantitative analysis of neuronal cytoplasmic and nuclear immunostaining (IS) for PAK7 in prefrontal cortex (PFC) and visual cortex (VC) primary cultures. A: PFC cytoplasmic IS; B: PFC nuclear IS; C: VC cytoplasmic IS; D: VC nuclear IS. Relative IS intensity is expressed as control fold $\pm$ standard error (SE). Differences between treatment groups were assessed by Kruskal-Wallis test followed by Mann-Whitney test.

For each immunofluorescence experiment in PFC, seven different animals were used to make cultures; while 9 different animals were tested for VC measurements. Some animals were used to make both PFC and VC experiments. In general, sufficient amount of good quality culture from one or the other cortical regions were obtained in order to be able to perform all

the different stimulation on cells belonging from the same animal. However, occasionally only some treatments, together with the control, were included in a single experiment. After immunofluorescence protocol, at least five fields of view were imaged for coverslip stimulated with individual treatments. Cytoplasmic and nuclear staining for PAK7 was measured from the same neurons in each experiment. Variability was present in the level of fluorescence resulting from each experiment, for example due to higher level of background staining present in culture made from one animal compared to another. In order to combine measured values from different treatment conditions, we measured for each animal the average value of IS (immunostaining) of control neurons, and we divided the IS value of each neuron measured within the same experiment for the average IS of control neurons. Since basal level of IS was variable among culture from different animals, this criterion of analysis allowed us to compare IS resulting from different treatments in individual experiments. Individual IS intensity values before normalization were plotted for each individual experiment in order to assess intra-animal and inter-animals biological variability. Give the large number of cells measured for each experiment, box plots were used to represent intra- and inter-experiments variability effectively. Box plots were made to represent first and third quartiles (top and bottom of box) and the second quartile (median) values of raw data; bottom and top whiskers represents 10th and 90th percentiles. Graphs show that variability changes between experiments for the same cortical area and among individually treated groups within the same experiments. Basic level of fluorescence is different experiments, therefore normalization was required in order to combine results from individual experiments. Additionally, variability within treated group within the same experiment appears high for some experiments and lower for others: in fact some experiments show similar degree of variability in each group, and others show high variability in some groups and low variability in others. However, the graphic representation of raw fluorescence measurements before normalization to the control are generally consistent with the results obtained after normalization. Plots relative to immunofluorescence experiments in PFC are shown in Figure 48 and 49; while plots relative to experiments in VC are reported by Figure 50 and 51. Averaged values of raw neuronal intensity data for each individual were plotted for each condition of stimulation in both PFC and VC cultures (Figure 52). The standard error values calculated from single neurons IS values were lower than the ones calculated from averages

values for each individual/treatment. Therefore, for data analysis normalized IS values of individual neurons were used as individual data points in both Kruskal-Wallis and Mann-Whitney tests. Table 12 reports details about number of cells analyzed, normalized IS intensity $\pm$ standard error and significance.



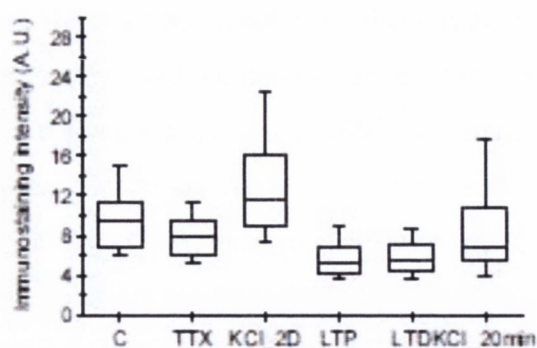
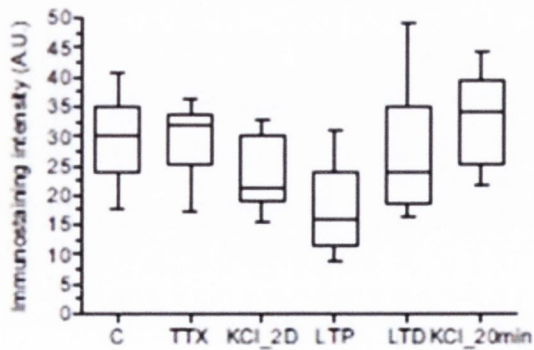
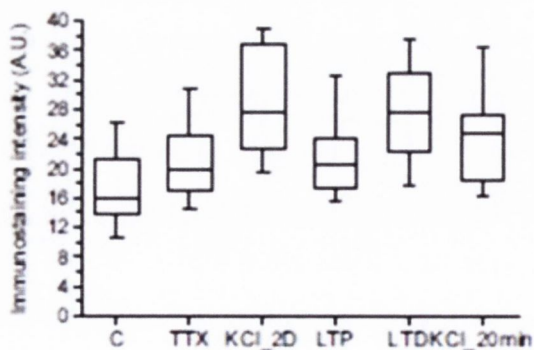
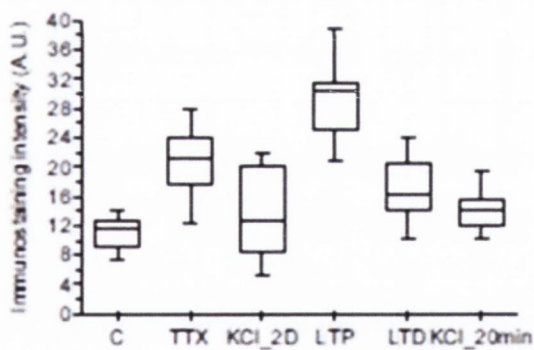
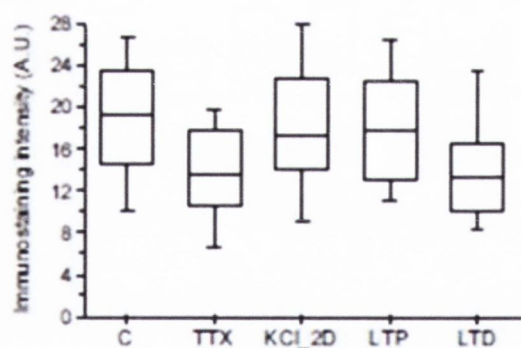


Figure 48. Box plots representing the distribution of PAK7 immunostaining intensity in the nuclear compartment of PFC neurons treated with TTX, 2 days KCl (KCl_2D), LTP, LTD, 20 minutes KCl (KCl_20min) and control neurons (C). Each graph represents an individual experiment, each experiment included cultures belonging from the same animal and treated with different stimulation protocols. Boxes and whiskers show the distribution of the single data points before normalization to the control. A.U. = arbitrary unit.



