

UNIVERSITY OF TRENTO

DOCTORAL THESIS

**Brain functional connectivity
and its aberrations in mouse models
of autism**

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*A thesis submitted in fulfillment of the requirements
for the degree of Doctor of Philosophy*

in the

Doctoral School in Cognitive and Brain Sciences, University of Trento
Center for Neuroscience and Cognitive Systems, Istituto Italiano di
Tecnologia

November 24, 2017

University of Trento

Abstract

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by Adam LISKA

Functional Magnetic Resonance Imaging (fMRI) has consistently highlighted aberrant functional connectivity across brain regions of autism spectrum disorder (ASD) patients. However, the manifestation and neural substrates of these alterations are highly heterogeneous and often conflicting. Moreover, their neurobiological underpinnings and etiopathological significance remain largely unknown. A deeper understanding of the complex pathophysiological cascade leading to impaired connectivity in ASD can greatly benefit from the use of model organisms where individual pathophysiological or phenotypic components of ASD can be recreated and investigated via approaches that are either off limits or confounded by clinical heterogeneity.

In this work, we first describe the intrinsic organization of the mouse brain at the macroscale as seen through resting-state fMRI (rsfMRI). The analysis of a large rsfMRI dataset revealed the presence of six distinct functional modules related to known brainwide functional partitions, including a homologue of the human default-mode network (DMN). Consistent with human studies, interconnected functional hubs were identified in several sub-regions of the DMN, in the thalamus, and in small foci within integrative cortical structures such as the insular and temporal association cortices.

We then study the effects of mutations in *contactin associated protein-like 2* (*Cntnap2*), a neurexin-related cell-adhesion protein, on functional connectivity. Homozygous mutations in this gene are strongly linked to autism and epilepsy in humans, and using rsfMRI, we showed that homozygous mice lacking *Cntnap2* exhibit aberrant functional connectivity in prefrontal and midline functional hubs, an effect that was associated with reduced social investigation, a core “autism trait” in mice. Notably, viral tracing revealed reduced frequency of prefrontal-projecting neural clusters in the cingulate cortex of *Cntnap2*^{-/-} mutants, suggesting a possible contribution of defective mesoscale axonal wiring to the observed functional impairments. Macroscale cortico-cortical white-matter organization appeared to be otherwise preserved in these animals. These findings revealed a key contribution of ASD-associated gene CNTNAP2 in modulating macroscale functional connectivity, and suggest that homozygous loss-of-function mutations in this gene may predispose to neurodevelopmental disorders and autism through a selective dysregulation of connectivity in integrative prefrontal areas.

Finally, we discuss the role mouse models could play in generating and testing mechanistic hypotheses about the elusive origin and significance of connective aberrations observed in autism and recent progress towards this goal.

Acknowledgements

The work presented in this thesis would not have been possible without the large contribution of many people around me. First of all, I would like to thank my advisor, Alessandro Gozzi, for all the guidance during the past few years. Working with him has been formative from personal, professional and scientific perspectives and it has also been a great experience. Moreover, the group of people that Alessandro brought together in the Functional Neuroimaging Lab will be difficult to beat elsewhere. Alberto Galbusera, Alessia de Felice, Carola Canella, Daniel Gutierrez Barragan, Ludovico Coletta and Marco Pagani are great colleagues and great friends and I really enjoyed working with them. A special shout-out goes to Alberto for collecting most of the MRI data that I have worked with. I would also like to thank all past members of the lab and other collaborators: Alice Bertero, Anna Aksiuto, Gergely David, Manuel Blesa, Nayome Rey Calvo and Richard Gomolka.

Pursuing a PhD, while stressful and often full of struggle, has also been a lot of fun, both inside the office and outside. I immensely enjoyed all the trips, hikes and aperitivi with my colleagues and other friends from CIMeC, IIT and around: Germán Kruszewski, Georgiana Dinu, Jan Bím, Nghia The Pham and many, many others with whom I have been lucky to share the beautiful city of Rovereto and the mountains around.

Many thanks go to the administration of the Doctoral School and the Center for Neuroscience and Cognitive Systems, namely to Leah Mercanti, Sara Maistrelli and Paola Battistoni, for making all the paperwork a (mostly) smooth ride. I would also like to thank the members of my oversight committee, Stefano Panzeri and Jorge Jovicich, for all the feedback they provided.

My parents, Hana and Zbyněk, and my brothers, Jan and Martin, provided a great amount of support over the past years. Their visits in Rovereto were always a source of joy!

Finally and above all, I would like to thank Angeliki, whose encouragements, laughs and support made the start of our journey extraordinary.

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List of Abbreviations

Acb	nucleus accumbens
Amy	amygdala
AO	anterior olfactory nucleus
AON	anterior olfactory nucleus
BF	basal forebrain module
CA1/3	CA1/3 fields of hippocampus
Cg	cingulate cortex
CM	central medial nucleus
dHc	dorsal hippocampus
DMN	default mode network
FrA	frontal association cortex
Hc	hippocampus/hippocampal module
Hypo	hypothalamus
Ins	insular cortex
LCN	lateral cortical network
M1/2	primary/secondary motor cortex
M2	secondary motor cortex
mPFC	medial prefrontal cortex
MS	medial septal nucleus
OFc	orbitofrontal cortex
P	pons
PtA	parietal association cortex
Rs	retrosplenial cortex
S1/2	primary/secondary somatosensory cortex
TeA	temporal association cortex
Thal	thalamus module
Th	thalamus
vHc	ventral hippocampus
VM	ventral midbrain module
vSub	ventral subiculum
VTA	ventral tegmental area

Publications

Parts of this thesis have appeared previously in the following publications:

- Adam Liska, Alberto Galbusera, Adam J. Schwarz, and Alessandro Gozzi (2015). “Functional Connectivity Hubs of the Mouse Brain”. In: *NeuroImage* 115, pp. 281–291. ISSN: 1095-9572. DOI: [10.1016/j.neuroimage.2015.04.033](https://doi.org/10.1016/j.neuroimage.2015.04.033)
- Adam Liska and Alessandro Gozzi (2016). “Can Mouse Imaging Studies Bring Order to Autism Connectivity Chaos?” In: *Frontiers in Neuroscience* 10, p. 484. DOI: [10.3389/fnins.2016.00484](https://doi.org/10.3389/fnins.2016.00484)
- Adam Liska, Alice Bertero, Ryszard Gomolka, Mara Sabbioni, Alberto Galbusera, Noemi Barsotti, Stefano Panzeri, Maria Luisa Scattoni, Massimo Pasqualetti, and Alessandro Gozzi (2017). “Homozygous Loss of Autism-Risk Gene CNT-NAP2 Results in Reduced Local and Long-Range Prefrontal Functional Connectivity”. In: *Cerebral Cortex*, pp. 1–13. DOI: [10.1093/cercor/bhx022](https://doi.org/10.1093/cercor/bhx022)

Chapter 1

Introduction

1.1 Motivation

1.1.1 What is the brain and why do we study it?

Humans have been fascinated by their own brains for centuries. The brain is the seat of our memories, thoughts, and perception; it is within the brain that our complex behaviours are generated. However, we are still far from understanding its workings. We know that the brain is composed of a very large number of specialised cells called neurons, which are interconnected and form a vast network. We know that neurons process our perceptual inputs and generate behaviour by passing information through this network, and we know that even a slight disruption of this network can have profound consequences on our lives and on the way we experience the world, as pathological conditions as diverse as Parkinson's disease, aphasia and schizophrenia are caused by specific changes that occur within the nervous system. Nevertheless, making sense of all of this knowledge, being able to interpret it and make predictions about future behaviour using one single model is still far beyond our reach.

Owing to continual technological advances, our knowledge of the brain has greatly advanced in the last few decades. We are now able to watch, record and alter the activity of single neurons, identify their interconnections and study how these relate to behaviour or how they are affected in brain disorders. In spite of this, treatment possibilities for many disorders remain limited and we still struggle at devising targeted interventions. There is, nevertheless, a general feeling among many in the field that we are currently on the cusp of a new big leap in our understanding of the brain and several large research initiatives focused on the brain have been recently unveiled to make this leap possible¹. These have complementary goals: Some focus on developing new techniques to study the structure and function of the brain, other on analytical procedures and data storage or on mapping the brain of a specific species in great detail.

During my doctoral studies, I focused on studying the activity and structure of the rodent brain using a medical imaging technique called magnetic resonance imaging (MRI), and in the following sections I will briefly introduce the type of questions that I have addressed in my research.

1.1.2 How do we measure brain activity?

MRI is a medical imaging technique that is capable of producing images of the whole brain. It does so by making use of magnetic properties of hydrogen nuclei present

¹Human Brain Project (<https://www.humanbrainproject.eu>), BRAIN Initiative (<https://www.braininitiative.nih.gov>), Brain/MINDS (<http://brainminds.jp/en>), among others.

throughout the body in the form of water molecules. The technique is non-invasive and its images have a very good contrast between soft tissues, such as between a tumour and its surroundings or between grey and white matter of the brain. These properties have made MRI popular among clinicians and the technique has become ubiquitous in modern day clinical practice (Westbrook and C. K. Roth, 2011).

However, neuroscientists are not interested only in the structure of brain; they are just as much interested in studying its activity and function: Is region A activated by a sensory stimulus B? Is the performance of a subject in task C affected when region D is stimulated or inhibited? Do people with disease E show reduced activity in region F?

A classical approach to measuring the activity of a neuron is to implant a micro-electrode into the brain and take advantage of how changes in the electric potential across the neuron's membrane encode the state of the neuron (Hubel and Wiesel, 1959; Adrian, 1928). However, this technique is invasive and records the activity of a single neuron. Another technique called electroencephalography (EEG) records the combined electrical activity of neurons at the surface of the skull. While this technique is non-invasive, its spatial resolution and sensitivity to neurons that are located deeper in the brain are limited (Jackson and Bolger, 2014). Currently, the most popular technique to study brain function is based on the following observation: When neurons in a particular region of the brain are active, the flow of oxygenated blood into that region increases in order to deliver the fuel required by the active neurons, and the change in the balance in concentration between oxygenated and deoxygenated blood results in an increase of the MR signal coming from that particular area (Ogawa et al., 1990). Therefore, in order to identify regions of high or low neuronal activity, we need to repeatedly and in short intervals acquire MR images of a subject's brain and track changes in MR signal across different brain structures. This approach was further developed and resulted in the introduction of blood-oxygen-level dependent functional MRI (BOLD fMRI) in the early 1990s (Bandettini et al., 1992; Kwong et al., 1992). In fMRI, the brain is divided into small cubes called voxels and one can infer the activation time series for each of these voxels individually. The spatial resolution of the method is therefore better than that of EEG and now reaches submillimeter values. However, its sampling frequency is relatively low at around one sample per second, and it can by no means measure the activity of a single neuron: Its signal is a proxy for the combined activity of all neurons in the selected voxel convolved with the complex hemodynamic response.

Functional MRI has quickly become popular among neuroscientists, especially because of its non-invasiveness and whole-brain coverage. A typical fMRI experimental paradigm aims at identifying associations between sensory stimulation or task performance and increased neuronal activity in specific brain regions (Friston, Jezzard, and Turner, 1994). Periods of activity are usually interleaved with periods of rest during which the subject lies in an MRI scanner with no overt task or sensory stimulation. By comparing the activity in individual brain regions in all segments of the acquisition, researchers can identify those regions that show statistically significant increases when the subject is performing the task compared to the resting baseline. A large number evoked activations were studied in this way and a number task- or sensory-specific networks of co-activating regions have been identified. These studies have greatly advanced our knowledge of the functional organization of the brain (Poldrack and Farah, 2015).

1.1.3 What does the brain do when we are at rest?

The type of experiments described in the preceding paragraph paints a very “reflexive” picture of the brain: It sits around not doing much unless it is externally stimulated or unless it is involved in an externally directed task. However, there are many observations that contradict such a view of brain function and instead suggest a brain that is constantly buzzing with activity (Raichle, 2010). One of these observations is the relatively high energy consumption of the brain that only fractionally increases during task performance (Raichle, 2015). Moreover, spontaneous activity is clearly visible on EEG and fMRI scans acquired during periods of rest and this activity shows signs of non-random organization across brain regions which goes beyond simple monosynaptic axonal connectivity (M. D. Fox and Raichle, 2007).

If the “reflexive” function of the brain constitutes only a small fraction of its activity, how can we uncover and interpret the intrinsic activity? This has been the object of research within the fMRI community ever since the first description of non-random organization of the resting-state fMRI (rsfMRI) signal within the human motor cortex (Biswal et al., 1995). An important step in this direction has been the observation that networks of regions whose activity time series are correlated at rest correspond to networks of co-activated regions elicited during task performance (Smith, P. T. Fox, et al., 2009; Crossley, Mechelli, Vértes, et al., 2013). Several explanations for this phenomenon have been put forward, including learning consolidation, preparation for task performance and synchronization based on Hebbian-style reinforcement mechanisms, although the evidence for the time being remains inconclusive (Power, Schlaggar, and Petersen, 2014). The observation of specifically organized brain activity at rest has nevertheless marked a paradigm shift in neuroimaging and there has been a continual increase in the application of rsfMRI to the study of brain organization (Snyder and Raichle, 2012).

1.1.4 What is a connectome? How is it affected in brain disorders? Can it help us better understand or diagnose these disorders?

The shift from task-evoked to resting-state fMRI has brought about also a shift in the way the fMRI signal is analysed. A growing number of studies started to study brain’s intrinsic activity from the perspective of mathematical graphs or networks: The brain is represented as a network of interconnected functional regions (Bullmore and Sporns, 2009). The strength of a connection between two regions can be expressed as the degree to which the intrinsic activity in those two regions is similar and is commonly referred to as “functional connectivity” (Friston, 2011). Such a representation of the brain is also referred to as the “connectome” (Smith, Beckmann, et al., 2013) and it is useful both from the data analysis perspective – the underlying field of network analysis has burgeoned recently, too, and it provides a large number of useful analytical tools – and from the interpretative perspective – as it is closer to the actual architecture of the brain.

Recent studies have revealed two defining characteristics of human functional connectomes. First, their nodes can be reliably partitioned into densely connected clusters called modules or communities, most of which typically replicate previously described functional systems of the brain (Power, Cohen, et al., 2011; Yeo et al., 2011). Second, there exist a limited set of nodes called hubs that act as integrators between these systems and that are crucial for the coordinated involvement of multiple systems in complex behaviors (van den Heuvel and Sporns, 2013).

Connectomic studies have not only provided insights into the organization of the healthy brain, but also led to observations of altered brain connectivity in a number of brain disorders: deficient connectivity of the prefrontal cortex in schizophrenia (Anticevic, Brumbaugh, et al., 2013), widespread over- and under-connectivity in autism spectrum disorders (Di Martino et al., 2014), and many others (Crossley, Mechelli, Scott, et al., 2014; Buckner et al., 2009). This area of research, also referred to as “pathoconnectomics” (Rubinov and Bullmore, 2013), has seen an especially large following, with the objective to help diagnosis, devise measurable biomarkers, and better understand their genetic or environmental underpinnings (Castellanos et al., 2013; Khalili-Mahani et al., 2017).

However, connectomic approaches to studying and diagnosing brain disorders still face several challenges before widespread adoption can take place. Moreover, many of these challenges have been used to argue against the use of neuroimaging-based findings in clinical practice (Weinberger and Radulescu, 2016). For example, there are inconsistencies across studies in connectivity alterations observed for a specific disorder. These disparities and potential lack of replicability may be attributable to several factors, including clinical heterogeneity of the patient groups, imaging parameters, analytical procedures and inadequate correction of other confounding factors such as motion or cross-site differences (Filippi and Rocca, 2016; Marchitelli et al., 2016; Power, Schlaggar, and Petersen, 2015). As pathoconnectomics is still a nascent field, several of these may come into play in a single study and therefore it is imperative that the community gives them full attention. Current developments, such as the use of registered reports (Chambers, 2013) and increased data and code sharing (Poldrack, Baker, et al., 2017), may help address some of the issues regarding the general replicability of findings, by allowing the readers to distinguish *a priori* from *post hoc* analyses and to potentially re-run some of the analyses, and by allowing the researchers to replicate their results on comparable datasets acquired elsewhere and to test their new analytical methods on larger datasets.