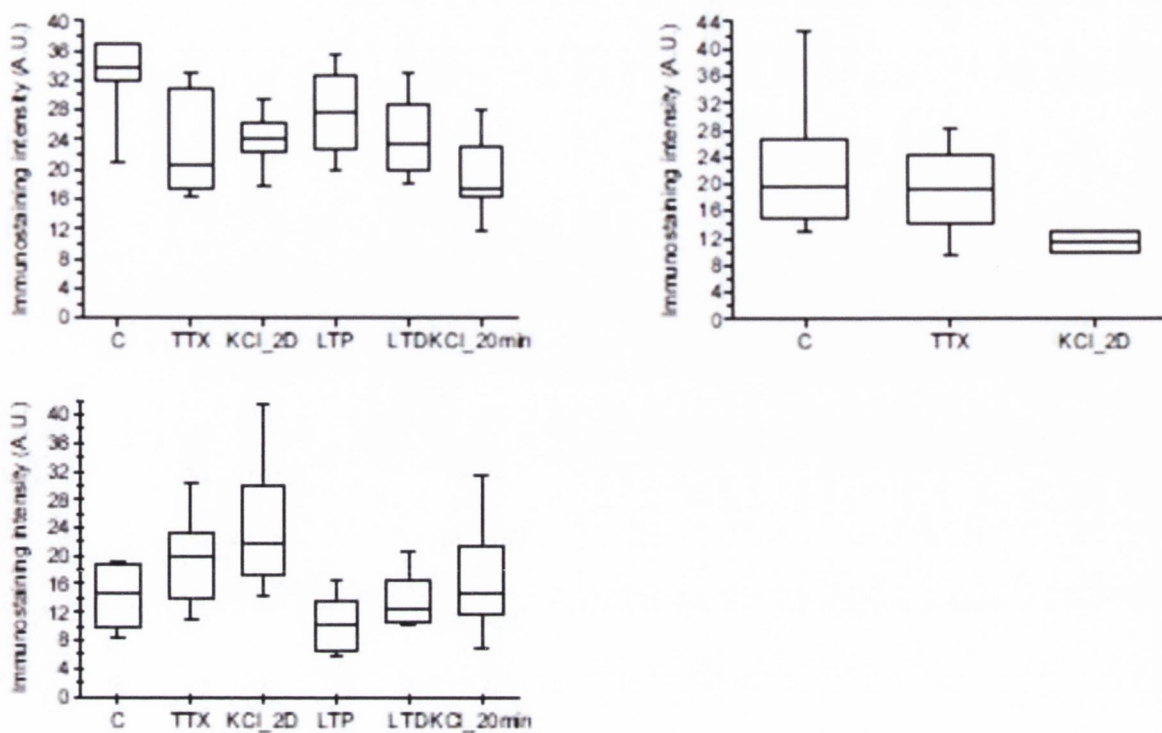
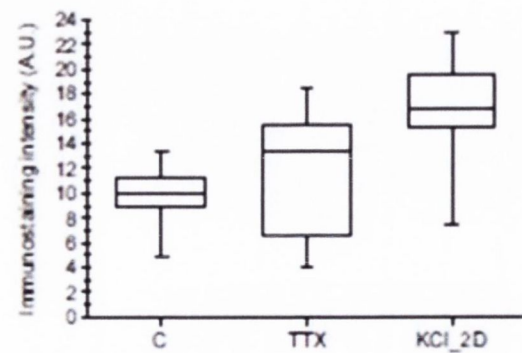
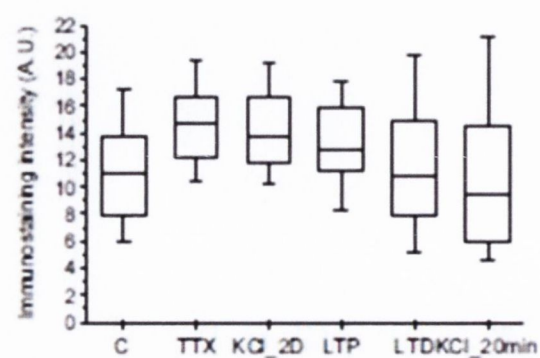
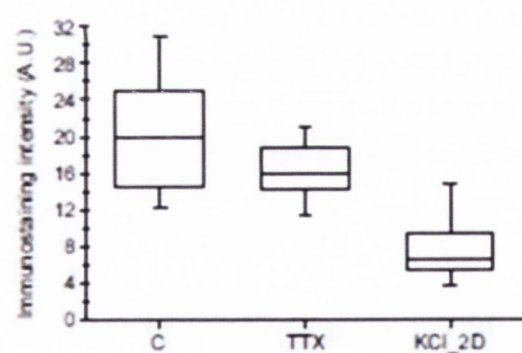
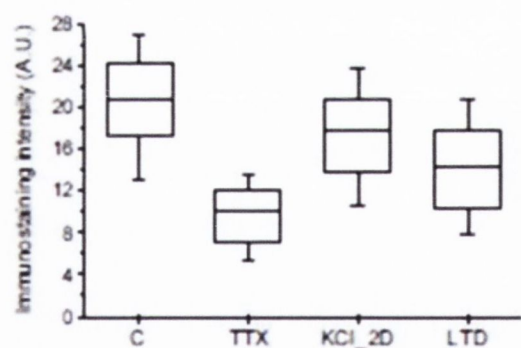
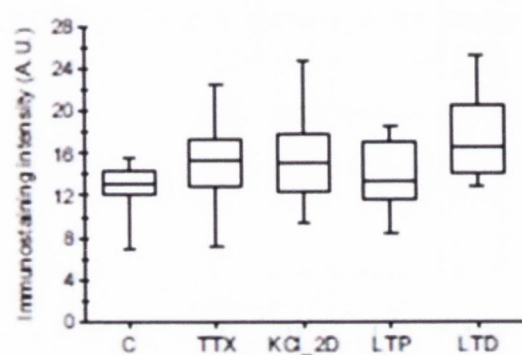
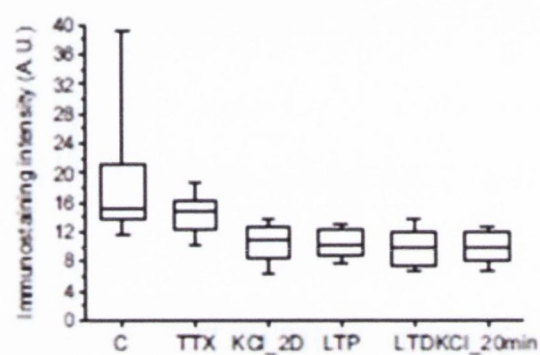
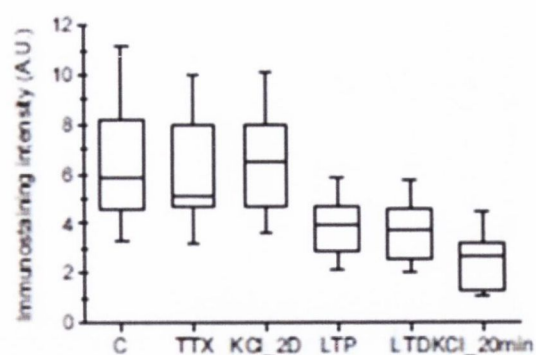
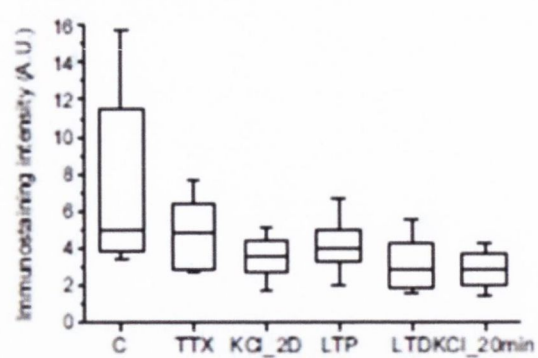


Figure 49. Box plots representing the distribution of PAK7 immunostaining intensity in the cytoplasmic compartment of PFC neurons treated with TTX, 2 days KCl (KCl_2D), LTP, LTD, 20 minutes KCl (KCl_20min) and control neurons (C). Each graph represents an individual experiment, each experiment included cultures belonging from the same animal and treated with different stimulation protocols. Boxes and whiskers show the distribution of the single data points before normalization to the control. A.U. = arbitrary unit.



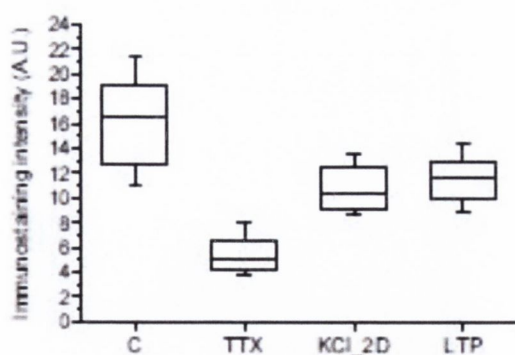
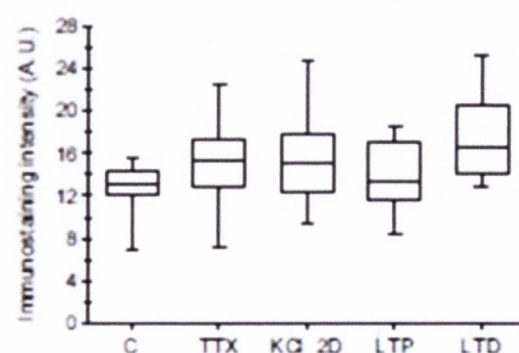
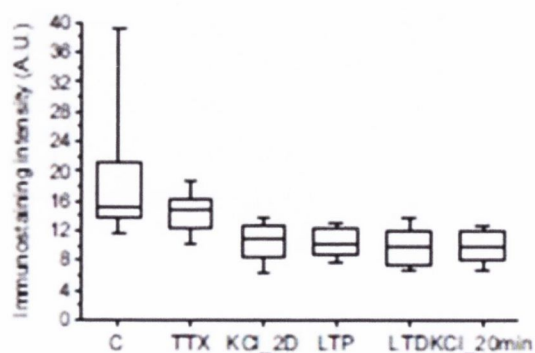
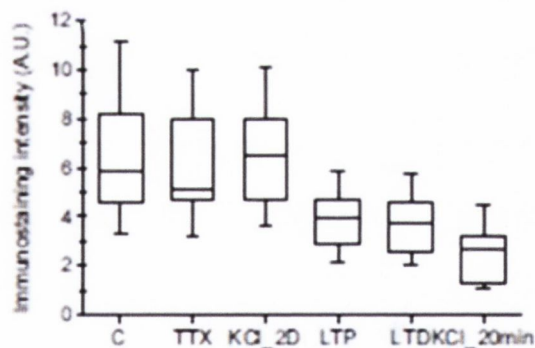
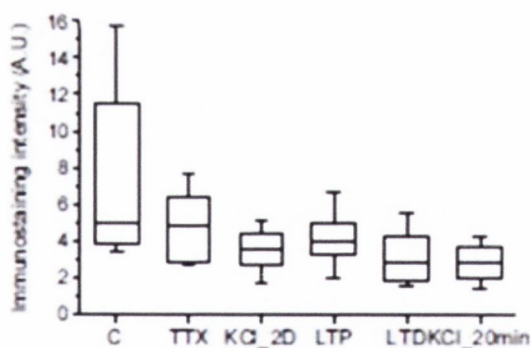


Figure 50. Box plots representing the distribution of PAK7 immunostaining intensity in the nuclear compartment of VC neurons treated with TTX, 2 days KCl (KCl_2D), LTP, LTD, 20 minutes KCl (KCl_20min) and control neurons (C). Each graph represents an individual experiment, each experiment included cultures belonging from the same animal and treated with different stimulation protocols. Boxes and whiskers show the distribution of the single data points before normalization to the control. A.U. = arbitrary unit.



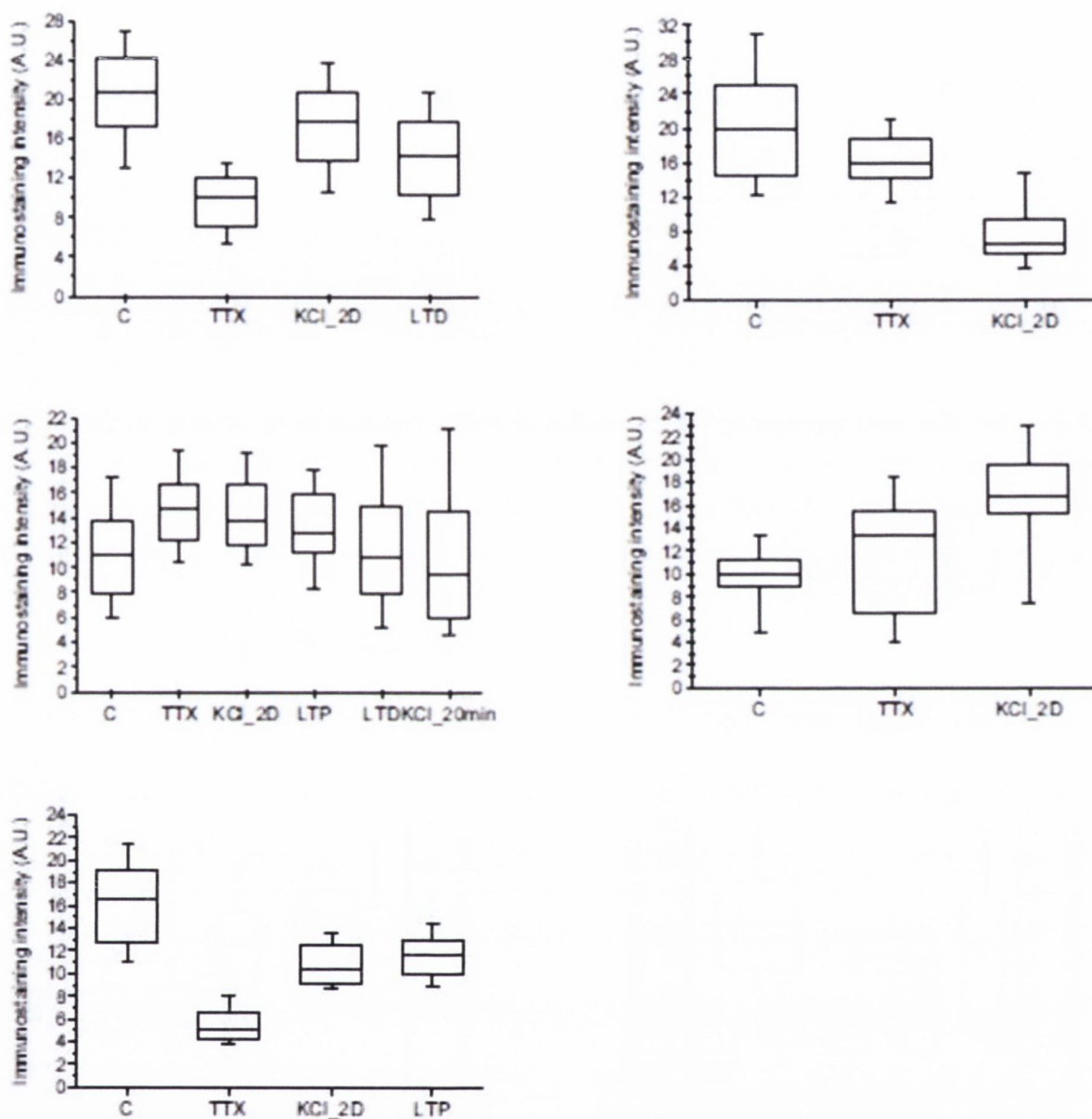


Figure 51. Box plots representing the distribution of PAK7 immunostaining intensity in the cytoplasmic compartment of VC neurons treated with TTX, 2 days KCl (KCl_2D), LTP, LTD, 20 minutes KCl (KCl_20min) and control neurons (C). Each graph represents an individual experiment, each experiment included cultures belonging from the same animal and treated with different stimulation protocols. Boxes and whiskers show the distribution of the single data points before normalization to the control. A.U. = arbitrary unit.