1.1.5 Autism spectrum disorders

While many brain disorders – neurological, psychiatric and developmental – have been studied using neuroimaging techniques, in this thesis we will focus specifically on autism spectrum disorders (ASD). Autism is a heterogeneous syndrome characterised by core behavioural features including deficits in social communication and interaction, as well as restricted, repetitive patterns of behaviour, interests and activities (Association, 2013). Individuals with ASD can also present several other comorbidities, such as intellectual disability and epilepsy (Zafeiriou, Ververi, and Vargiami, 2007; Bauman, 2010). While the typical age of diagnosis used to be around 3-4 years, current efforts aim at a much earlier diagnosis in order to allow for early intervention (Lai, Lombardo, and Baron-Cohen, 2014). Nevertheless, the disorder continues to represent a considerable burden to affected individuals, their immediate family and public healthcare systems.

Although a primary and unitary aetiology for ASD has not been identified, its high heritability has been consistently documented across a series of twin studies (Tick et al., 2016), revealing a contribution of complex and highly heterogeneous genetic mutations (Geschwind, 2009; Geschwind and State, 2015; Sanders et al., 2015). Remarkably, although previously identified mutations, genetic syndromes and *de novo* copy number variations (CNVs) account for about 10–20 % of ASD cases, none

of these single known genetic causes accounts for more than 1–2 % of cases [reviewed in (Abrahams and Geschwind, 2008)], making heterogeneity a major hallmark of the disorder (Betancur, 2011). Nevertheless, the advances in identification of autism-risk genes led to renewed interest in studying the underlying neurobiology of the disorder and several major cellular pathways have been shown to be affected, including transcription and chromatin regulation, synapse development, and signal transduction (Sanders, 2015; Kleijer et al., 2017).

Concurrently, the advent of non-invasive brain imaging raised hopes that the clinical heterogeneity of ASD could be narrowed down to a smaller number of identifiable “imaging endophenotypes” that could help ASD diagnosis, patient stratification, and possibly provide clues as to the elusive aetiology of this group of disorders (Geschwind and State, 2015; Gottesman and Gould, 2003). While several initial studies focused on brain overgrowth as a potential early indicator of autism (Courchesne, 2002; Lange et al., 2015; Ecker, Schmeisser, et al., 2017), a large number of studies have since considered functional connectivity disruptions in individuals with ASD, following early reports of reduced brain connectivity identified using positron-emission tomography [PET, (Horwitz et al., 1988)], later corroborated by investigations with task-based (Just, Cherkassky, et al., 2004) and resting-state fMRI (Assaf et al., 2010; Cherkassky et al., 2006; Kennedy and Courchesne, 2008). The extensive literature published to date points at the presence of major functional connectivity alterations in ASD populations, although the identified regional patterns vary considerably across studies and patient cohorts (Ameis and Catani, 2015; Bernhardt et al., 2016; Ecker and D. Murphy, 2014; Ecker, Bookheimer, and D. G. M. Murphy, 2015; Kana, Libero, and Moore, 2011; Müller, 2014; Vasa, Mostofsky, and Ewen, 2016). These results are intriguing as they are consistent with the observation that many autism-risk genes are involved in synapse development and function: Aberrations in these genes might lead to perturbations of neural connectivity, which in turn could result in aberrant large-scale functional connectivity (Ecker, Schmeisser, et al., 2017).

1.1.6 Can animal research be of any value in pathoconnectomics?

Do connectivity disruptions cause brain disorders or are they a mere by-product of brain dysfunction? Can we identify possible genetic contributions to this phenomenon? What is the neural basis that underpins connectivity disruptions? Is functional connectivity disruption associated with aberrant structural connectivity?

Despite intensive human connectomic research, many fundamental questions about the nature of connectivity disruptions in brain disease remain open. This may be due to the fact that there currently exists a disconnect between the studies of large-scale organization of the human brain with techniques such as MRI and EEG, and the research at the level of genes, neurons and microcircuits performed in laboratory animals. Let us consider the case of ASD: Despite some obvious limitations in reliably modeling the full phenotypic spectrum of such a complex developmental disorder, mouse models have played a central role in advancing our basic mechanistic and molecular understanding of the disorder (Silverman, Yang, et al., 2010; de la Torre-Ubieta et al., 2016). Therefore, a deeper understanding of the complex pathophysiological cascade leading to aberrant connectivity in ASD can greatly benefit from the use of the mouse, where individual pathophysiological or phenotypic components of the spectrum can be recreated and investigated via approaches that are either off limits or confounded by clinical heterogeneity (Nestler and Hyman,

2010). The use of the mouse may also help us address some of the more technical challenges of human connectomic research discussed above: motion can be very well controlled and rs-fMRI measurements can be complemented by other brain activity readouts, such as by measuring local field potentials (LFP). Moreover, there is an increasing number of publicly available resources with rich data about the mouse brain, including high-resolution gene expression and neural circuitry datasets produced by the Allen Institute for Brain Science² (Lein et al., 2007; Oh et al., 2014), which pave the way for multimodal investigations and more refined biological interpretations of connectomic findings (Konopka, 2017; van den Heuvel, Scholtens, et al., 2016). Taken together, research in the mouse allows for combined investigations of microscopic and macroscopic findings, which are currently called for in the field of autism research (Ecker, Schmeisser, et al., 2017).

However, while initial rsfMRI experiments in the mouse proved promising (Sforzini, Schwarz, et al., 2014), before we can readily translate the knowledge across the two species, we need to learn more about the large-scale organization of the mouse brain and ascertain that – at least to some extent – it does replicate fundamental features of the human brain connectome, such as the presence of functional networks and hubs and their impairment in pathological states (van den Heuvel, Bullmore, and Sporns, 2016). Similarly, methods that are used in human research to identify points of disruptions need to be first tested in the mouse before they can be applied to study disease models. It is these research questions that formed the basis of my doctoral studies and that are discussed in the following chapters of this thesis.

1.2 Structure and main contributions of the thesis

Chapter 2 We build upon initial applications of rsfMRI to the mouse by describing large-scale functional organization of a mouse brain. Specifically, we apply a fully-weighted network analysis (1) to map whole-brain intrinsic functional connectivity (i.e., the functional connectome) at a high-resolution voxel scale and (2), to spatially locate functional connectivity hubs in the mouse brain. Analysis of this large rsfMRI dataset revealed the presence of six distinct functional modules related to known large-scale functional partitions of the brain, including a default-mode network (DMN). Consistent with human studies, highly-connected functional hubs were identified in several sub-regions of the DMN, including the anterior and posterior cingulate and prefrontal cortices, in the thalamus, and in small foci within well-known integrative cortical structures such as the insular and temporal association cortices. According to their integrative role, the identified hubs exhibited mutual preferential interconnections. These findings highlight the presence of evolutionarily-conserved, mutually-interconnected functional hubs in the mouse brain, and may guide future investigations of the biological foundations of aberrant rsfMRI hub connectivity associated with brain pathological states.

Chapter 3 We apply the methods developed in the previous study to investigate functional connectivity, its aberrations and their potential neurobiological underpinnings in mice lacking contactin associated protein-like 2 (*Cntnap2*). Homozygous mutations in CNTNAP2, a neurexin-related cell-adhesion protein, are strongly linked to autism and epilepsy in humans. Importantly, the mouse model employed in this study recapitulates not only the high-confidence ASD mutation, but also

²<http://www.brain-map.org/>

many behavioural and neurobiological traits seen in human carriers of the same mutation. Moreover, common genetic variants in CNTNAP2 were recently described to be associated with impaired frontal lobe connectivity in humans, allowing therefore for a direct comparison of our investigations with findings in humans. Here we used rsfMRI to show that homozygous mice lacking *Cntnap2* exhibit reduced long-range and local functional connectivity in prefrontal and midline brain “connectivity hubs.” Long-range rsfMRI connectivity impairments affected heteromodal cortical regions and were prominent between fronto-posterior components of the mouse default-mode network, an effect that was associated with reduced social investigation, a core “autism trait” in mice. Notably, viral tracing revealed reduced frequency of prefrontal-projecting neural clusters in the cingulate cortex of *Cntnap2*^{-/-} mutants, suggesting a possible contribution of defective mesoscale axonal wiring to the observed functional impairments. Macroscale cortico-cortical white-matter organization appeared to be otherwise preserved in these animals. These findings reveal a key contribution of ASD-associated gene CNTNAP2 in modulating macroscale functional connectivity, and suggest that homozygous loss-of-function mutations in this gene may predispose to neurodevelopmental disorders and autism through a selective dysregulation of connectivity in integrative prefrontal areas.

Chapter 4 We discuss recent progress in mouse brain connectivity mapping via rsfMRI in the context of autism connectivity research and show its growing potential to generate and test mechanistic hypotheses about the elusive origin and significance of connectional aberrations observed in autism. Furthermore, we describe initial examples of how the approach can be employed to establish causal links between ASD-related mutations, developmental processes, and brain connectional architecture.

Chapter 5 In this final chapter we summarize the findings of this thesis and discuss future directions.

Chapter 2

Functional connectivity hubs of the mouse brain

This chapter has been published as:

Adam Liska, Alberto Galbusera, Adam J. Schwarz, and Alessandro Gozzi (2015). "Functional Connectivity Hubs of the Mouse Brain". In: *NeuroImage* 115, pp. 281–291. ISSN: 1095-9572. DOI: [10.1016/j.neuroimage.2015.04.033](https://doi.org/10.1016/j.neuroimage.2015.04.033)

2.1 Background

Resting-state BOLD functional magnetic resonance imaging (rsfMRI) has been widely employed to investigate the intrinsic functional organization of the human brain (Bullmore and Sporns, 2009). Graph theory representations of rsfMRI networks, whereby brain connectivity is conceptualized as a set of nodes (neuronal elements) and edges (their interconnections), have demonstrated that the human brain has topological features recapitulating the defining characteristics of complex networks (Watts and Strogatz, 1998), including the presence of functionally specialised modules encompassing well-characterized neurofunctional systems (Fair et al., 2009; Meunier et al., 2009; Power, Cohen, et al., 2011). In order to account for the brain's ability to simultaneously coordinate multiple networks systems and ensure efficient communication, the presence of functional hub nodes serving as integrators of distinct neuronal systems has been hypothesized. Numerous rsfMRI studies have indicated the presence of highly-connected cortical regions as putative functional hubs for the human brain, most of which appear to exhibit overlap with sub-regions of the default mode network (DMN) (Cole, Pathak, and Schneider, 2010; Tomasi and Volkow, 2011; Zuo et al., 2012). Importantly, the integrative role of these hub regions renders them points of potential vulnerability to dysfunction in brain disorders. Consistent with this notion, aberrant rsfMRI connectivity profiles have been described for several hub regions in pathological conditions such as autism, schizophrenia and neurodegenerative disorders (Buckner et al., 2009; van den Heuvel and Sporns, 2013). However, fundamental issues related to the etiopathological and biological foundations of these alterations remain to be addressed. For one, the neurophysiological cellular underpinnings of functional hub derangement observed in neuropsychiatric disorders remain largely unknown. It is also unclear whether these alterations are patho-physiologically relevant, or just epiphenomenal to underlying brain disorders.

Functional hub identification in preclinical species like the mouse, where genetic, cellular and molecular underpinnings of several brain disorders can be reproduced in controlled conditions and manipulated with cellular specificity (Deisseroth, 2011), may offer new critical insight into the above-mentioned issues. Initial attempts to unravel the rodent's brain functional topology have been carried out in rats (D'Souza et al., 2014; Liang, King, and N. Zhang, 2011; Liang, King, and N. Zhang, 2012) and more recently in mice (Mechling et al., 2014; Stafford et al., 2014). By using independent-component analysis (ICA) decomposition of rsfMRI signals in awake rats, Liang, King, and N. Zhang (2011) reported the presence of three large modules, covering cortical areas, prefrontal and limbic hippocampal regions and basal forebrain structures, respectively. Using anatomically-defined labels, D'Souza et al. (2014) identified six communities in medetomidine sedated rats, including two purely cortical systems (i.e. frontal and somatosensory) together with four mixed communities involving hippocampal and peri-hippocampal cortices, basal ganglia, thalamic nuclei and pons. ICA-based decomposition has also been recently applied to mouse rsfMRI datasets acquired under isoflurane anaesthesia (Mechling et al., 2014), leading to the identification of a basal ganglia module plus four other composite communities which included complex combinations of cortical and subcortical systems. Two of the above studies also report attempts to identify inter-connecting hub regions. D'Souza et al. (2014) attributed a putative integrative function to the hippocampus, striatum plus all cortical subdivision, with the sole exception of visual, primary motor and parietal cortices. These latter regions are part of a set of eleven putative hub regions described by Mechling et al. (2014) in the mouse brain, which also included somatosensory, frontal as well as subcortical diencephalic structures and the striatum. Collectively, while these initial studies led to the identification of seemingly stable functional partitions, substantial heterogeneity exists in their anatomical composition, as well as in the location of integrative structures, a finding that may reflect discrepant experimental procedures (e.g. anaesthesia, preprocessing procedures) and is probably exacerbated by heterogeneity in the regional parcellation schemes (coarse ICA-based, or anatomical volumes) and network thresholding strategies employed. Moreover, none of the functional partitions described so far can be straightforwardly related to known distributed human networks (e.g. DMN), which is a limiting factor in the translation of preclinical research to human condition.

Employing rigorous control of motion and potential physiological confounds (Ferrari et al., 2012), we recently demonstrated the presence of robust distributed rsfMRI networks in the mouse brain (Zhan et al., 2014), including functional precursors of the human salience and default mode networks (Sforazzini, Schwarz, et al., 2014; Sforazzini, Bertero, et al., 2016), an observation recently replicated by an independent group (Stafford et al., 2014). Our datasets offer the opportunity to spatially locate functional hubs in the mouse brain and relate them to known network system of the human brain, which greatly enhances the translational value of this approach. To this purpose, here we applied a computationally unbiased, fully-weighted network analysis of rsfMRI connectivity at a voxel scale in a large cohort of adult mice. We show the presence of six large-scale functional partitions, and anatomically localise mutually inter-connected hubs in several sub-regions of the DMN as well as in several cortical association areas of the mouse brain. These bear a strong resemblance to findings in the human brain, suggesting the presence of evolutionarily conserved cortical regions serving as integrators of segregated brain systems in the mouse, and supporting the use of this species to investigate aberrant rsfMRI hub connectivity associated to brain pathological states.

2.2 Materials and methods

Il in vivo studies were conducted in accordance with the Italian law (DL 116, 1992 Ministero della Sanità, Roma) and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal research protocols were also reviewed and consented to by the animal care committee of the Istituto Italiano di Tecnologia (permit 07-2012). All surgical procedures were performed under anaesthesia.

2.2.1 Animal preparation

MRI experiments were performed on male 20-24 week old C57BL/6J (B6) mice (n=41, Charles River, Como, Italy). The animal preparation protocol was recently described in detail (Ferrari et al., 2012; Sforazzini, Schwarz, et al., 2014; Sforazzini, Bertero, et al., 2016; Zhan et al., 2014). Briefly, mice were anaesthetized with isoflurane (5% induction), intubated and artificially ventilated (2% maintenance). The left femoral artery was cannulated for continuous blood pressure monitoring and blood sampling. At the end of surgery, isoflurane was discontinued and substituted with halothane (0.75%). Functional data acquisition commenced 45 min after isoflurane cessation. Mean arterial blood pressure was recorded throughout the imaging sessions. Arterial blood gases (paCO₂ and paO₂) were measured at the end of the functional time series to exclude non physiological conditions. Mean paCO₂ and paO₂ levels recorded were 20 ± 5 and 257 ± 33 mmHg, respectively, well within the physiological range.

2.2.2 Image data acquisition

All in vivo experiments were performed using a 7.0 Tesla MRI scanner (Bruker Biospin, Milan). Transmission and reception were achieved using a 72 mm bird-cage transmit coil and a custom-built saddle-shaped four-channel solenoid coil for signal reception. Shimming was performed on a $6\text{mm} \times 6\text{mm} \times 6\text{mm}$ region, using a FASTMAP protocol. For each session, high-resolution anatomical images were acquired with a fast spin echo sequence (RARE, hennig1986) with the following parameters: repetition time (TR)/echo time (TE) 5500/60 ms, matrix 192×192 , field of view $2 \times 2 \text{ cm}^2$, 24 coronal slices, slice thickness 0.50 mm. Co-centred single-shot BOLD rsfMRI time series were acquired using an echo planar imaging (EPI) sequence with the following parameters: TR/TE 1200/15 ms, flip angle 30° , matrix 100×100 , field of view $2 \times 2 \text{ cm}^2$, 24 coronal slices, slice thickness 0.50 mm, 300 volumes and a total rsfMRI acquisition time of 6 min.

2.2.3 Image data preprocessing

Image preprocessing was carried out using tools from FMRIB Software Library (FSL, v5.0.6; <http://fsl.fmrib.ox.ac.uk/fsl/>) (Jenkinson et al., 2012) and AFNI (v2011_12_21_1014; <http://afni.nimh.nih.gov/afni/>). RsfMRI time series were despiked (AFNI/3dDespike), corrected for motion (AFNI/3dvolreg), and spatially normalized to an in-house C57Bl/6J mouse brain template (Sforazzini, Bertero, et al., 2016) (FSL/FLIRT, 12 degrees of freedom). The normalized data had a spatial resolution of $0.2 \times 0.2 \times 0.5 \text{ mm}^3$ ($99 \times 99 \times 24$ matrix). Head motion traces and mean ventricular signal (averaged fMRI time course within a manually-drawn ventricle mask) were regressed out of each of the time series (AFNI/3dDeconvolve). To

assess the effect of global signal removal, separate rsfMRI time series with the whole-brain average time course regressed out were also generated. All rsfMRI time series were spatially smoothed (AFNI/3dmerge, Gaussian kernel of full width at half maximum of 0.5 mm) and band-pass filtered to a frequency window of 0.01-0.08 Hz (AFNI/3dBandpass) (Sforazzini, Bertero, et al., 2016).

2.2.4 Functional network formation

Time courses from all voxels in a brain tissue mask associated with the anatomical template were extracted and a 16135×16135 connectivity matrix was calculated for each subject using Pearson product-moment correlation coefficient as a measure of inter-voxel connectivity (Bullmore and Sporns, 2009), resulting in subject-wise functional connectivity networks. In contrast to the vast majority of network analyses of rsfMRI data, the connectivity matrix was not subject to any further arbitrary thresholding and/or binarisation (Bullmore and Sporns, 2009). Separate connectivity matrices were created for the rsfMRI dataset with global signal regression.

2.2.5 Module detection

Most of network attributes used to identify functional hubs rely on a prior detection of modules that accurately describe the topological organization of brain networks (Sporns, 2013). To this purpose, standard approaches in human and rodent brain analyses employ a modular partition based on a connectivity network averaged across a large number of subjects (D'Souza et al., 2014; Liang, King, and N. Zhang, 2011; Liang, King, and N. Zhang, 2012; Mechling et al., 2014; Power, Cohen, et al., 2011; Power, Schlaggar, et al., 2013; Rubinov and Sporns, 2010; Yeo et al., 2011; Zuo et al., 2012). Accordingly, the subject-wise connectivity matrices were first transformed to z scores using Fisher's r-to-z transform, averaged across all animals and transformed back to r values to create the average functional network.

The average functional network was then partitioned into non-overlapping modules by maximizing the modularity of the final partition (Newman and Girvan, 2004) using the Louvain algorithm (Blondel et al., 2008), as implemented in Brain Connectivity Toolbox (BCT) (Rubinov and Sporns, 2010). An asymmetric measure of modularity incorporating both positive and negative weights was employed (Rubinov and Sporns, 2011). Corresponding average null networks, against which we compared the resulting modularity value (Guimerà, Sales-Pardo, and Amaral, 2004), were created from subject-wise null networks, each matching the covariance structure of a single subject connectivity matrix (Zalesky, Fornito, and Bullmore, 2012).

The robustness of the resulting modules was further assessed by taking advantage of the non-deterministic nature of the Louvain algorithm (Blondel et al., 2008) and investigating the presence of competing maxima, whose presence is suggestive of an absence of a clear modular structure (Gfeller, Chappelier, and De Los Rios, 2005; Karrer, Levina, and Newman, 2008; Massen and Doye, 2006; Wilkinson and Huberman, 2004). To this purpose, we performed 100 independent iterations of the algorithm, each with a randomized order of nodes on input, and created iteration stability maps of modules by calculating for each node the proportion of iterations in which it was assigned to each module. These iterations yielded a consistent output and, as further analyses required one single modular structure, a reference partition of the mouse functional network was created by assigning each voxel to the module to which it belonged in more than 50 % of iterations. This procedure was carried out on rsfMRI datasets with and without global signal regression.

The two cortical modules identified in our study, the default mode network (DMN) and lateral cortical network (LCN), have been previously shown to be anticorrelated in both mice and rats (Schwarz, Natalia Gass, et al., 2013; Sforazzini, Bertero, et al., 2016). To investigate the presence of analogous anticorrelations in the present dataset upon global signal regression, we extracted the mean signals from the identified cortical modules and correlated them with all voxels within the brain to obtain T statistics maps.

To assess inter-subject variability of the modular structure, subject-wise connectivity matrices were partitioned using the same method and the similarity of each pair of individual partitions was quantified with the variation of information (VI) metric (Rubinov and Sporns, 2011), achieving a mean VI value of 0.2412 ($\sigma = 0.0203$). The same procedure was repeated for subject-wise null networks, constructed as described above, achieving a mean VI value of 0.2889 ($\sigma = 0.0116$). A paired t-test between the corresponding VI values confirmed that the level of reproducibility is highly statistically significant ($p < 0.00001$). Moreover, the effect size obtained (2.9) was of similar order of magnitude to a recent rat study (D'Souza et al., 2014).

In order to assess the impact of spatial smoothing and voxel "adjacency" on the detection of functional modules (Power, Cohen, et al., 2011), we created two additional functional networks in which we removed connections shorter than 0.5 mm and 1.0 mm, respectively. We then separately identified modules in these two additional networks for comparisons with original functional partitions.

2.2.6 Global and module hub identification

Functional hubs have commonly been defined as nodes with a high density of connections across the whole network (Bullmore and Sporns, 2009). However, consideration of node connectivity distributions within and between the different component modules allows a more nuanced view of topological function and node roles within the overall network (Guimerà and Amaral, 2005; van den Heuvel and Sporns, 2013; Zuo et al., 2012). In particular, it allows candidate hubs to be defined based on high connectivity within the overall network, within their own module and to nodes in other modules.

Normalized positive connection strength of a node in a weighted network (also referred to as strength) quantifies the overall density of its connections across the whole network and is defined as the sum of all positive connections of the node:

$$s_i = \frac{\sum_{w_{ij}>0} w_{ij}}{N-1},$$

where w_{ij} is the weight of the connection between nodes i and j , and N is the number of nodes in the network (Rubinov and Sporns, 2011).

Conversely, connection diversity of a node assesses the distribution of its connections across modules, i.e., whether the node preferentially connects only to a limited subset of modules (low diversity) or whether its connections are spread evenly across the whole network (high diversity) (Rubinov and Sporns, 2011). The values of connection diversity are in the range of $[0, 1]$ and the measure is formally defined as:

$$h_i = -\frac{1}{\log M} s_i(u) \log s_i(u),$$

where M is the number of modules and $s_i(u)$ is the strength of node i within module u . The diversity parameter captures, for complete weighted networks, topological

functionality analogous to the participation coefficient in binary networks (Guimerà and Amaral, 2005).

The strength of node i within module u is defined as:

$$s_i(u) = \frac{\sum_{w_{ij}>0} w_{ij} \delta_u(j)}{N - 1},$$

where $\delta_u(j) = 1$ when j is part of module u , and $\delta_u(j) = 0$ otherwise (i.e., only connections of node i to nodes j within module u contribute to the summation) (Rubinov and Sporns, 2011). Within this framework, we refer to the strength of a node within its own module as the within-module strength of the node.

Guimerà and Amaral (2005) elaborated a number of node roles in a “functional cartography” of the within- vs. between-module connectivity landscape of binary networks. While this presents an appealing conceptual framework, the proposed definitions were based on somewhat arbitrary (although intuitive) divisions of the parameter space. Analogous parameter-space divisions for fully weighted networks of functional connectivity have yet to be defined, and should meaningfully reflect both the network characteristics and underlying biology. A critical first step in elucidating the connectivity landscape of these neurobiological networks is to localise and understand the behaviour of the extreme nodes, i.e. those with maximal connection strength or diversity.

To identify and characterise extreme nodes, we implemented the statistical “top percentage” threshold approach (Cole, Pathak, and Schneider, 2010), which identifies the highest strength and diversity regions and at the same time quantifies inter-subject consistency and avoids arbitrary strength or diversity thresholding. Briefly, this approach consists in calculating connection strength, connection diversity and within-module strength maps separately for each subject, converting them to standard scores and performing a series of one-tailed one-sample t-tests for each network attribute, comparing the value of the given attribute at each voxel to zero (its mean value). This results in a statistical map expressing the probability that the value of a given network attribute at a given voxel is higher than the average. A statistical threshold is then selected for each attribute such that only 10 % of voxels remain. The reported threshold p-values were corrected using the false discovery rate (FDR) approach (Genovese, Lazar, and Nichols, 2002); however, as it was already noted in (Cole, Pathak, and Schneider, 2010), this approach does not suffer from the multiple comparisons problem as it does not rely on the use of statistical probabilities for threshold selection and FDR correction is applied in order to remain statistically conservative.

We identified as global hubs those nodes that exhibited high connection strength or connection diversity. Furthermore, we identified as module hubs those nodes that exhibited high within-module strength (Guimerà and Amaral, 2005). To enable a direct comparison of our data with human and primate studies, where module and hub connectivity maps are typically reported for cortical areas, high connection diversity nodes in the two cortical modules were mapped separately.

In order to evaluate the reproducibility of the results with a smaller number of animals in an unbiased manner, 100 random subsets were created, each with exactly $N = 10$ animals. Hub regions were mapped independently for each group and we calculated the number of times out of 100 in which each voxel was identified as a hub of a given type. Furthermore, to assess the impact of higher temporal signal-to-noise ratio (tSNR) in cortical areas consequent to the use of surface coils (Kalthoff et al., 2011) on the network measure of connection strength, subject times series were corrupted with random pink noise throughout the brain such to achieve homogenous

tSNR levels (≈ 25) equalling values observed in deep subcortical areas. Average tSNR values in representative regions of interest before pink noise corruption were as follows: 40.1 ± 3.5 in somatosensory cortex, 38.9 ± 2.9 in dorsal hippocampus, 35.7 ± 2.3 in cingulate cortex, 26.8 ± 1.8 in ventral thalamic areas, 23.5 ± 2.0 in hypothalamus. After pink noise correction, tSNR values were 25.0 ± 1.1 in somatosensory cortex, 24.7 ± 0.4 in dorsal hippocampus, 24.8 ± 0.3 in cingulate cortex, 26.8 ± 1.5 in thalamus and 22.8 ± 0.8 in hypothalamus. The high strength global hub analysis was subsequently repeated for these time series after applying pre-processing steps described above.

2.2.7 Hub connectivity analysis

To assess whether the identified hubs are preferentially and mutually interlinked, we analysed their connectivity relationships using representative single-voxel seeds, each displaying the largest value of the network attribute in question for the given hub region. We first mapped the strongest connections (thresholded at 90th percentile) of each candidate hub within each of the component modules. The presence of overlap between these hub "seed maps" and module hub foci would suggest that the identified hubs exhibit reciprocal and preferential high strength connections, corroborating a role of these nodes as functional inter-module integrators.

The nature of the interconnected hub "backbone" of the mouse functional connectome was then assessed directly by considering the network comprising only connections between the seeds. Mean hub-hub correlation values were extracted from the connection weights of the average functional network and the group-level significance of each connection was assessed using one-sample t-tests on z-transformed versions of the correlation coefficients. The tests were corrected for multiple comparisons using the Benjamini-Hochberg method and a false discovery rate of 0.01. A graph representation of the connections surviving statistical thresholding was displayed using the graph embedder (GEM) algorithm (Frick, Ludwig, and Mehldau, 1994), as implemented in the Network Workbench package¹. The connectivity profile of each candidate hub was further assessed by computing the proportion of its connection strength into each module within the network.

2.3 Results

2.3.1 The mouse brain can be partitioned into six neurofunctional modules, including a default-mode cortical network

The network attributes used to identify functional hubs rely on a prior detection of modules that accurately describe the topological organization of brain networks. To map functional connectivity modules of the mouse brain at a high resolution and high degree of confidence, we computed average inter-voxel rsfMRI connectivity in 41 male C57Bl/6J mice, and partitioned the resulting functional network into modules using a modularity-based algorithm (Blondel et al., 2008; Rubinov and Sporns, 2011). This approach led to the identification of five core cortical and sub-cortical functional modules, each manifesting a remarkably stable anatomical distribution across all repeated runs of the partitioning algorithm, and a single weaker module, composed of various thalamic nuclei, which appeared as an autonomous module in

¹<http://nwb.cns.iu.edu/>

60 % of iterations and was split across neighbouring modules in the remaining iterations (Fig. 2.1). The mean modularity of the functional network partitions (mean modularity $Q = 0.094729$, $\sigma = 0.000322$) was significantly higher than that of a corresponding null model (mean modularity $Q = 0.021335$, $\sigma = 0.000137$). Although we imposed no prior anatomical constraints, all six modules evidenced bilateral symmetry and strong correspondence with distributed functional and anatomical systems of the mammal brain. Specifically, the largest cortical module we identified extended along prefrontal midline structures to include bilateral posterior parietal and temporal association regions (Fig. 2.1A, Module 1). In the light of its remarkable similarity to the rodent precursor of the DMN (Lu et al., 2012; Schwarz, N. Gass, et al., 2013; Schwarz, Gozzi, Chessa, et al., 2012), a distributed cortical network recently described also in mice using seed-based correlations (Sforazzini, Bertero, et al., 2016; Stafford et al., 2014), this module has been referred to as "DMN". A second cortical module, referred to as "lateral cortical network" (LCN), and including frontal association, anterior somatosensory, motor and insular cortices (Fig. 2.1A, Module 2), was identified. A similar network has been reliably identified in mice and rats using seed-based correlations (Schwarz, Natalia Gass, et al., 2013; Sforazzini, Bertero, et al., 2016), and is topologically reminiscent of the human central executive network (Menon, 2011). The remaining three core modules consist mostly of well-characterised subcortical neuro-anatomical systems of the mammal brain. The first of these modules encompassed dorsal and ventral hippocampal regions as well as a minor involvement of ventral retrosplenial areas (Fig. 2.1A, Module 3). A "basal forebrain" module was also apparent, including striatal and septal regions, the nucleus accumbens and anterior olfactory nucleus (Fig. 2.1A, Module 4). A fifth "ventral midbrain" module was identified to comprise several ventral brain regions including the amygdala, hypothalamus, and ventral tegmental area (Fig. 2.1A, Module 5). Finally, thalamic areas emerged as a clearly defined sixth module, although with lower inter-iteration stability (Fig. 2.1A, Module 6). Importantly, the partitioning of the functional network created from the same rsfMRI dataset upon global signal regression yielded consistent network modules (mean modularity $Q = 0.278539$, $\sigma = 0.001541$), with an increased stability of the thalamic module (Fig. 2.2), corroborating the robustness of the methodological approach and overall stability of the identified functional modules. Consistent with human data, the proportion of negative connections in the functional network upon global signal regression was increased from 13 % to 52 % (K. Murphy et al., 2009; Weissenbacher et al., 2009). Correlation analysis of the mean signals from the two cortical modules (DMN and LCN) in global signal regressed rsfMRI timeseries highlighted the presence of robust anticorrelations between these two modules (Fig. 2.3), thus providing additional empirical evidence of intrinsic anticorrelations between the two modules, a finding recently described in both mice and rats (Schwarz, Natalia Gass, et al., 2013; Sforazzini, Bertero, et al., 2016).