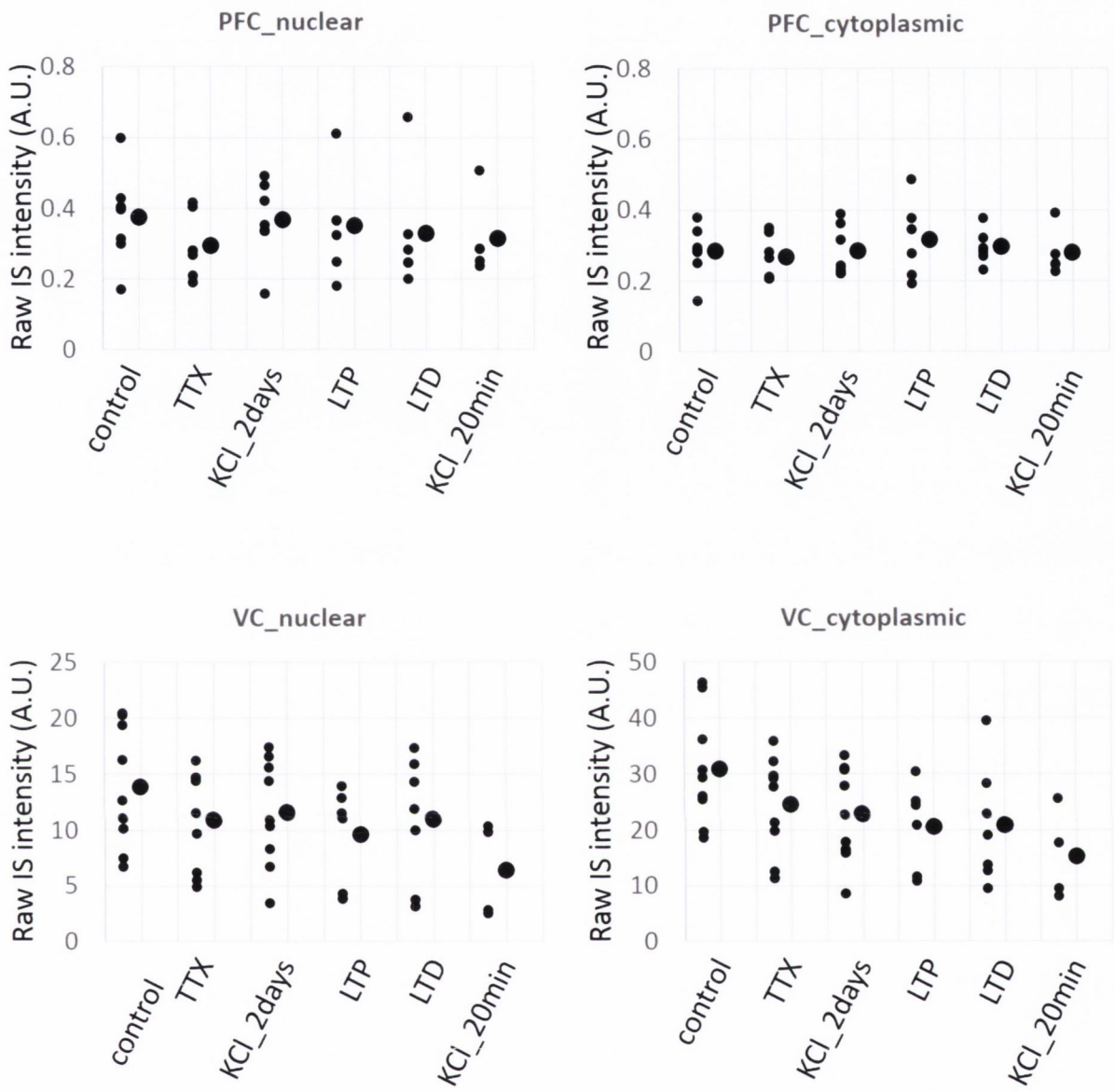


Figure 52. Distribution of averaged neuronal raw IS values for stimulation (TTX, KCl_2 days, LTP, LTD or KCl_20 minutes) and control treatments. Small dots represent averages of IS (immunostaining) for each individual and treatment before normalization to the control. Means of IS averages are also shown (big dots).

Treatment	Brain area	Subcellular compartment	Number of cells	IS intensity $\pm$ SE	<i>P</i> value
C	PFC	CYTOPLASMIC	151	1 $\pm$ 0.02	-
		NUCLEAR	151	1 $\pm$ 0.03	-
	VC	CYTOPLASMIC	205	1 $\pm$ 0.02	-
		NUCLEAR	205	1 $\pm$ 0.03	-
TTX	PFC	CYTOPLASMIC	128	1.01 $\pm$ 0.04	NS
		NUCLEAR	128	0.97 $\pm$ 0.04	NS
	VC	CYTOPLASMIC	174	0.85 $\pm$ 0.03	<10E-4
		NUCLEAR	174	0.84 $\pm$ 0.03	<10E-3
KCl 2 days	PFC	CYTOPLASMIC	129	1.01 $\pm$ 0.04	NS
		NUCLEAR	129	1.15 $\pm$ 0.05	0.01
	VC	CYTOPLASMIC	167	0.78 $\pm$ 0.03	<10E-9
		NUCLEAR	167	0.92 $\pm$ 0.04	NS
LTP	PFC	CYTOPLASMIC	114	1.06 $\pm$ 0.05	NS
		NUCLEAR	114	0.98 $\pm$ 0.05	NS
	VC	CYTOPLASMIC	142	0.75 $\pm$ 0.02	<10E-9
		NUCLEAR	142	0.77 $\pm$ 0.03	<10E-7
LTD	PFC	CYTOPLASMIC	120	1.08 $\pm$ 0.05	NS
		NUCLEAR	120	1.07 $\pm$ 0.05	NS
	VC	CYTOPLASMIC	114	0.79 $\pm$ 0.04	<10E-15
		NUCLEAR	114	0.79 $\pm$ 0.04	<10E-4
KCl 20 minutes	PFC	CYTOPLASMIC	95	1.09 $\pm$ 0.04	NS
		NUCLEAR	95	1.07 $\pm$ 0.04	NS
	VC	CYTOPLASMIC	87	0.57 $\pm$ 0.03	<10E-25
		NUCLEAR	87	0.53 $\pm$ 0.04	<10E-18

Table 12. Details about immunocytofluorescence for PAK7 in primary neuronal cultures. Semi-quantitative analysis of neuronal cytoplasmic and nuclear immunostaining (IS) for PAK7 in prefrontal cortex (PFC) and visual cortex (VC) primary cultures. Treatments, brain area, subcellular compartment, number of cells analyzed, relative immunostaining (IS) intensity $\pm$ standard error (SE) and significance (*P* value) are listed. Relative IS intensity is expressed in control fold $\pm$ standard error (SE). Differences between treatment groups were assessed by Kruskal-Wallis test followed by Mann-Whitney test.