To further confirm the robustness of our modular partition, and rule out bias from spatial smoothing and voxel adjacency artefacts (Power, Schlaggar, et al., 2013) we carried out a modular partition of functional network in which all connections shorter than 0.5 mm (approximately 2.5 voxels in plane) were removed, leading to the identification of a set of modules very consistent with those observed with full network (Fig. 2.4A). With a much more stringent selection (i.e. removal of connections shorter than 1 mm, ca. 5 voxels in plane) modular instability was observed for subcortical modules, with evidence of stable partitioning of the DMN and thalamic modules as a single joint community (Fig. 2.4B). This modular structure is consistent with previous seed-based rsfMRI studies of the mouse brain, in which thalamic areas

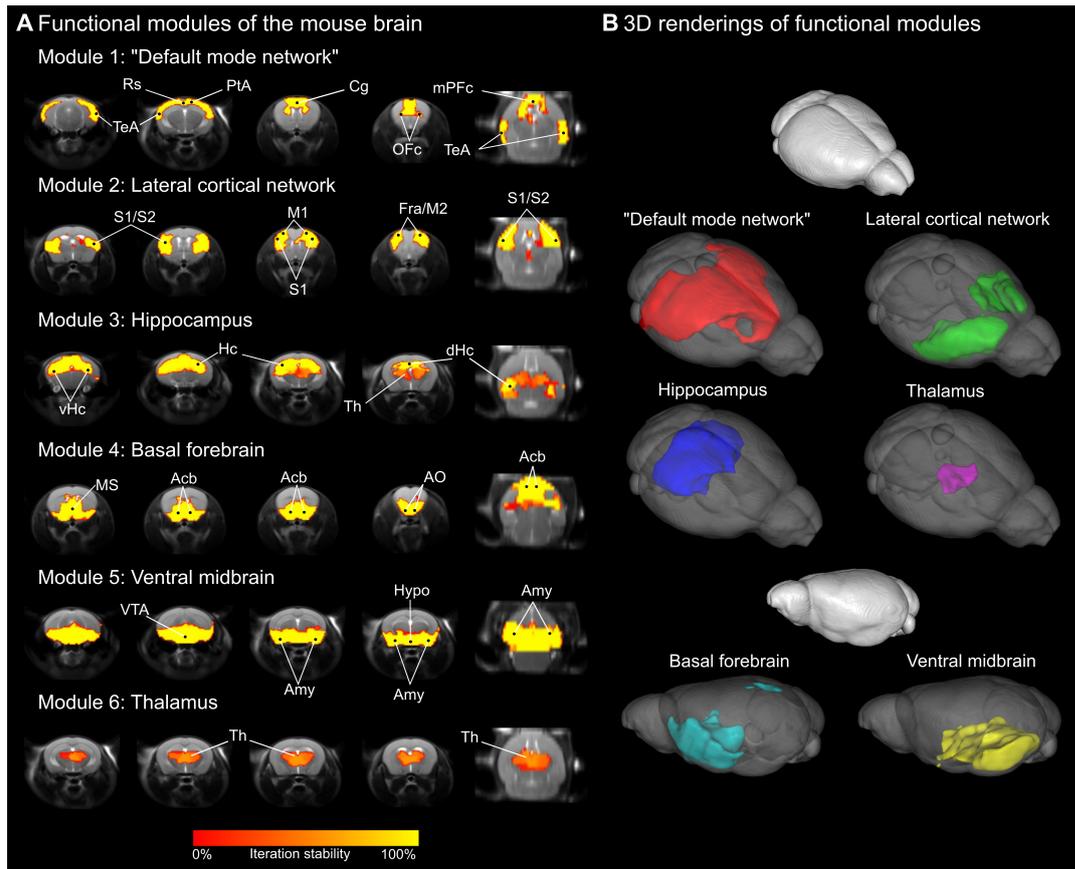


FIGURE 2.1: Functional modules of the mouse brain. (A) Module stability maps (100 iterations, $N=41$ subjects) overlaid on the anatomical template. For each module, four representative coronal slices (left) and one image in the horizontal plane (right) are shown. (B) Three-dimensional renderings of the reference partition within a transparent brain template. Opaque renderings show brain orientation.

appear to be strongly correlated with cingulate and retrosplenial cingulate cortices (Sforazzini, Bertero, et al., 2016). The appearance of subcortical modular instability upon removal of 1 mm connections is not unexpected, because 1 mm long connections cover the anatomical extension of some of the anatomical structures that constitute individual functional modules (e.g. radial hippocampus, or thalamus) (Paxinos and Franklin, 2004).

2.3.2 Global functional hubs are located in cingulate and prefrontal cortex

To identify functional hubs at a voxel scale, we first mapped connection strength values for all nodes in the functional network (Rubinov and Sporns, 2011). In agreement with human studies (Tomasi and Volkow, 2011), cortical and subcortical regions appeared to have distinct connective profiles, with the former exhibiting much higher strength overall (Fig. 2.5A). Anatomical maps of the voxels exhibiting the highest strength ($p < 0.0001$, FDR corrected) revealed foci of high connection strength in several sub-regions of the DMN network, including prefrontal, anterior and posterior cingulate cortex as well as parietal association regions (Fig. 2.5B).

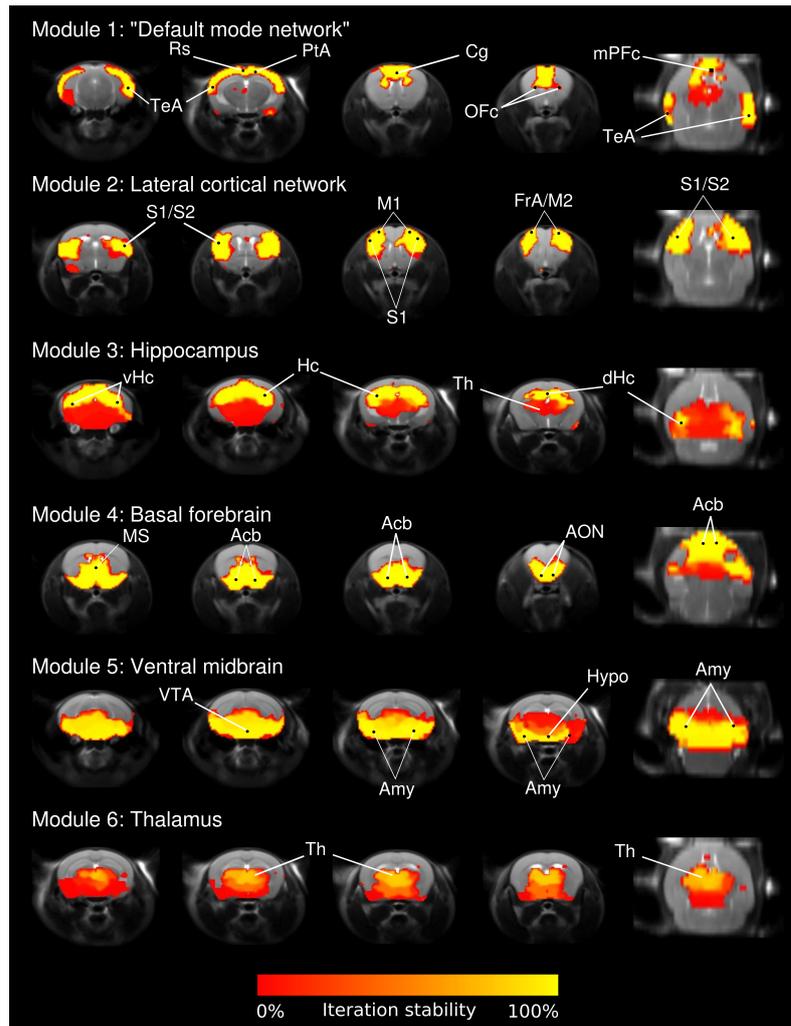


FIGURE 2.2: Functional modules of the mouse brain computed after global signal regression. Module stability maps (100 iterations, $N=41$ subjects) overlaid on the anatomical template. For each module, four representative coronal slices (left) and one image in the horizontal plane (right) are shown.

To account for potential bias induced by coil-induced regional variation in temporal signal to noise ratio (tSNR), we performed connection strength mapping on rsfMRI timeseries corrupted with random pink noise such to achieve homogenous tSNR levels equalling values observed in deep subcortical areas (≈ 25). The results of this analysis confirmed the original hub locations ($p < 0.0038$, FDR corrected, Fig. 2.6) thus ruling out a significant contribution of coil-related bias on high strength connection maps.

2.3.3 High connection diversity hubs are located in the thalamus and associative cortical areas

Connection diversity is a network attribute used to identify nodes participating in multiple functional sub-networks (Power, Schlaggar, et al., 2013; Rubinov and Sporns, 2011). Whole-brain mapping of nodes exhibiting high connection diversity ($p < 0.001$, FDR corrected) revealed a prominent involvement of thalamic areas

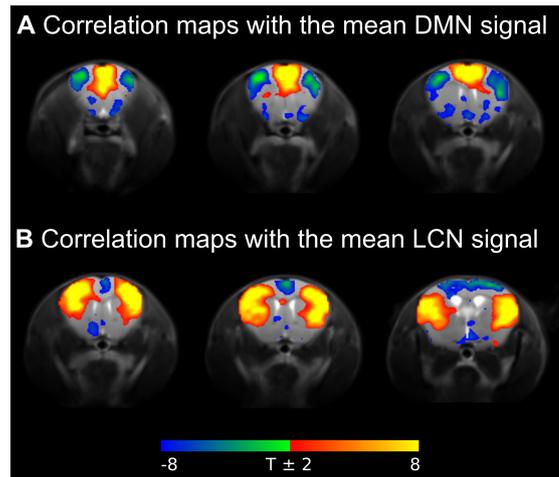


FIGURE 2.3: The two cortical modules identified in the study, the default mode network (DMN) and lateral cortical network (LCN), are anticorrelated in the dataset with global signal regression.

(Fig. 2.5A,C), a finding consistent with the integrative and relay functions subserved by this region (Draganski et al., 2008).

To extrapolate and compare our results with human studies, where topological analyses are typically limited to cortical regions, we also generated a map of high connection diversity voxels within the identified neocortical modules (Fig. 2.7). As recently described in humans (Power, Cohen, et al., 2011), nodes within the DMN module exhibited low average connection diversity, suggesting an extensive internal integration of this module and its function as a highly efficient “processing” system. Importantly, the approach also led to the identification of spatially restricted foci of high connection diversity the temporal association cortex ($p < 0.001$, FDR corrected), a cortical area serving prominent integrative roles. Consistent with recent human studies (Power, Schlaggar, et al., 2013), foci of high connection diversity were also found in the anterior insular cortex ($p < 0.032$, uncorrected), although in this region the effect appeared to be less robust and did not survive FDR correction ($p < 0.2905$, FDR corrected).

2.3.4 Intra-module mapping of high connection hubs

To further investigate the topological organization of the individual sub-networks, we mapped, for each of the identified modules, voxels characterised by high within-module connectivity strength, which we refer to as “module hubs” (Fig. 2.8). The top 10 % voxels were statistically highly significant for all the modules, with the exception of the ventral midbrain module, where the FDR corrected p-value was, however, very close to significance level (DMN: $p < 0.000011$, LCN: $p < 0.00039$, Hc: $p < 0.0016$, basal forebrain: $p < 0.0068$, ventral midbrain: $p < 0.0572$, thalamus: $p < 0.0000096$, all FDR corrected). Module hub mapping in the DMN and lateral cortical networks highlighted high within-module strength foci in the anterior cingulate cortex, and frontal association cortices, respectively. Additional candidate module hubs were identified in the dorsal hippocampus (hippocampal module), nucleus accumbens and olfactory nuclei (basal ganglia), pons/ventral subiculum (ventral midbrain), and centromedial thalamic nuclei (thalamus).

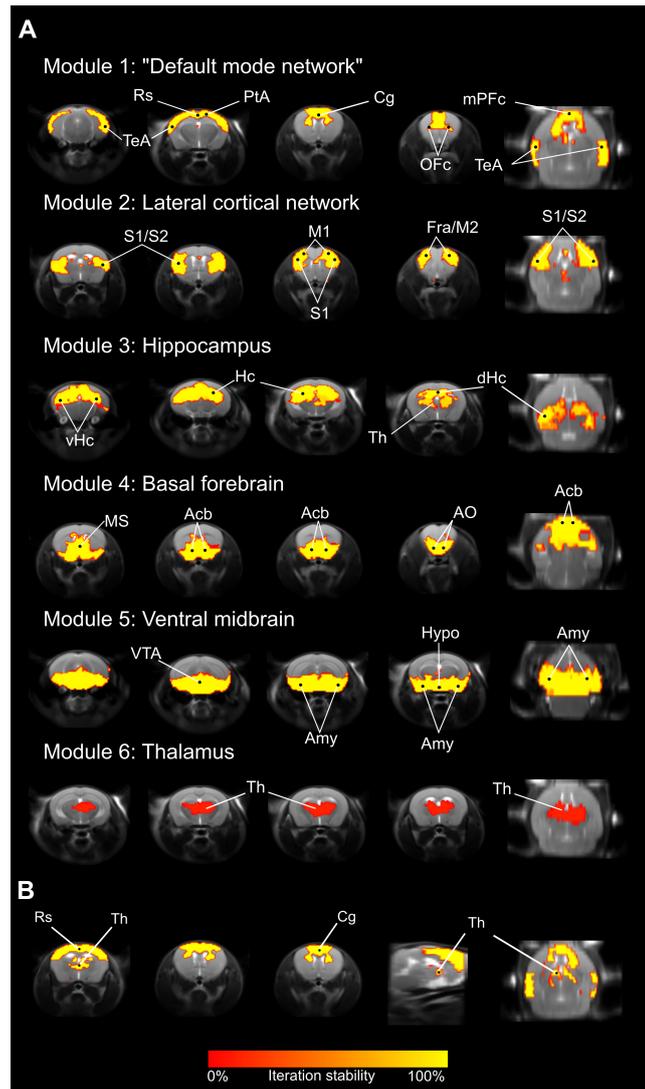


FIGURE 2.4: Modules of the mouse brain upon removal of short connections. (A) Connections shorter than 0.5 mm were removed. (B) DMN module in the functional network upon removal of connections shorter than 1.0 mm. Module stability maps (100 iterations, N=41 subjects) are overlaid on the anatomical template.

2.3.5 Reproducibility of global and intra-module hub mapping on smaller subject cohorts

In order to evaluate the reproducibility of global and intra-module hub mapping on smaller subject cohorts, 100 random subject subsets each with exactly N=10 animals were generated, and global and intra-module hub regions were mapped independently for each group. The results show robust conservation of most hub locations across the vast majority of randomly-generated 10-subject groups for global and module hubs (Fig. 2.9). Diversity hubs within the two cortical modules exhibited lower conservation, reflecting intrinsic lower stability and significance levels these integrative locations as reported above.

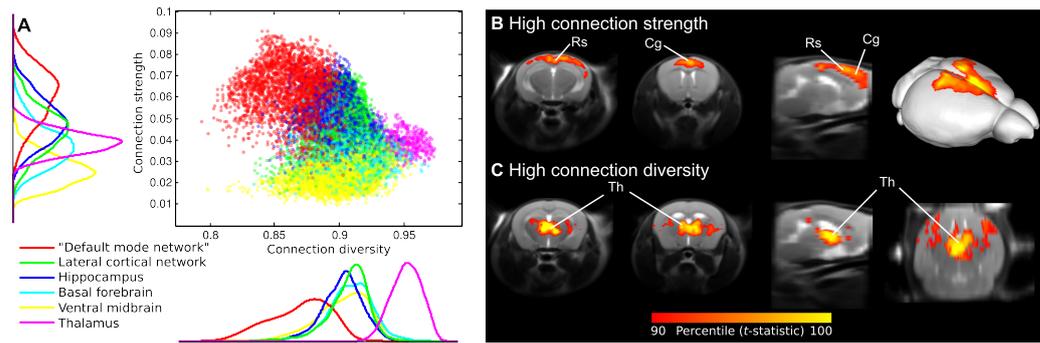


FIGURE 2.5: Global hubs of the mouse brain. (A) Connection diversity and connection strength values are plotted for all nodes in the average functional network. Nodes are colour-coded according to their module. (B) Nodes surviving the top percentage threshold for connection strength are shown on two images in the coronal view (left), one image in the sagittal view (middle), and on a three-dimensional cortical surface rendering. (C) Nodes surviving the top percentage threshold for connection diversity are shown on two images in the coronal view, one image in the sagittal view (left), one image in the sagittal view (middle), and on a three-dimensional cortical surface rendering.