4.4 Functional investigation of the effect of the human *PAK7* mutation in primary neuronal cultures

A rare tandem duplication overlapping the *PAK7* gene on chromosome 20 was recently proved to increase the risk of SCZ. The duplicated fragment is about 149kb big, and contains the first two exons of the gene, which span over regulatory sequences (Morris et al. 2014). The brain-specific expression of *PAK7* makes it challenging to investigate the neurobiological consequences of the duplication in humans: the consequences of the human duplication in terms of proteins expression are unknown. In order to get insight on the pathophysiology of such duplication in neurons, we used *Pak7* KO mice. These mice have been generated by inserting a stop codon in the exon 3 of *Pak7* (Li & Minden 2003). As a result, they express only exons 1 and 2 of the gene, which -similarly to the human sequence- have not coding potential. We confirmed the lack of PAK7 protein expression using two different methods of immunodetection: western blot and immunocytochemistry (Figure 52 and Figure 53 respectively). We propose a model that replicates the *Pak7* duplication *in vitro*: our system might be used as a powerful tool to clarify the molecular events that occur as a consequence of the duplication. Primary neuronal cultures are prepared from *Pak7* KO pups and transfected using a plasmid containing the full length *Pak7* cDNA fused with GFP (green fluorescent protein). The rate of transfection is about 10%; therefore transfected cells that express the *Pak7* plasmid can be recognized and selected through detection of green fluorescent given by the expression of GFP. We used MAP2 immunostaining (IS) to compare development of dendrites in transfected and untransfected neurons in the same neuronal culture. Microtubule associated protein 2 (MAP2) is a neuronal-restricted protein associated with neurofilaments that marks neuronal cell bodies and dendrites. Figure 54 shows how neurons transfected with the *Pak7*/GFP plasmid can be visualized using a confocal microscope taking advantage of the green fluorescence provided by the expression of GFP (Figure 54-B); PAK7 immunostaining (Figure 54-C) confirms the expression of PAK7 as a consequence of the transfection, while MAP2 co-staining of the same cultures allows to detect neuronal bodies and dendrites (Figure 54-D). Preliminary results show that this system allows to measure the length of neuronal dendrites of *Pak7* KO neurons, and to compare it with the one belonging from neurons transfected with the *Pak7*/GFP plasmid and untransfected cells. Additionally, the analysis of MAP2 immunostaining density might be useful to evaluate

differences in dendritic thickness among neurons of different genotypes (Figure 52-53). However, additional experimental replicates are required to provide statistically meaningful results.

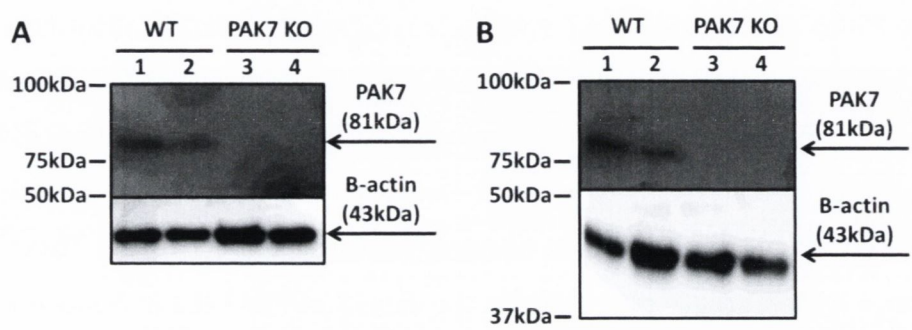


Figure 53. Western blot analyses of PAK7 expression in wild type (WT) and *Pak7* KO. Total brain extracts from adult WT (lanes 1 and 2) and *Pak7* KO (lanes 3 and 4) animals were run in double and membranes were probed with two different antibodies against PAK7. Membranes were also probed against β -actin to ensure equal total protein load for each lane. A: membrane probed with polyclonal antibody anti-PAK7 raised in goat (sc-22155 used at 1:500); no PAK7 is found in the brain of *Pak7* KO mice. B: membrane probed with polyclonal antibody anti-PAK7 raised in rabbit (SAB-3500335 used at 1:1000); no PAK7 is found in the brain of *Pak7* KO mice

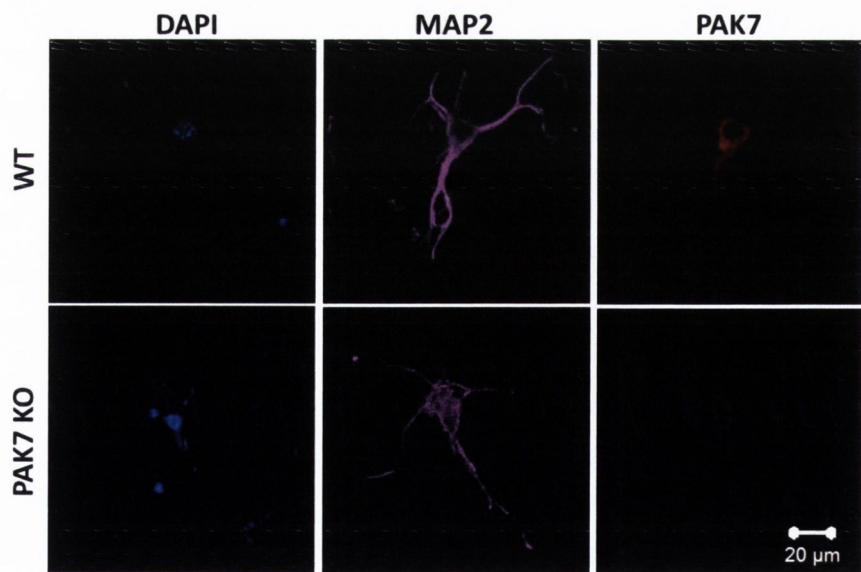


Figure 54. Immunocytochemistry staining of PAK7 expression in wild type (WT) and *Pak7* KO. Primary neuronal cultures from WT and *PAK7* KO pups underwent immunostaining (IS) for DAPI (nuclear marker), MAP2 (neuronal marker) and PAK7 (SAB-3500335 used at 1:5000). No PAK7 specific IS immunostaining found in neurons from *Pak7* KO mice.

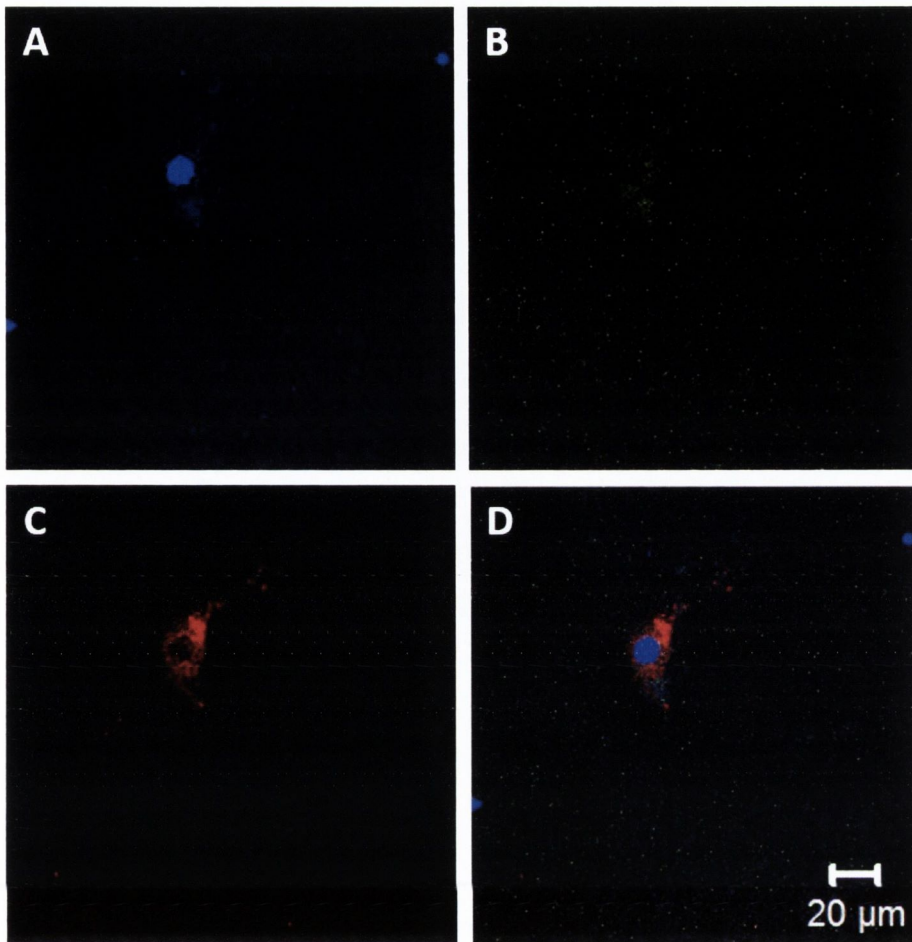


Figure 55. Immunocytochemistry for PAK7 on primary neuronal cultures from *Pak7* KO pups transfected with *Pak7*/GFP plasmid. A: DAPI immunostaining labels cellular nuclei (blue); B: GFP fluorescence labels transfected cells expressing *Pak7*/GFP plasmid (green); C: PAK7 immunostaining (red) in combination with GFP confirms expression of the *Pak7*/GFP plasmid; D: merge.