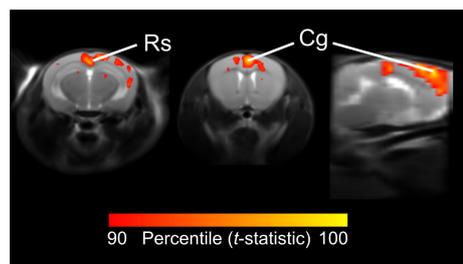


FIGURE 2.6: High-strength nodes in rsfMRI time series after tSNR corruption with pink noise. The final timeseries had tSNR values similar to those observed in deep brain areas furthest to the surface coil array (≈ 25). In spite of this, cingulate and retrosplenial areas emerged as regions with highest global connectivity strength.

2.3.6 The identified hubs are mutually and preferentially interconnected

To assess the presence of mutual inter-module connections between the identified hubs, the anatomical correspondence between the strongest connections of each source hub seed (Fig. 2.10) and the independently determined hub foci in other modules was investigated (Fig. 2.11). For the majority of the candidate hub pairs, the strongest connections of the source hub overlapped with voxels identified above as foci of maximal within module strength or connection diversity. This finding of robust and preferential hub-hub connections suggests that these brain regions act as a tightly interconnected sub-network within the mouse brain (Fig. 2.12A,C), underpinning cross-module integrative functions.

The interconnections between the eight candidate hubs were then characterised directly to better elucidate the module connectivity that they subserved (Fig. 2.12). Many, but not all, of the hub connections were significant, with the cingulate node (DMN module) having the highest number of significant connections (6) to other candidate hubs, and the temporal association cortex node (DMN) exhibiting the statistically strongest connections, namely to the cingulate node (within-module) and to the frontal association cortex node (across-modules, LCN). The ventral subiculum

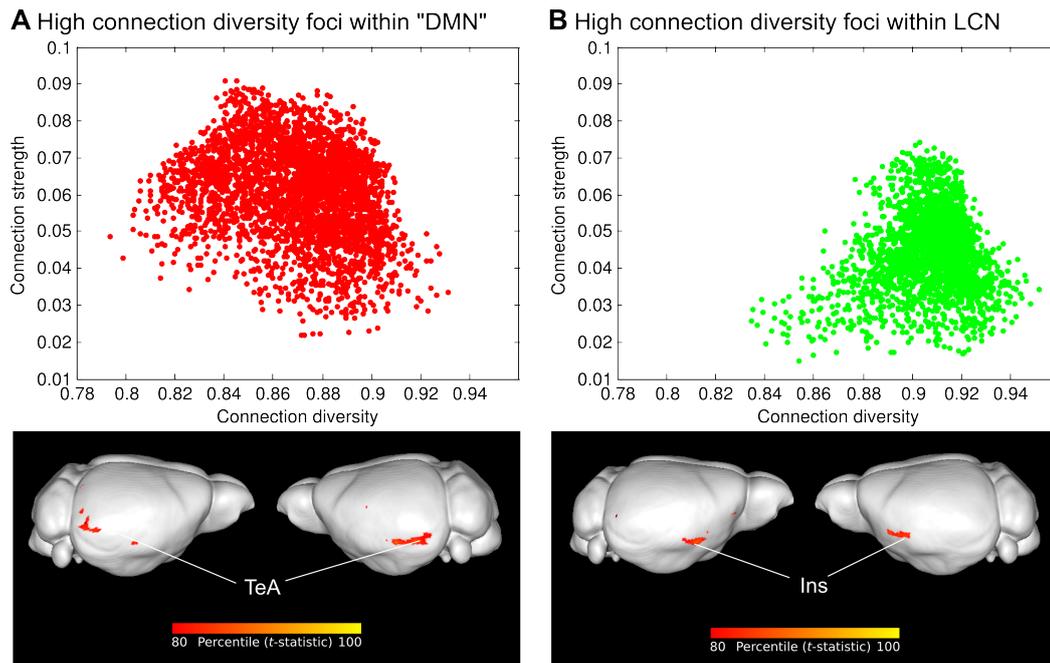


FIGURE 2.7: High connection diversity regions within cortical modules. Connection diversity and strength values (calculated in the average functional network) are plotted for all nodes in the “default mode network” (A) and the lateral cortical network (B). Bottom panels highlight brain nodes surviving the top percentage threshold within each of the two cortical. The nodes are shown as three dimensional renderings on the cortical surface.

node (VM module) had the least number (2) of significant connections to other candidate hubs, to the cingulate cortex and hippocampal nodes (both across-modules, DMN and Hc modules respectively). Notably, both the DMN and LCN modules each featured two putative cortical hubs, highlighting a key contribution of cortical hubs within these circuits (i.e. cingulate, temporal, frontal association, and insular cortices) as prominent integrative nodes of rsfMRI connectivity networks in the mouse brain.

The connectional profiles of candidate hubs attest to the widespread connectivity of hubs both within their own module and across the whole functional network (Fig. 2.12B). Interestingly, a prominent integrative role of the DMN module was apparent, as this region receives the largest share of the connection strength from all hubs (excepting connections within a hub’s own module), although it is only second in size to the ventral midbrain module.

2.4 Discussion

We have demonstrated the presence of distinct functional modules in the mouse brain, and a set of anatomically localised, mutually interconnected candidate hub regions acting as cross-module functional integrators. Our approach provides a fine-grained description of the mouse functional connectome that can serve as a reference and complement ongoing research in the meso- and large-scale connective architecture of this species (Oh et al., 2014; Stafford et al., 2014; Zingg et al., 2014). It also opens the way to targeted manipulations of hub nodes in mouse models of

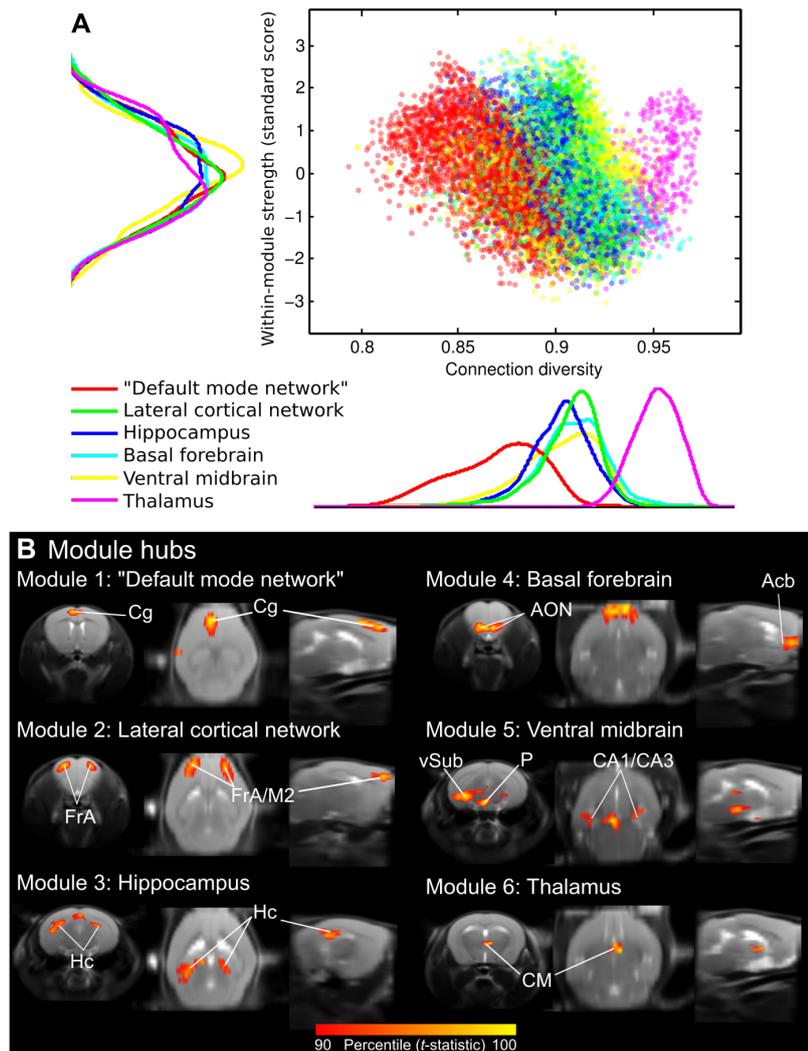


FIGURE 2.8: Module hubs. (A) Connection diversity and normalised (z) scores of within-module strength plotted for all nodes in the average functional network. Nodes are colour-coded according to their module. (B) For each module, nodes surviving the top percentage threshold are shown on images in representative axial, horizontal and sagittal views of the mouse brain.

brain pathology, a line of research that may advance our understanding of the elusive role of functional hub regions in neuropsychiatric states (van den Heuvel and Sporns, 2013). Importantly, we interrogated the mouse connectome at a high, voxel-scale spatial resolution and worked with fully-connected, fully-weighted networks, hence minimising bias induced by parcellation schemes and issues associated with arbitrary thresholding and/or binarisation (Bullmore and Sporns, 2009).

Modular organization is central to functional segregation in the brain, whereby distinct neuronal processing is performed by regions organized in functional modules (Sporns, 2013). Studies of functional modular organization in the human brain have consistently reported the presence of distinct distributed modules corresponding to known functional brain systems, such as the default mode, dorsal attention or somato-motor networks (Meunier et al., 2009; Power, Cohen, et al., 2011; Yeo et al., 2011). In keeping with this, the mouse brain functional networks identified here

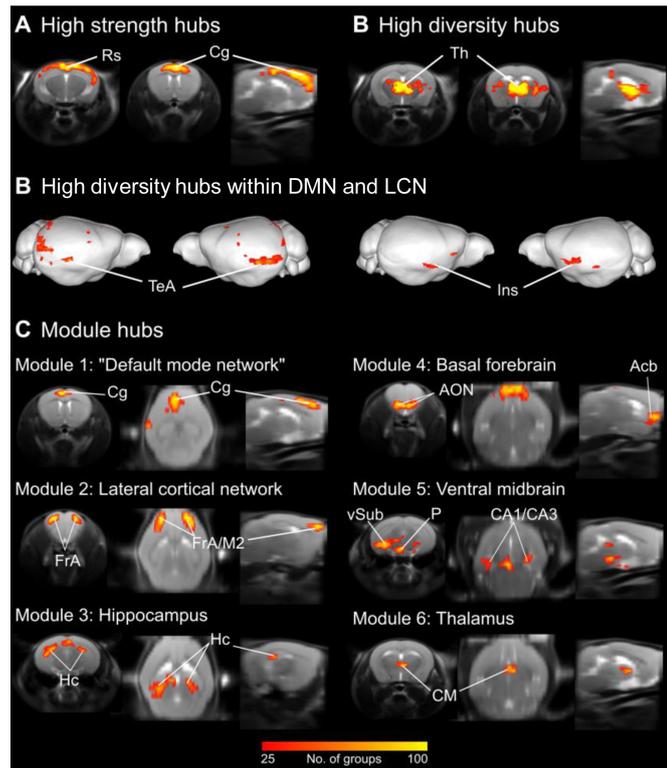


FIGURE 2.9: Reproducibility of functional hubs in random sub-groups of 10 animals. The voxel maps express the number of groups (out of 100 random 10-animal partitions of the original 41-subject cohort) in which a given voxel was identified as belonging to a hub of the given type.

can be reliably related to established large-scale neuro-functional and neuroanatomical systems of the mammal brain. The detection of a DMN module using graph-based approaches is in good agreement with the results of classic (ICA- and seed-based) rsfMRI network mappings in the rodent brain (Schwarz, Natalia Gass, et al., 2013; Schwarz, N. Gass, et al., 2013; Sforazzini, Bertero, et al., 2016; Stafford et al., 2014) and underscores the pivotal role of this integrative network across mammal brain evolution (Lu et al., 2012). Similarly, the presence of a lateral cortical module is in agreement with recent seed-correlation and ICA rsfMRI studies in mice and rats where the presence of a similar DMN-anticorrelated systems has been described (Schwarz, Natalia Gass, et al., 2013; Schwarz, N. Gass, et al., 2013; Sforazzini, Bertero, et al., 2016), thus leading to the hypothesis that such a network could be a precursor of lateralised “task-positive” executive modules present in humans and primates (M. D. Fox, Snyder, et al., 2005). Importantly, the identification of functionally-distinct antero-posterior distributed cortical module components is in excellent agreement with recent cortical connectivity mapping obtained with tracer injections in the mouse cortex. Indeed, by applying graph-based analyses of tracer-based structural connectivity, Zingg et al. (2014) identified two major neocortical clusters (i.e., somatic sensorimotor and medial antero-posterior networks) that exhibit remarkable neuroanatomical overlap with our LCN and DMN modules. Similarly, the same authors also identified two lateral integrative subnetworks in the cortex (anterior insular and posterior temporal) that can be related to the high connection diversity cortical hub nodes identified in the present work. Collectively,

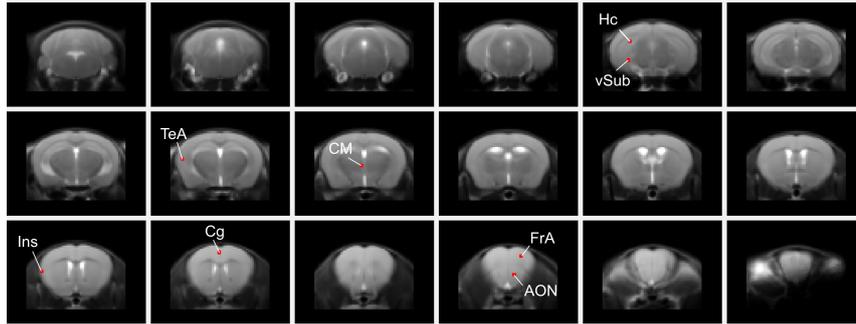


FIGURE 2.10: Locations seeds used for inter-module hub connectivity analysis, each displaying the largest value of the network attribute in question for the given hub regions, overlaid on the anatomical template.

these findings corroborate the emerging view that functional correlations in spontaneous brain activity are constrained and guided by patterns of anatomical connectivity (Honey et al., 2009; Sui et al., 2014), a notion that has been more recently demonstrated also for the mouse brain (Stafford et al., 2014).