5. Discussion

5.1 Developmental pattern of expression of synaptic and neuronal markers in *Disc1-L100P* mutant mice

The functional investigation of neurodevelopmental effects of high penetrant mutations discovered in humans might strongly contribute to elucidate the etiology of the SCZ, providing a molecular profile of the disorder. However, the majority of studies have been centered on adult rodent models. For the first time here, we investigate the neurodevelopmental pattern of expression of synaptic proteins and cellular mechanisms involved in synaptic transmission, neuronal function and the activity-dependent reorganization of synaptic connections in *Disc1-L100P* mutants at critical ages. Figure 56 provides a schematic representation of the synaptic and neuronal proteins studied in the experiments described in this thesis project.

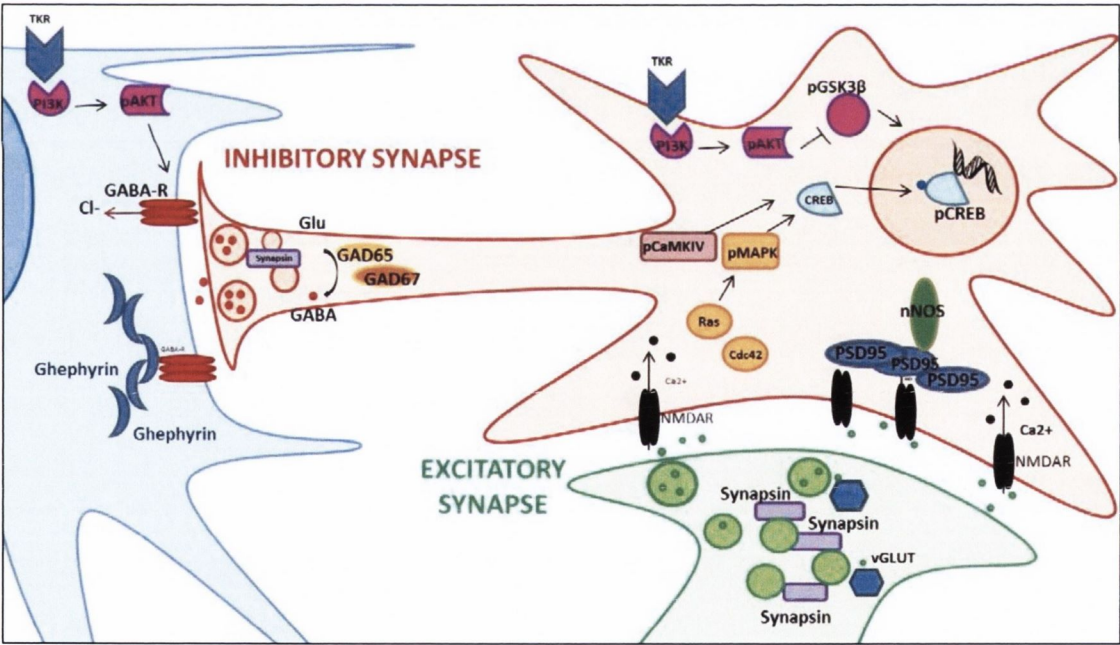


Figure 56. Schematic representation of synaptic and neuronal proteins and pathways explored.

5.1.1 Alterations of synaptic architecture in *Disc1*-L100P mutants during postnatal development

Using semi-quantitative analyses we demonstrate that DISC1-dependent alterations of synaptic architecture determine disruption of the excitatory/inhibitory balance; we provide evidence that this process starts early during postnatal development when a pathological phenotype is usually undetectable. The findings at P8-10 suggest the incapacity of the system to establish new mature synapses in spite of the increasing attempts, and deficits in providing proper level of brain inhibition in response to sustained excitation. At the age of pruning (P30-40) we show a decreased number of excitatory synapses, while in the brain of adult mutants we show significantly decreased levels of components of inhibitory synapses. Proper GABAergic transmission is essential to regulate neural synchrony necessary for normal cognitive function, which is impaired in SCZ (Gonzalez-Burgos et al. 2011). These results are consistent with previous studies on L100P and other *Disc1* rodent models (Hikida et al. 2007; Lee et al. 2013) and post-mortem studies in SCZ patients (Akbarian et al. 1995; Volk et al. 2000); but they go further, and they provide a new molecular model active during development.

5.1.2 The cellular mechanisms affected in brain are different at each age studied

Having identified impairment of synaptic architecture in mutants, we tested the mutant brains for active levels of CREB that are supposed to be changed in our model (Millar et al. 2005; Clapcote et al. 2007). The finding that levels of pCREB are globally unchanged in mutants drove us to investigate on the cellular mechanisms affected by synaptic impairment and putative pathways that may compensate for the activation of CREB in mutants. We show that the cellular mechanisms are developmentally regulated, with distinct kinases dysregulated at different postnatal stages: decreased levels of pCaMKIV at P8-10, decreased levels of pAKT and pGSK3 β at P>60. Reduced levels of pCaMKIV, together with decreased levels of GAD65, might contribute to establish the aberrant synaptic architecture observed at P8-10. In neurons the pCaMKIV- dependent phosphorylation of CREB is required for long term potentiation (LTP) maintenance, as well as formation of glutamatergic synapses and spines between P7 and P28 (Miyamoto 2006; Zhou et al. 2008). Thus, prolonged decrease of pCaMKIV levels in mutant brains at critical ages may produce the compensatory reduction

of spines observed at the age of pruning in our model. In P>60 mutant brains the major cellular changes are related to pAKT and pGSK3 β . It must be said that pGSK3 β can directly activate the transcriptional factor CREB; therefore, the effect of the robust decrease of pAKT on CREB activation is controversial. Levels of both pAKT and pGSK3 β are decreased in post-mortem brain samples of SCZ patients (Zheng et al. 2012); accordingly, some antipsychotic drugs act by increasing AKT phosphorylation. pAKT - together with pGSK3 β - regulates NMDA receptor-dependent synaptic plasticity by promoting the trafficking of PSD95 to spines (Yoshii & Constantine-Paton 2007). We do not find decreased levels of PSD95 in total brain extracts at P>60: this result does not contradict previous studies on post-mortem brains of SCZ patient that show reduced number of spines occurring selectively in specific brain areas (Kjaerby et al. 2013). The whole brain preparation used for WB does not allow to discriminate changes in specific brain areas. While decreased levels of pAKT might contribute to determine a decreased number of spines in specific brain areas, they might also play a role in determining the general deficit in inhibitory transmission observed in adult mutant brains. In fact, pAKT phosphorylates type A GABA receptors increasing the trafficking of those at the post- synaptic membrane (Wang et al. 2003). Since AKT is not exclusively expressed by neurons within the brain, additional experiments will be required in the future to clarify the association between reduced pAKT levels and SCZ.

5.1.3 The cellular mechanisms affected in cerebellum are different at each age compared with brain

The pattern of alterations changes in CRB: this indicates that not only different kinases are dysregulated at different developmental stages, but also the changes are brain area-specific. Indeed, pERK1/2 levels are increased at both P30-40 and P>60, while pAKT and pGSK3 β are decreased at P30-40 in mutant extracts (Table 13). Increased levels of cerebellar pERK1/2 have been found in post-mortem SCZ brains (Kyosseva et al. 1999), as well as another rodent model of SCZ (Kyosseva et al. 2001). The measurement of synaptic proteins in CRB was not carried out due to time limitation.

5.1.4 Molecular alterations are brain area-specific

Results from CRB measurement revealed that additional differences between wild types and mutants exist and are restricted to selected brain areas. Therefore, the next step on this research is a comprehensive developmental analysis of the expression of the markers in histological sections from prefrontal cortex (PFC), hippocampus (HP) and CRB; which are all involved in higher brain functions that are affected by SCZ. Comprehensive investigation of the expression of synaptic and neuronal markers in individual brain areas will allow clarifying the spatial distribution of alterations during postnatal development. In this study we started to investigate whether brain area-specific changes occur in the brain of P>60 mutants. Preliminary results show the following changes in mutant brains: decreased levels of synapsin in the HP, but not PFC and CRB, and reduced levels of pCREB in PFC and CRB (Table 14). However the results collected to date are partial; only two animals per genotype have been compared for the antigens studied, which are not sufficient to identify significant trends of expression. In future studies we will complete the histological analysis of the brain areas in adult brains and at earlier postnatal stages in order to achieve a better comprehension of the developmental profile.

Δ DISC1 vs WT		P8-10 WB	P30-40 WB	P>60 WB
Synapsin		↑	↓	↓
PSD95		=	↓	=
Gephyrin		=	=	↓
GAD67		=	=	↓
GAD65		↓	=	↓
pCREB		=	=	=
totCREB		=	=	=
pAKT	BRAIN	=	=	↓
	CRB	=	↓	=
pGSK3 β	BRAIN	na	=	↓
	CRB	na	↓	=
pCaMKIV		↓	=	=
pERK1/2	BRAIN	=	=	=
	CRB	=	↑	↑
PAK7	synaptic	↓	=	=
	cytoplasmic	=	↓	=
nNOS		=	=	=

Table 13. Summary table of the results obtained from WB experiments. The table show variation of the expression of each measured marker in *Disc1* mutants (Δ DISC1) compared to WT. Increase (↑), decrease (↓) or not statistically different expression (=) are reported for each postnatal stage studied (P8-10, P30-40, P>60). na = not applicable; experiments were not performed.

ΔDISC1 vs WT	BRAIN AREA	P>60
Synapsin	PFC	=
	HP (CA1)	↓
	HP (CA3)	↓
	CRB	=
PSD95	PFC	=
	HP (CA1)	=
	CRB	=
GAD65	PFC	=
	HP (CA1)	=
	CRB	=
pCREB	PFC	↓
	HP (CA3)	=
	CRB	↓
total CREB	PFC	=
	HP (CA1)	=
	CRB	na
PDE4b	PFC	=
	HP (CA1)	=
	CRB	=
cAMP	PFC	=
	HP (CA1)	=
	CRB	=

Table 14. Summary table of the results obtained from immunostaining of tissue sections of PFC, HP CA1 and/or CA3 and CRB. The table show variation of the expression of each measured marker in *Disc1* mutants (ΔDISC1) compared to WT. Decrease (↓) or similar expression (=) are reported for P>60 animals. na = not applicable; statistical analysis was not performed.

5.1.5 Summary and future directions

The present study provides the first evidence that synaptic alterations occur early during postnatal development in the brain of *Disc1*-L100P mutant mice, and that a different trend of expression of synaptic proteins exists at ages crucial for brain development (synaptogenesis and pruning) as well as in adulthood. We show that such changes involve distinct cellular mechanisms at each age - implicating dysregulation of a variety of cellular pathways - and different cellular mechanisms are disrupted in brain and cerebellum at each age studied. Prefrontal cortex (PFC), hippocampus (HP) and cerebellum (CRB) have been previously related to SCZ. In order to test our hypothesis that developmental alterations are brain area-specific, we started to study the expression of selected markers for neuronal and synaptic functions in PFC, HP and CRB of adult *Disc1* mutants. Preliminary results collected by testing adult brains seem to confirm our hypothesis; however, additional experiments are required to drive robust conclusions on the spatial specificity of alterations in these model. Future studies in these direction are guaranteed. Once completed, these studies will provide a comprehensive view of the spatial-temporal profile of alterations in this mouse model.

It must be noted that the WB experiments were conducted using protein extracts of brains tissue, from which CRB was previously separated. Measurement from WB and histological experiments on CRB tissue can be combined to obtain a global view of the changes occurring in this area in mutant brains (Tables 13 and 14). Using western blot, we identified robust decrease of molecular components of inhibitory synapses in total brain extracts from P>60 mutant brains (synapsin, gephyrin, GADs); no change of pCREB expression was found by screening of total brain extracts, but preliminary measurements from brain sections from P>60 brains, suggest that a decrease of pCREB might occur selectively in PFC and CRB of mutants. Although histological results are preliminary, we can make speculation to reconcile them to the molecular trends observed in WB, if they were to be confirmed by additional experiments. Measured levels of GAD65 and pCREB in isolated brain areas might appear inconsistent compared to the ones from WB analyses; we cannot exclude the presence of differences in the markers' expression in all the other brain areas that we did not consider in our study. Expression of synapsin appears reduced in HP of mutant mice; although this result would follow the trend of the WB results, a similar decrease of synapsin was not found in PFC and CRB in the preliminary experiments. If this result was confirmed, the decrease of

synapsin observed in whole brain extracts of mutant mice must be determined by alterations in other brain areas that we did not test. Taken together, WB results from brain and CRB, and preliminary results from histological experiments, point out that additional brain areas - together with the ones traditionally associated to SCZ - might be involved in determining the behavioral changes observed in *Disc1*-L100P mutant mice. This idea is supported by previous literature that reported molecular aberrations in a different brain area of *Disc1*-L100P mutants (Lipina et al. 2010). Overall our WB results in from P>60 brains are consistent with data from other rodent models of SCZ and with some of the most reproduced molecular features observed in post- mortem brains of SCZ patients, validating the *Disc1*-L100P mutant mouse as a rodent model of SCZ. Measurements on separated CRB tissue confirm the involvement of multiple cellular mechanisms in specific brain areas to pathophysiological mechanisms underlying the phenotype of the L100P mutant.

This work provides new contribution in the field of study: we show that alterations in brain of adult *Disc1* mutant animals result from synaptic and neuronal dysregulation starting early during postnatal development, when aberrant behavioral phenotypes are not yet observed. The finding that molecular alterations take place according to a determined temporal pattern in the brain has important implications for the future of molecular psychiatry, pointing out the necessity to investigate disorders like SCZ from a developmental perspective in order to understand underlying pathophysiological mechanisms and design effective diagnostic strategies and treatment. Additionally, the completion of the histological screening during postnatal development will provide clear comprehension of the brain areas affected in the L100P model at each given age; this might provide clues for new clinical approaches.