The correspondence between our cortical modules and analogous functional networks of the human brain is of high translational relevance, as the approach permits to identify key topological landmarks that can guide cross-species extrapolation of neural circuit research in health and pathology. In this respect, our work represents a significant advance over previous graph-based attempts to unravel the rodent's functional topology (Bifone, Gozzi, and Schwarz, 2010; D'Souza et al., 2014; Liang, King, and N. Zhang, 2011; Liang, King, and N. Zhang, 2012; Schwarz, Gozzi, and Bifone, 2008; Schwarz, Gozzi, and Bifone, 2009). Indeed, while these previous studies identified plausible functional modules, including large cortical partitions (Liang, King, and N. Zhang, 2011) and some subcortical networks similar to those described here (e.g. basal ganglia and hippocampus) (D'Souza et al., 2014; Liang, King, and N. Zhang, 2011), they did not to reveal antero-posterior cortical networks like the rat's DMN module, or the lateral cortical system, a finding that could reflect discrepant experimental procedures as well as heterogeneity in the regional parcellation schemes (coarse ICA-based or anatomical volumes) and network thresholding strategies employed, or the fact that the initial graph-based parcellation used cross-subject analyses of responses to pharmacological stimuli (Bifone, Gozzi, and Schwarz, 2010; Schwarz, Gozzi, and Bifone, 2008; Schwarz, Gozzi, and Bifone, 2009). Likewise, the results of a recent attempt to map functional modules and hubs in the mouse employing ICA-based functional parcellation (Mechling et al., 2014) resulted in a coarse modular organization that includes some of the modules identified in this study (e.g. basal ganglia and hippocampus), as well as a combination of cortical and subcortical structures encompassing multiple neurofunctional systems of the brain (e.g. sensory motor and limbic areas), which corroborate the underlying modular structure of the mouse brain, but cannot be directly related to analogous functional modules of the human brain. The identification of neuro-biologically interpretable functional modules is also key to the identification of candidate hub regions deemed to link and integrate specialized functional systems (Sporns, 2013). Using graph-based methods, numerous studies in humans have converged on a limited set of regions that occupy a central position in the functional topology of the human brain. These regions include anterior and posterior cingulate cortices, the insular cortex, and portions of superior frontal cortex, temporal cortex and lateral parietal cortex

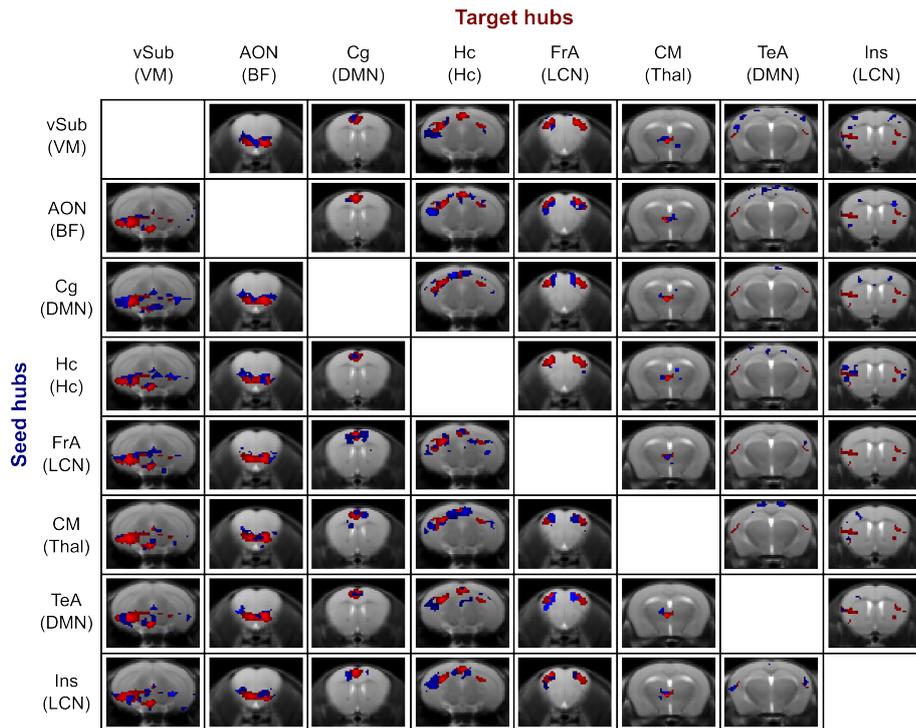


FIGURE 2.11: Functional hubs are mutually interlinked. The strongest connections of each source hub to modules of target hubs (thresholded at 90th percentile for each module, in blue) are overlaid on top of target hub regions (in red). The results are shown on a representative coronal slice for each of the hub-module pair.

(Cole, Pathak, and Schneider, 2010; Sporns, 2014; Tomasi and Volkow, 2011; van den Heuvel and Sporns, 2013). Importantly, the very same regions have also been shown to be implicated in the anatomy of various brain disorders, such as schizophrenia and Alzheimer's disease, which can be investigated and modelled in the mouse (Buckner et al., 2009; Crossley, Mechelli, Scott, et al., 2014). Consistent with human findings (Cole, Pathak, and Schneider, 2010), we identified high strength nodes in the mouse brain located in midline regions within the DMN module, with a predominant involvement of integrative areas such as the prefrontal, anterior and posterior cingulate cortex. Notably, a striking neuroanatomical correspondence also exists between our high connection strength hubs, and high degree structural connectivity hubs of the mouse based axonal tracing (Stafford et al., 2014), a finding that recapitulates a fundamental neuro-architectural feature of the human brain (van den Heuvel and Sporns, 2013). Similarly, high connection diversity regions were identified in the temporal association cortex and, albeit with a lower degree of statistical confidence, also in the anterior insula, two areas classically implicated in multimodal integration (Gogolla et al., 2014). Furthermore, the same areas have been recently described in the human brain as regions of high participation coefficient, a binary network counterpart to connection diversity (Power, Schlaggar, et al., 2013). Importantly, most of the hub regions we identified in the mouse brain exhibit robust and specific mutual inter-connections, a finding which is consistent with an integrative functional role of these nodes, and which argues against a predominant confounding contribution of the correlational nature of rsfMRI-based networks (Power, Schlaggar, et al., 2013). Collectively, these correspondences underscore the translational relevance of

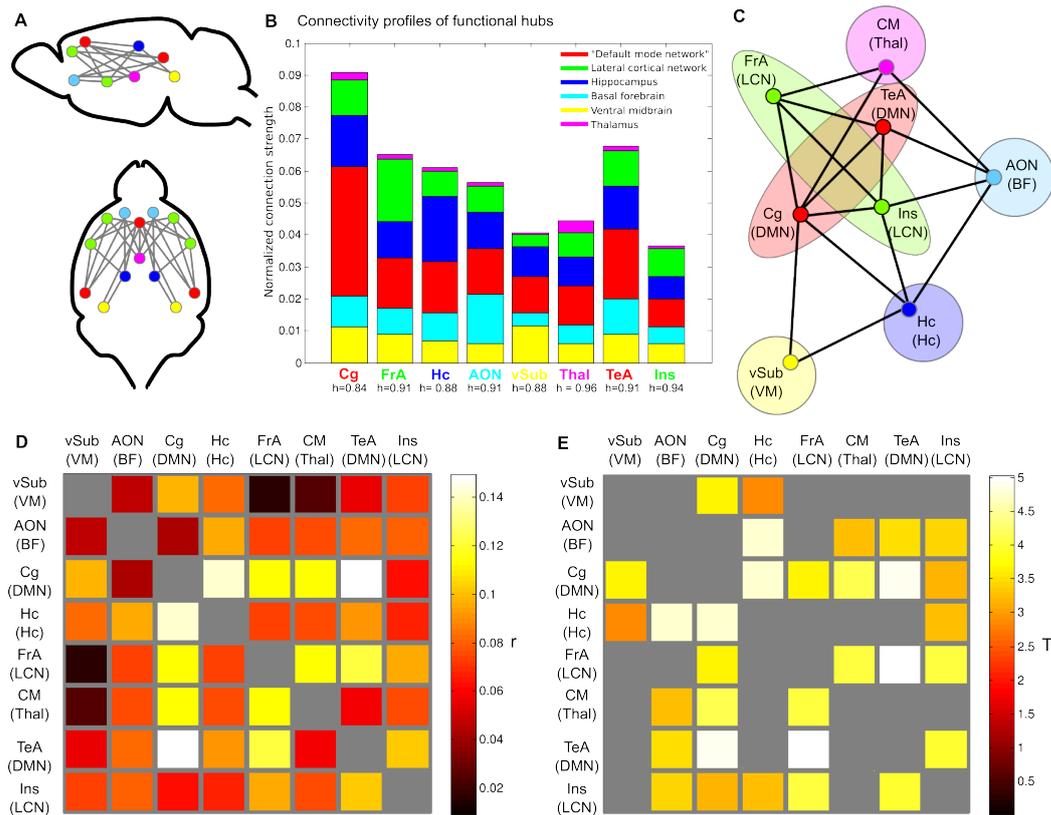


FIGURE 2.12: (A) Approximate locations of candidate hubs of the mouse brain. Connections surviving statistical thresholding are indicated by a link between nodes (B) Connectivity profiles of candidate hubs, showing the proportion of their strength across all modules. (C) Graph representation of the connections surviving statistical thresholding, with node positions determined using the GEM algorithm. (D) Average correlation matrix for all pairs of identified hubs. (E) One sample t-tests for all pairs of identified hubs; non-significant connections (after FDR correction) are shown in grey.

our findings, and support the notion that the mouse brain contains evolutionary-conserved cortical foci serving as integrators of segregated systems in the mammal brain.

The fact that our experiments were performed in anaesthetized animals raises the question as to the degree to which the observed effects reflect the functional architecture of the mouse brain in conscious states. Two recent mouse rsfMRI studies have highlighted different connectivity signatures and reduced inter-hemispheric connectivity as a function of anaesthetic regimen (Grandjean et al., 2014; Jonckers, Delgado y Palacios, et al., 2014). The present work was performed in halothane-anaesthetised animals, a regimen that appears to be particularly suited to map distributed rsfMRI circuits in this species for several reasons. First, halothane ensures motion control and stable hypnosis while preserving cerebral blood flow autoregulation (Gozzi, Ceolin, et al., 2007) and cortical electrical responsiveness (Orth et al., 2006) without the occurrence of burst suppression activity, a phenomenon associated with significant rsFC alterations (Liu et al., 2011). Consistent with this, our recent work (Sforazzini, Schwarz, et al., 2014; Sforazzini, Bertero, et al., 2016; Zhan et al., 2014) demonstrates the presence of (1) robust homotopic inter-hemispheric functional connectivity in both cortical and subcortical areas, and (2) distributed networks remarkably similar to those seen in conscious (and lightly anaesthetised)

rats and primates, anatomically homologous to the human salience network (SN) and default-mode network (DMN) (Hutchison et al., 2010; Lu et al., 2012; Rilling et al., 2007; Schwarz, N. Gass, et al., 2013; Schwarz, Gozzi, Chessa, et al., 2012; Vincent et al., 2007). Importantly, the observation of a DMN-like network in the mouse has been recently replicated by an independent group (Stafford et al., 2014) using a different anaesthetic (isoflurane), a finding that corroborates a neurobiological foundations of this cortical module. Moreover, BOLD fMRI oscillations in the DMN-like network exhibit anti-correlations with neighbouring fronto-parietal areas, a cardinal feature of the human and primate DMN (M. D. Fox, Snyder, et al., 2005). By showing analogous networks using cerebral blood volume weighted signals, we also demonstrated that these spontaneous fluctuations are not significantly contaminated by large blood vessels (Sforazzini, Bertero, et al., 2016). Finally, we recently demonstrated excellent spatial correspondence between rsfMRI signals obtained during light anaesthesia and electrophysiological coherence signals in freely-behaving animals, suggesting that the anaesthetic protocol negligibly influences intrinsic rsfMRI connectivity profiles (Zhan et al., 2014). Collectively, the identified rsfMRI networks exhibit significant correspondence with analogous measurements in awake habituated rats and human studies, thus legitimating the extrapolation of our results to conscious states. Consistent with this notion, global topological features of rsfMRI networks were found to be well maintained in the anesthetized rat brain when compared to awake (restrained) states, despite the use of much higher (2.25-fold) minimal alveolar concentration levels of anaesthetic than the present work (Eger et al., 2003; Liang, King, and N. Zhang, 2012; Sonner, Gong, and Eger, 2000). The remarkable overlap between modules and hubs identified in this work and recent tract tracing mapping in the mouse (Zingg et al., 2014), as well as analogous graph-based mappings in conscious human brain provide further empirical support to a marginal confounding contribution of anaesthesia to our findings.

2.5 Conclusion

In conclusion, our results describe topologically distinct neuro-functional modules of the mouse brain, including a DMN-like module, and identify a set of mutually-interconnected functional hubs that include well-characterised integrative cortical structures. These findings reveal the presence of evolutionarily conserved functional modules and integrative hubs in the mouse brain, and support the use of this species to investigate the elusive neurobiological underpinnings of the functional hub aberrations described for several pathological states. Importantly, our approach also provides a fine-grained description of the mouse functional connectome that complements and integrates ongoing research in the large-scale connective architecture of this species.

Chapter 3

Reduced connectivity in *Cntnap2*-null mice

This chapter has been published as:

Adam Liska, Alice Bertero, Ryszard Gomolka, Mara Sabbioni, Alberto Galbusera, Noemi Barsotti, Stefano Panzeri, Maria Luisa Scattoni, Massimo Pasqualetti, and Alessandro Gozzi (2017). “Homozygous Loss of Autism-Risk Gene CNTNAP2 Results in Reduced Local and Long-Range Prefrontal Functional Connectivity”. In: *Cerebral Cortex*, pp. 1–13. DOI: [10.1093/cercor/bhx022](https://doi.org/10.1093/cercor/bhx022)

3.1 Background

Neuroimaging and post-mortem studies have consistently revealed impaired or atypical connectivity across brain regions of autistic spectrum disorders (ASD) patients (Evdokia Anagnostou and Taylor, 2011). These findings have led to the hypothesis that aberrant connectivity patterns might represent a common final pathway or neurobiological pathogenetic correlate of the autistic phenotype to which different ASD etiologies may converge (Just, Keller, et al., 2012). Although great heterogeneity exists in the sign and distribution of abnormal connectivity across studies and imaging modalities, consistent features indeed appear to emerge, including reduced functional coherence of long-range intra-hemispheric cortico-cortical default mode circuitry, impaired inter-hemispheric regulation and possible increase in local and short-range cortico-subcortical coherence (Rane et al., 2015). However, the neurophysiological underpinnings of these connectional derangements are largely unknown, and a causal etiopathological contribution of specific genetic variants to impaired connectivity in ASD remains to be firmly established.

Mouse lines recapitulating high-confidence ASD mutations (Sanders et al., 2015) have been employed to understand how specific genetic alterations translate into relevant changes in cells and circuits (Auerbach, Osterweil, and Bear, 2011). The recent optimization of neuroimaging readouts of functional connectivity such as resting-state functional MRI (rsfMRI) in the mouse (Sforazzini, Schwarz, et al., 2014) permits to extend this paradigm to the investigation of the elusive genetic and neurobiological foundations of aberrant connectivity observed in ASD (Liska and Gozzi, 2016). The approach leverages on the identification of robust homotopic and distributed rsfMRI connectivity networks in the mouse, including possible homologues of distributed human rsfMRI systems like the salience and default mode (DMN) networks (Gozzi and Schwarz, 2016), and the observation that cyto-architecturally conserved heteromodal cortices in cingulate and retrosplenial regions exhibit similar “hub-like” topological properties in both species (Cole, Pathak, and Schneider, 2010;

Liska, Galbusera, et al., 2015). Importantly, as mouse rsfMRI measurements rest on the same biophysical principles as corresponding human neuroimaging readouts, this approach has the merit of providing a direct translational bridge across species.

Homozygous loss-of-function mutations in Contactin Associated Protein-like 2 (CNTNAP2) encoding CASPR2, a neurexin-related cell-adhesion molecule, are strongly linked to autism and epilepsy in consanguineous families (Strauss et al., 2006; Alarcón et al., 2008; Rodenas-Cuadrado, Ho, and Vernes, 2014). Loss of *Cntnap2* in mice leads to abnormal neuronal migration, reduced GABAergic neurons, spontaneous seizures, and behavioural traits consistent with ASD symptoms in humans (Peñagarikano et al., 2011), an ensemble of traits that phenocopy major neuropathological features observed in cortical dysplasia-focal epilepsy (CDFE) syndrome, a rare neuronal migration disorder associated with a recessive mutation in CNTNAP2 (Strauss et al., 2006). Interestingly, common genetic variants in CNTNAP2 were recently described to be associated with impaired frontal lobe connectivity in humans (Scott-Van Zeeland et al., 2010). However, a causal relationship between ASD-related loss-of-function mutations in CNTNAP2 and functional connectivity remains to be firmly established. Moreover, the role of CNTNAP2 in shaping macroscale circuit assembly, and the specific substrates affected, remain largely unknown.

To address these questions, we used BOLD rsfMRI, diffusion-weighted MRI and retrograde viral tracing to map large-scale functional connectivity and white matter topology in homozygous *Cntnap2*-null mice (*Cntnap2*^{-/-}). We document that loss of *Cntnap2* results in local and long-range connectivity reductions affecting prefrontal regions that act as “functional connectivity hubs” in the mouse brain (Liska, Galbusera, et al., 2015), and that fronto-posterior hypo-connectivity is associated with impaired social behaviour. The presence reduced prefrontal-projecting neuronal frequency in the cingulate cortex of *Cntnap2*^{-/-} mutants suggest a possible contribution of defective mesoscale axonal wiring to the observed functional connectivity impairments. Collectively, these results reveal a role of autism-risk gene CNTNAP2 in modulating functional network assembly between key integrative connectivity hubs of the mammalian brain. The observed long-range prefrontal hypo-connectivity in *Cntnap2*^{-/-} mice recapitulates imaging findings in autism and adds to the construct validity of this mouse line to model ASD-related phenotypes.

3.2 Materials and methods

3.2.1 Ethical statement

All in vivo studies were conducted in accordance with the Italian law (DL 116, 1992 Ministero della Sanità, Roma) and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal research protocols were also reviewed and consented to by the animal care committee of the Istituto Italiano di Tecnologia. The Italian Ministry of Health specifically approved the protocol of this study, authorization no. 07753 to A.G. All surgical procedures were performed under anaesthesia.