Additional experiments will allow achieve full characterization of the mutant model used for this study: first, mutant brains might be screened for additional markers of interest such as calcium channels subunits, cellular phosphatases that - together with kinases- contribute to determine levels of activation of transcriptional factors like CREB, small GTPases and microglial markers; second, functional assessment using optogenetics approaches will permit to test *Disc1* mutants for impairments in specific brain circuits and selected cell types *in vivo*. Previous literature show that the course of illness is different in males and females SCZ patients (McGrath et al. 2008; Saha et al. 2005). In our experiments the sex of animals used was not monitored, therefore sex-specific experiments to be conducted in the future might

gather different results from the ones presented here. Previous studies reported brain alterations in *Disc1*-L100P mutants including defective adult neurogenesis, neuronal maturation and migration (Chandran et al. 2014); some of them investigated mechanisms at earlier stages of life, finding disrupted dynamics in the development of interneuronal circuitry (Lee et al. 2013), and altered expression of selected synaptic markers (Brown et al. 2011) during embryonic and early postnatal stages. None of the studies investigating cellular and molecular effects of the L100P mutation on neuronal and brain development describe sex-monitored use of the mutants in their experiments (Lee et al. 2011; Lee et al. 2013; Brown et al. 2011; Chandran et al. 2014). Some studies available in literature exclusively tested male L100P mutants, finding impaired locomotor activity (Shoji et al. 2012) and increased striatal dopamine activity (Lipina et al. 2010) compared to wild type males. Other two studies employed sex-balanced groups of mice: although behavioral differences were reported as an effect of the L100P mutation, no sex-specific effects have been described (Clapcote et al. 2007; Lipina et al. 2012). One study performed a comprehensive investigation of the exploration behavior of *Disc1*-L100P mutants: although behavioral impairment was found in both sexes, some of the identified abnormalities were significantly more pronounced in mutant males (Walsh et al. 2012). These findings suggest the existence of sex-specific molecular alterations in the brain of *Disc1*-L100P mutants. In our study sex of animals used for experiments was not monitored, therefore we cannot make any statement related to putative sex-specific effects of the L100P mutation on the expression of the markers that we measured: such effects might be covered in some of the tested groups of mice; conversely, we cannot establish whether or not the age-specific alterations that we observe may be confounded by sex-differences between groups. Future studies employing sex-monitored groups of mice are needed to clarify whether molecular alterations in the brain of the *Disc1*-L100P mutants are sex-specific.

5.2 Functional investigation of PAK7

5.2.1 Functional interactions among risk factors for psychosis

Different risk genes for SCZ are associated with different aspects of brain development, and may modulate a number of symptoms. Most of these genes are not exclusively related to SCZ having also been associated with other neurodevelopmental disorders. Developmentally

regulated gene interactions are likely to underlie the etiology of specific disorders. We assessed the interaction of DISC1 with nNOS and PAK7 during postnatal development. Both nNOS and other members of the PAK7 family are involved in activity-dependent spine dynamics mechanisms regulated by DISC1 (Hayashi-takagi et al. n.d.; Nikonenko et al. 2008). The role of PAK7 is unknown, and it might be involved in brain processes relevant to the disease, likely through the interaction with other susceptibility factors. Our findings confirm the hypothesis of developmental interaction between risk factors: PAK7 expression is altered in *Disc1* mutant brains according to a spatial-temporal regulated pattern, suggesting that DISC1 might regulate the activation of PAK7 and -similarly to what happens for other PAK isoforms- this process might contribute regulate synaptic mechanisms required for the refinement of neuronal circuitry during postnatal development. We confirm this hypothesis by histology and co-IP experiments: in fact, DISC1 and PAK7 colocalize at hippocampus and cortex, and they interact at the synaptic level at P30-40 and P>60. Assessment of the DISC1/PAK7 interaction at P8-10, as well as co- IP experiments in *Disc1*-L100P mutant brain extracts, would also be informative.

5.2.2 PAK7 levels are modulated by neuronal activity

In order to investigate its role in the activity-dependent reorganization of synaptic transmission, we measured by immunocytofluorescence the levels of PAK7 in response to neuronal stimulation. For the first time here, we apply chemical and pharmacological stimulations able to induce different types of synaptic plasticity in primary neurons in culture. In cultures from PFC (prefrontal cortex) PAK7 levels increase only as a result of prolonged stimulation with KCl, a treatment that eventually produce feedback neuronal depression. Strikingly, the increase of PAK7 takes place at the level of the nucleus. Previous studies described different neuronal functions of the protein, associated to specific subcellular localizations (Dan et al. 2002a; Wu & Frost 2006; Cotteret & Chernoff 2006; Strohlic et al. 2012). With these results, we suggest that PAK7 might be not only implicated in synaptic remodeling mechanisms (through its interaction with DISC1), but it might also act in the nucleus of PFC neurons, mediating activity-dependent long term effects, which require regulation of genetic transcription. These effects might be indirect or direct, through PAK7-mediated activation of nuclear targets.

5.2.3 Summary and future directions

Our study contributes to the functional investigation of PAK7 leads to three main important findings. First, the levels of expression of PAK7 is reduced in DISC1 mutants; second, PAK7 interacts with DISC1 at synapses in wild type brain; third, in PFC the levels of expression of PAK7 change in response to term pharmacological treatments that induce homeostatic plasticity. Future studies might be performed in order to study the discovered interaction from physiological and functional points of view, and to address further questions as follows: First- The relation between DISC1 and PAK7 might be direct or indirect as part of a bigger protein complex, thus it would be interesting to clarify this point for example by designing a FRET (fluorescence resonance energy transfer) assay, which provides a better spatial resolution. Second- The techniques used so far do not allow us to obtain a precise description of the relationship between neuronal activity and the PAK7/DISC1 interaction. It would be interesting to clarify how different patterns of neuronal stimulation change the interactions between DISC1 and PAK7, and how stable in time are these interactions. In order to clarify these mechanisms, we would combine a super-resolution approach with voltage-sensitive dye imaging; this would allow monitoring the relationship between neuronal firing pattern and DISC1/PAK7 interaction. The voltage-sensitive dyes can undergo rapid conformational change in response to membrane depolarization; their use can allow resolving individual action potentials at the synapse. Super- resolution approaches like PALM (photoactivated localization microscopy) and STORM (stochastic optical reconstruction microscopy) can be used to label and track the movement of individual DISC1 and PAK7 molecules. By combining these new technologies we could also determine whether the protein-protein interaction differ within and across dendritic processes of a neuron. This type of investigation would shed light on the synaptic plasticity dynamics within neuronal cells. Third- Additional proteins are likely to modulate the DISC1/PAK7 interaction. Since this project started, further risk mutations have been confirmed that might provide target for functional analyses. A number of SCZ risk genes cluster in groups with neurobiological meaning such as the synaptic genes group, which include components of the postsynaptic density. Future studies are guaranteed that will test the neurobiology of other risk factors, particularly by investigating their role in protein networks and pathways regulated by DISC1 and PAK7.

This would allow revealing the involvement of broader molecular mechanisms underlying the pathophysiology of SCZ.

5.3 Functional investigation of the human *PAK7* duplication

5.3.1 Investigation of the effect of the duplication in murine primary neuronal cultures

We previously identified a tandem duplication of the first two exons of the *PAK7* gene as a risk factor for psychosis (Morris et al. 2014). The consequences of the duplication are unknown; the brain-restricted expression of *PAK7* makes it difficult to investigate the neurobiological effect of such mutation in humans. We set up a system that can be used as a powerful method to investigate the molecular events that occur as a consequence of the human *PAK7* duplication in neurons. We show that it is possible to transfect the full sequence of the *Pak7* gene, in primary cultures made from *Pak7* KO mice, which express exon 1 and 2 of the gene. This strategy reproduces the human *PAK7* mutation *in vitro*, and could contribute to reveal the associated synaptic and neuronal dynamics. However, this system does not guarantee absolute reproducibility of the human mutation in murine primary neuronal cultures, due to inability to control the position of the insert.

5.3.2 Future directions

Human induced pluripotent stem cells (iPSC) obtained from patients might represent one of the next tools to investigate the effect of the *PAK7* duplication *in vitro*. A programmatic approach would be particularly beneficial: indeed neurons obtained from duplication carriers can be phenotypically characterized for structural and electrical features and compared with neurons derived from non-mutant individuals. This would best allow not only elucidating cellular and molecular defects that take place as a consequence of the duplication, but also establishing which cellular types are affected in humans.

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