3.2.2 Animals

Cntnap2-null (*Cntnap2*^{-/-}) and control “wild-type” (*Cntnap2*^{+/+}) breeding pairs were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and bred locally. Mice were housed by sex in mixed genotype groups, with temperature maintained at $21 \pm 1^\circ\text{C}$ and humidity at $60 \pm 10\%$. All experiments were performed on adult male

mice between 12-16 week of age, corresponding to young maturity. The specific age-range for each experimental activity is reported below. No onset of spontaneous seizures was observed in any of the *Cntnap2* mutants or control mice tested in behavioural, imaging or tracing studies. This is consistent with previous reports showing propensity for spontaneous epileptic episodes in *Cntnap2*^{-/-} to occur only after 6 months of age (Peñagarikano et al., 2011).

3.2.3 Social interaction

For behavioural testing, 12-week-old *Cntnap2*^{-/-} and control *Cntnap2*^{+/+} mice ($n = 13$ each group), were evaluated in the male-female social interaction test during the light phase, as previously described (Scattoni, Ricceri, and Crawley, 2011; Scattoni, Martire, et al., 2013). An unfamiliar stimulus control female mouse in estrous was placed into the home-cage of an isolated test male mouse, and social behaviour were recorded during a 3-min test session. Scoring of social investigation parameters was conducted using Noldus Observer 10XT software (Noldus Information Technology, Leesburg, VA, USA). Social interactions were defined as number of events (frequency) and duration of the following behavioural responses performed by the test mouse: anogenital sniffing (direct contact with the anogenital area), body sniffing (sniffing or snout contact with the flank area), head sniffing (sniffing or snout contact with the head/neck/mouth area), locomotor activity, rearing up against the wall of the home-cage, digging in the bedding, and grooming (self-cleaning, licking any part of its own body). Social investigation is defined as the sum of sniffing and following behaviours (Scattoni, Gandhi, et al., 2008). No observations of mounting, fighting, tail rattling, and wrestling behaviours were observed. Scoring was rated by two investigators blind to genotype. Inter-rater reliability was 98 %. To measure ultrasound vocalization recordings, an ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany) was mounted 20 cm above the cage and the USVs recorded using Avisoft RECORDER software version 3.2. Settings included sampling rate at 250 kHz; format 16 bit. The ultrasonic microphone was sensitive to frequencies between 10 and 180 kHz. For acoustical analysis, recordings were transferred to Avisoft SASLabPro (version 4.40) and a fast Fourier transformation (FFT) was conducted as previously described (Scattoni, Gandhi, et al., 2008). Start times for the video and audio files were synchronized.

3.2.4 Resting-state fMRI

rsfMRI experiments were performed on the same experimental cohorts employed in the behavioural tests ($n = 13$ *Cntnap2*^{+/+}; $n = 13$ *Cntnap2*^{-/-}). At the time of imaging, mice were 13-14 weeks old. The animal preparation protocol was recently described in great detail (Ferrari et al., 2012; Sforazzini, Bertero, et al., 2016). Briefly, mice were anaesthetized with isoflurane (5 % induction), intubated and artificially ventilated (2 % maintenance). The left femoral artery was cannulated for continuous blood pressure monitoring and terminal arterial blood sampling. At the end of surgery, isoflurane was discontinued and substituted with halothane (0.75 %). Functional data acquisition commenced 45 min after isoflurane cessation. Mean arterial blood pressure was recorded throughout imaging sessions. Arterial blood gases (paCO₂ and paO₂) were measured at the end of the functional time series to exclude non-physiological conditions. Mean paCO₂ and paO₂ levels recorded were

17 ± 3 and 250 ± 29 mmHg in *Cntnap2*^{+/+} and 15 ± 3 and 231 ± 38 mmHg in *Cntnap2*^{-/-}. Possible genotype-dependent differences in anaesthesia sensitivity were evaluated using Student's two-sample t-test applied to two independent readouts previously shown to be linearly correlated with anaesthesia depth: mean arterial blood pressure and amplitude of cortical BOLD signal fluctuations (M. A. Steffey, Brosnan, and E. P. Steffey, 2003; Liu et al., 2011; Zhan et al., 2014).

rsfMRI images were acquired with a 7.0 Tesla MRI scanner (Bruker Biospin, Milan) as previously described (Liska, Galbusera, et al., 2015), using a 72 mm birdcage transmit coil and a four-channel solenoid coil for signal reception. For each session, high-resolution anatomical images were acquired with a fast spin echo sequence (repetition time (TR)/echo time (TE) 5500/60 ms, matrix 192×192 , field of view $2 \times 2\text{cm}^2$, 24 coronal slices, slice thickness 0.50 mm). Co-centred single-shot BOLD rsfMRI time series were acquired using an echo planar imaging sequence with the following parameters: TR/TE 1200/15 ms, flip angle 30° , matrix 100×100 , field of view $2 \times 2\text{cm}^2$, 24 coronal slices, slice thickness 0.50 mm, 500 volumes and a total rsfMRI acquisition time of 10 min. Readers can contact the corresponding author for access to the MRI raw data, templates and code employed to generate the functional maps.

3.2.5 Functional connectivity analyses

The first 20 volumes of the rsfMRI data were removed to allow for T1 equilibration effects. The time series were then despiked, corrected for motion and spatially normalized to an in-house mouse brain template (Sforazzini, Schwarz, et al., 2014). The normalised data had a spatial resolution of $0.1042 \times 0.1042 \times 0.5\text{ mm}^3$ ($192 \times 192 \times 24$ matrix). Head motion traces and mean ventricular signal (averaged rsfMRI time course within a reference ventricular mask) were regressed out of each of the time series. No inter-group differences in ventricular volume was observed as measured by the dimension of individual ventricular masks (t-test, $p = 0.31$). All rsfMRI time series were spatially smoothed (full width at half maximum of 0.6 mm) and band-pass filtered to a frequency window of 0.01-0.1 Hz.

To obtain an unbiased identification of the brain regions exhibiting genotype-dependent differences in functional connectivity, we implemented recently developed aggregative metrics for these parameters (Cole, Pathak, and Schneider, 2010; Maximo et al., 2013; Liska, Galbusera, et al., 2015) and calculated local and global connectivity maps for all subjects. This metric considers connectivity of a given voxel to a subset of all other voxels within the brain mask by computing average connectivity strength. Specifically, we employed the weighted connectivity method, in which individual r-scores are first transformed to z-scores using Fisher's r-to-z transform and then averaged to yield the final connectivity score. Local connectivity strength was mapped by limiting this measurement to connections within a 6-voxel radius sphere (0.6252 mm in plane), while long-range connectivity was computed by considering only connections to voxels outside this sphere. The radius employed represents approximately half the thickness of mouse anterior cortex (Dodero et al., 2013) and is a good approximation of the overall average cortical thickness (Braitenberg and Schüz, 1998; Sun and Hevner, 2014). The use of this value ensures that the employed local connectivity metric reflects purely intra-cortical effects at least in outmost cortical voxels and in thicker fronto-cortical regions. This value is proportionally much lower than what is commonly employed in human local connectivity mappings, where values as large as 14 mm (i.e., 4/5-fold mean human cortical thickness) have been employed [reviewed by (Maximo et al., 2013)].

Voxelwise inter-group differences in each of these parameters were mapped using a two-tailed Student's *t*-test ($p < 0.05$ FWE cluster corrected with cluster-defining threshold of $t_{24} > 2.06$, $p < 0.05$, as implemented in FSL). The effect was also quantified in volumes of interest (VOIs). The anatomical location of the examined VOIs is reported in Fig. 3.1. Region identification and naming follow classic neuroanatomical labelling described in (Paxinos and Franklin, 2004). Many of these regions have recently been reclassified according to their cytoarchitectural properties such to match analogous regions in human and primates (Vogt and Paxinos, 2014). According to this scheme, the mouse prelimbic cortex corresponds to Brodmann area 32 (A32), cingulate cortex area 1 (anterior cingulate cortex) to Brodmann area A24b, infralimbic cortex to A24a, retrosplenial cortex to areas A30 and A29. In keeping with this and the comparative work of other authors (Ongür and Price, 2000), in this paper we define the mouse prefrontal cortex (PFC) as an anatomical ensemble of regions including prelimbic, infralimbic and anterior cingulate cortex, corresponding to Brodmann areas A24a/b, A32, and A10.

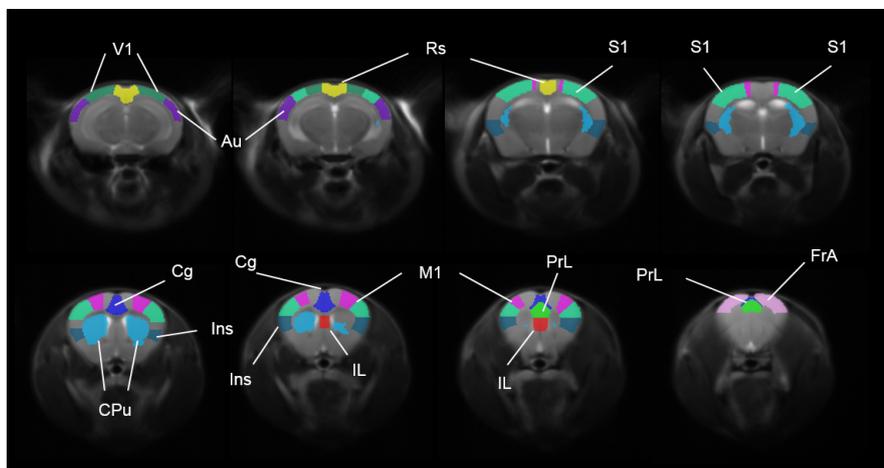


FIGURE 3.1: V1, primary visual cortex; Au, auditory cortex; Rs, retrosplenial cortex; S1, primary somatosensory cortex; Cg, cingulate cortex; CPu, caudate-putamen; Ins, insular cortex; IL, infra-limbic cortex; M1, primary motor cortex; PrL, prelimbic cortex; FrA, frontal association cortex.

Inter-group differences in the extension and intensity of long-range rsfMRI correlation networks were mapped using seed-based approach as previously described (Sforazzini, Bertero, et al., 2016). Small a priori seed regions of $3 \times 3 \times 1$ voxels were chosen to cover antero-posterior cortical networks and representative heteromodal cortical structures (Fig. 3.2). The mean time courses from the unilateral (medial, Rs, PrL) and bilateral seeds (TeA, Pt, and vHC) were used as regressors for each voxel. Group level differences in connectivity distributions were mapped using two-tailed Student's *t*-tests ($p < 0.05$ FWE cluster corrected with cluster-defining threshold of $t_{24} > 2.06$, $p < 0.05$, as implemented in FSL).

Alterations in inter-hemispheric functional connectivity were assessed by computing correlation coefficients of inter-hemispheric VOI pairs depicted in Fig. 3.1. The statistical significance of inter-group correlation strength in each VOI was assessed with a two-tailed Student's *t*-test ($t_{24} > 2.06$, $p < 0.05$) and corrected for multiple comparisons using a false discovery rate $q = 0.05$ according to the Benjamini-Hochberg procedure.

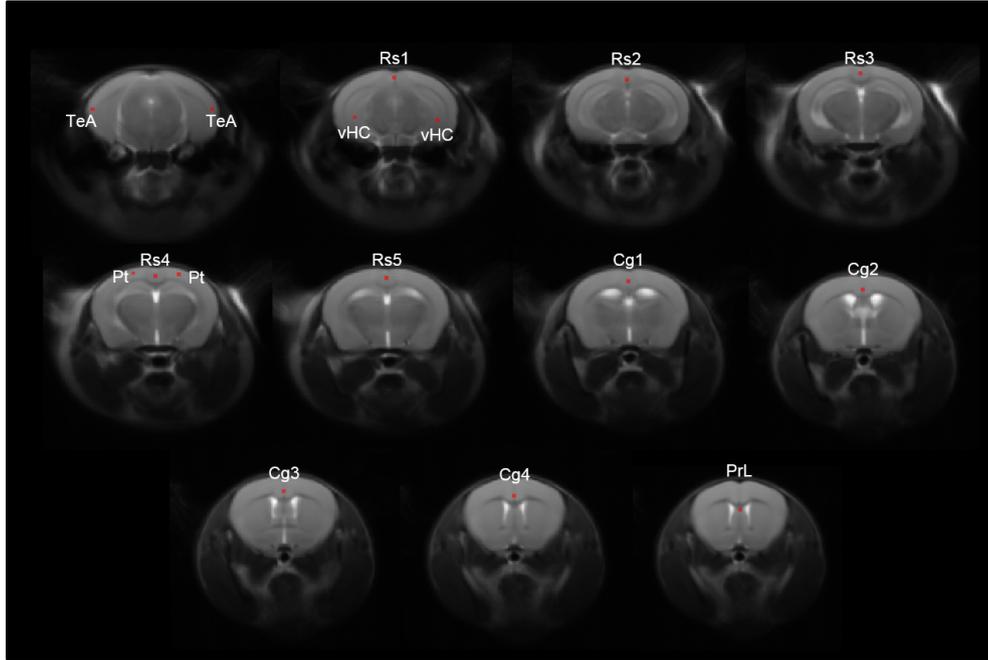


FIGURE 3.2: Location of seeds used in mapping anteroposterior DMN connectivity. TeA, temporal association cortex (bilateral); Rs, retrosplenial cortex; Cg, cingulate cortex; Pt, posterior parietal association cortex (bilateral); PrL, prelimbic cortex; vHC: ventral hippocampus.

Anteroposterior DMN connectivity was mapped by computing seed-to-VOI correlations. Prelimbic and cingulate cortex were employed as prefrontal volumes of interest. The location of seeds employed for mapping are indicated in Fig. 3.2. The statistical significance of inter-group effects was quantified using a two-way repeated-measures ANOVA, where seed location and genotype were used as variables.

3.2.6 Diffusion MRI

Ex vivo diffusion-weighted (DW) MRI was carried out on paraformaldehyde fixed specimens as previously described (dodero2013). At the end of the rsfMRI experiments, mice were transcardially perfused with 4deep isoflurane anaesthesia. Brains were imaged inside intact skulls to avoid post-extraction deformations. Each DW dataset was composed of 8 non-diffusion-weighted images and 81 different diffusion gradient-encoding directions with $b = 3000 \text{ s/mm}^2$ ($\delta = 6 \text{ ms}$, $\Delta = 13 \text{ ms}$) acquired using an EPI sequence with the following parameters: TR/TE = 13500/27.6 ms, field of view $1.68 \times 1.54 \text{ cm}^2$, matrix 120×110 , in-plane spatial resolution $140 \times 140 \mu\text{m}^2$, 54 coronal slices, slice thickness 280 μm , number of averages 20. Three mice were discarded from the analyses owing to the presence of large susceptibility distortions in the DW images due to the presence of air bubbles following imperfect perfusion procedure. As a result of this, the final number of subjects per group was $n = 13$ and $n = 10$, for *Cntnap2*^{+/+} and *Cntnap2*^{-/-}, respectively.

3.2.7 White-matter fibre tractography

The DW datasets were first corrected for eddy current distortions (FSL/eddy_correct) and skull-stripped ((Oguz et al., 2014)). The resulting individual brain masks were

manually corrected using ITK-SNAP ((Yushkevich et al., 2006)). Whole brain tractography was performed using MRtrix3 (J-Donald Tournier, Calamante, and Connelly, 2012) using constrained spherical deconvolution [$l_{\max} = 8$, (J.-Donald Tournier, Calamante, and Connelly, 2007)] and probabilistic tracking (iFOD2) with a FOD amplitude cut-off of 0.2. For each dataset, the whole brain mask was used as a seed, and a total of 100,000 streamlines were generated.

The corpus callosum and cingulum were selected as tracts of interest, given their major cortico-cortical extension and direct involvement in prefrontal-posterior connectivity (Vogt and Paxinos, 2014). The tracts were virtually dissected with waypoint VOIs described in Fig. 3.3 using TrackVis (<http://www.trackvis.org/>). Inter-group differences in streamline counts of the tracts were evaluated using a two-tailed Student's t-test ($t_{21} > 2.08$, $p < 0.05$). To provide a visual assessment of fibre distribution across groups, voxelwise parametric fibre density maps were generated using DiPy (Garyfallidis et al., 2014), by determining for each voxel the number of subjects in which at least one streamline of the fibre tract of interest passes through the voxel. For visualization purposes, both the dissected tracts and group fibre density maps were transformed to the Allen Mouse Common Coordinate Framework, Version 3 (<http://www.brain-map.org/>).

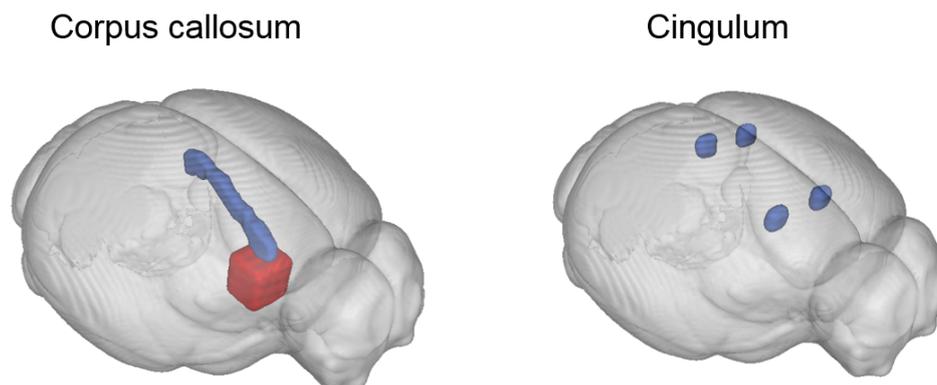


FIGURE 3.3: Location of waypoint ROIs used for virtual dissection of corpus callosum and cingulum tracts from whole-brain white matter tractography. Inclusion ROIs are indicated in blue, exclusion ROIs are indicated in red.

3.2.8 Rabies virus production and injection

Unpseudotyped recombinant SAD Δ G-mCherry rabies virus (RV) was produced as described by Osakada and Callaway (2013). Briefly, B7GG packaging cells, which express the rabies envelope G protein, were infected with unpseudotyped SAD Δ G-mCherry-RV, obtained by courtesy of Prof. Edward Callaway from the Salk Institute. After five to six days, the virus was collected, filtrated with $0.45 \mu\text{m}$ filter and concentrated by two rounds of ultracentrifugation. The titer of the SAD Δ G-mCherry-RV preparation was established by infecting Hek-293T cells (ATCC cat no. CRL-11268) with tenfold serial dilution of viral stock, counting mCherry expressing cells 3 days after infection. The titer was calculated as 2×10^{11} Infective Units (IU)/ml, and the stock was therefore considered suitable for in vivo microinjection. Intracortical rabies virus injections were carried out as previously described (Sforazzini, Bertero,

et al., 2016) in adult (12-16 week-old) male *Cntnap2*^{-/-} and control *Cntnap2*^{+/+} littermates (n = 6, each group). To this purpose, mice were deeply anesthetized with avertin (250 mg/kg) and firmly stabilized on a stereotaxic apparatus (Stoelting Inc.). A micro drill (Cellpoint Scientific Inc.) was used to drill holes through the skull. Injections were performed with a Nanofil syringe mounted on an UltraMicroPump UMP3 with a four channel Micro4 controller (World Precision Instruments), at a speed of 5 nl/s, followed by a 5–10 minutes waiting period, to avoid backflow of viral solution and unspecific labelling. One μ l of viral stock solution was injected unilaterally in the left anterior prefrontal cortex using the following coordinates for injections, expressed in mm from bregma: 1.42 from anterior to posterior, 0.3 lateral, -1.6 deep (Paxinos and Franklin, 2004)

3.2.9 Quantification of retrogradely labelled cells

RV-labelled cell quantification and histological analyses were carried out by an operator (A.B.) blind to genotype. After 7 days from viral injection, the animals were transcardially perfused with 4 % paraformaldehyde (PFA), brains were dissected, post-fixed over night at 4°C and vibratome-cut (Leica Microsystems). RV-infected cells were detected by means of immunohistochemistry performed on every other 100 μ m thick coronal section, using rabbit anti-red fluorescent protein (RFP) primary antibody (1:500, AbCam), and goat anti-rabbit HRP secondary antibody (1:500, Jackson ImmunoResearch), followed by 3-3' diaminobenzidine tetrahydrochloride (DAB, Sigma Aldrich) staining. Imaging was performed with MacroFluo microscope (Leica). Each picture was then superimposed onto the corresponding Paxinos Atlas table (Paxinos and Franklin, 2004), and cell bodies were plotted according to their anatomical localization. The cells were then assigned to their corresponding brain regions, and final region-based cell population counts were expressed as fraction of the total amount of labelled cells.

3.2.10 Histological and immunohistochemical analysis of white matter

To histologically assess the presence of microstructural white matter alterations, we examined immunofluorescence-enhanced coronal brain sections covering anterior callosal regions from adult (12 week-old) male *Cntnap2*^{-/-} and control *Cntnap2*^{+/+} littermates (n = 5, each group) after incubation with rat anti-myelin basic protein (MBP) primary antibody (1:1000, AbCam), followed by donkey anti-rat 594 secondary antibody (1:500, Thermo scientific). We also quantified MBP levels as previously described (Mottershead et al., 2003; Richetto et al., 2017). Briefly, three representative random images in anterior callosal regions characterized by parallel or transversal fiber extension with respect to the image plane (corpus callosum and forceps minor of the corpus callosum, respectively) were acquired on a Nikon A1 confocal system, equipped with 561 laser diode and appropriate filter for Texas Red fluorophore. Z-stack images (1.5 μ m thick) were acquired using an oil-immersion 60x plan-apochromat objective at 1024x1024 pixel resolution. Callosal image fields were also qualitatively inspected for the presence of inter-group differences in white matter organization or reduced neuronal packing/density. MBP content was empirically quantified by summing MBP-immunoreactive areas expressed as number of pixels whose values were above the background threshold, calculated as pixel intensity values in areas with no detectable immunostaining, such as cell nuclei or MBP-devoid background.

3.3 Results

3.3.1 Reduced local and long-range connectivity in fronto-cortical regions of *Cntnap2*^{-/-} mice

To obtain an unbiased mapping of genotype-dependent differences in functional connectivity, we implemented recently developed aggregative metrics for local and long-range functional connectivity. This analysis revealed foci of significantly reduced local and long-range connectivity in *Cntnap2*^{-/-} mutants with respect to wild-type control subjects (t-test, $p < 0.05$ FWE cluster-corrected, with cluster-defining threshold of $t_{24} > 2.06$, $p < 0.05$; Fig. 3.4) encompassing prefrontal (prelimbic and cingulate) and retrosplenial cortices. These same brain regions have been classified both in mice and in humans as “high strength” functional connectivity hubs (Buckner et al., 2009; Cole, Pathak, and Schneider, 2010; Liska, Galbusera, et al., 2015), and as such are thought to play a key integrative role in distributed functional networks. Local connectivity reductions appeared to be more widespread than corresponding long-range connectivity deficits (Fig. 3.4a, c), encompassing involvement of supplementary motor areas surrounding cingulate cortex. The observed local and long-range connectivity reductions were statistically significant also when integrated over a large volume of interest encompassing the whole cingulate cortex (local connectivity: Cg, t-test, $t_{24} = 3.11$, $p = 0.005$, Fig. 3.4b; long-range connectivity: Cg, t-test, $t_{24} = 2.26$, $p = 0.03$, Fig. 3.4d).

3.3.2 Long-range connectivity impairments in *Cntnap2*^{-/-} mice affect heteromodal cortical regions and the DMN

To identify regional targets of the observed long-range connectivity deficits, we probed rsfMRI networks previously shown to involve prefrontal, cingulate and retrosplenial regions (Sforazzini, Schwarz, et al., 2014; Gozzi and Schwarz, 2016). Seed-based mapping of retrosplenial and anterior cingulate/prelimbic cortex highlighted foci of reciprocal long-range hypoconnectivity along the midline brain axis in *Cntnap2*^{-/-} mutants (t-test, $p < 0.05$ FWE cluster-corrected, with cluster-defining threshold of $t_{24} > 2.06$, $p < 0.05$; Fig. 3.5a, b). We also probed connectivity of putative lateral components of the rodent DMN such as the posterior parietal and temporal association/auditory cortices, and postero-ventral hippocampus (Gozzi and Schwarz, 2016). Parietal cortical mapping revealed foci of reduced local and long-range (middle cingulate) connectivity in *Cntnap2*^{-/-} mice (t-test, $p < 0.05$ FWE cluster-corrected, with cluster-defining threshold of $t_{24} > 2.06$, $p < 0.05$; Fig. 3.5c). In the same animals, temporal association areas appeared to be widely hypo-connected to retrosplenial, cingulate and prefrontal regions (t-test, $p < 0.05$ FWE cluster-corrected, with cluster-defining threshold of $t_{24} > 2.06$, $p < 0.05$; Fig. 3.5d). We also observed foci of long-range hypo-connectivity between ventral hippocampal and ventral prefrontal (infralimbic) regions (t-test, $p < 0.05$ FWE cluster-corrected, with cluster-defining threshold of $t_{24} > 2.06$, $p < 0.05$; Fig. 3.5e). Inter-hemispheric connectivity in subcortical or motor-sensory networks appeared to be overall largely preserved. A reduction in inter-hemispheric connectivity was observed in primary motor areas and visual cortex when quantified in anatomical volumes of interest (Fig. 3.6), although the effect did not survive false discovery rate correction.

Importantly, no genotype-dependent differences in anaesthesia sensitivity were detected as seen with mean arterial blood pressure mapping (t-test, $t_{24} = 0.17$, $p = 0.87$; Fig. 3.7a) and amplitude of cortical BOLD signal fluctuations (t-test, $t_{24} =$

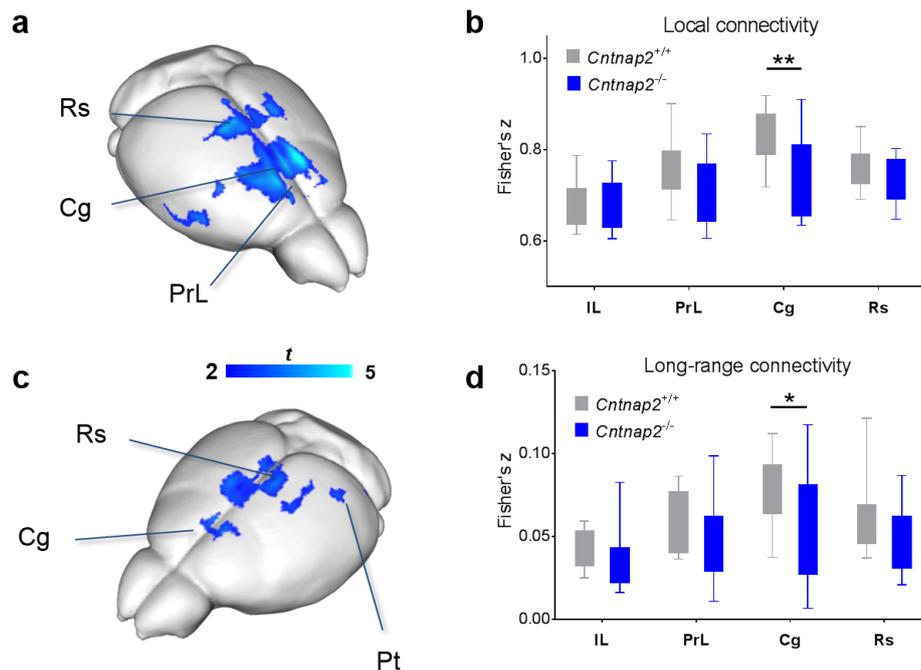


FIGURE 3.4: Reduced local and long-range connectivity in *Cntnap2*^{-/-} mutants. (a) Foci of reduced local connectivity in *Cntnap2*^{-/-} vs. control *Cntnap2*^{+/+} littermates (t-test; $t_{24} > 2.06$, $p < 0.05$; cluster corrected with cluster-level $p < 0.05$). (b) Mean local connectivity in regions of interest (t-test; Cg: $t_{24} = 3.11$, $p = 0.005$). (c) Foci of reduced long-range connectivity in *Cntnap2*^{-/-} vs. control *Cntnap2*^{+/+} littermates (t-test; $p < 0.05$ FWE cluster-corrected, with cluster-defining threshold of $t_{24} > 2.06$, $p < 0.05$). (d) Mean long-range connectivity in regions of interest (t-test; Cg: $t_{24} = 2.26$, $p = 0.03$). IL, infra-limbic cortex; PrL, prelimbic cortex; Cg, cingulate cortex; Rs, retrosplenial cortex, * $p < 0.05$, ** $p < 0.01$.

0.72, $p = 0.48$; Fig. 3.7b), two independent readouts previously shown to be linearly correlated with anaesthesia depth (M. A. Steffey, Brosnan, and E. P. Steffey, 2003; Liu et al., 2011). Together with the observation of region-dependent alterations, as opposed to the global reduction described with increased anaesthesia dosing (Nasrallah, Tay, and Chuang, 2014), these findings strongly argue against a confounding contribution of anaesthesia to the observed hypo-connectivity.

3.3.3 Hypoconnectivity in the mouse DMN is associated with impaired social behaviour

Recent human imaging studies in socially-impaired patients have revealed a putative association between long-range DMN hypo-connectivity and social competence (Schreiner et al., 2014). Based on these findings, we hypothesized that reduced long-range DMN connectivity in *Cntnap2*^{-/-} mice could be associated with impaired social behaviour. To test this hypothesis, we first corroborated DMN hypoconnectivity by quantifying functional connectivity along the dorsal midline axis of this network (anterior/middle cingulate cortex and retrosplenial cortex) using multiple seed-to-VOI measurements (Fig. 3.8). A clear dysconnection between posterior (retrosplenial) and middle/anterior portions of the DMN (cingulate, prelimbic cortex) was apparent (retrosplenial to cingulate cortex: two-way repeated-measures ANOVA,

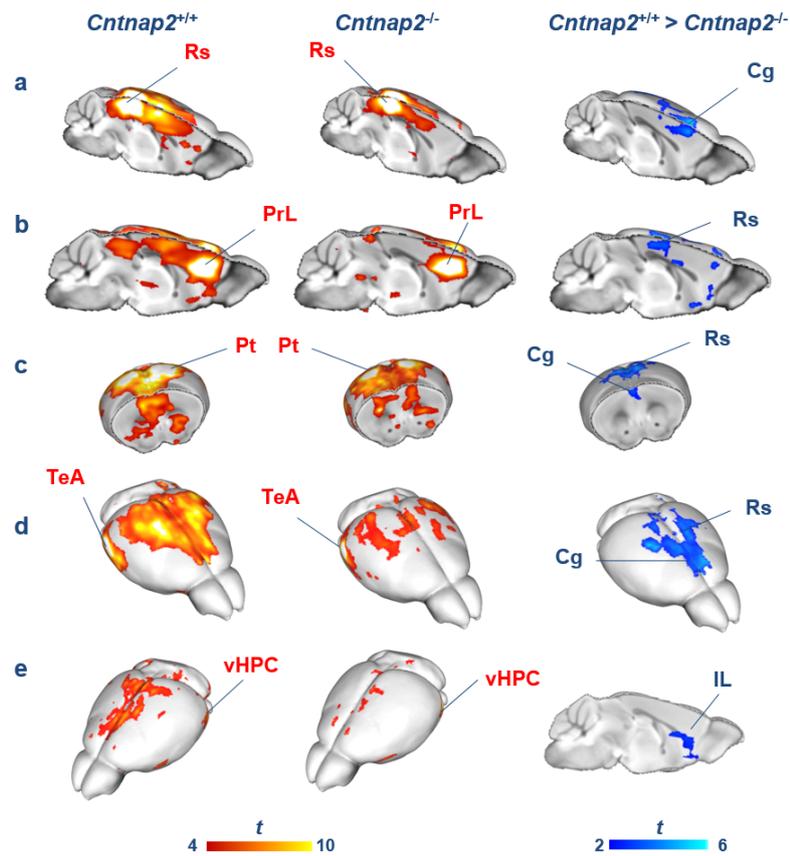


FIGURE 3.5: Reduced long-range connectivity in *Cntnap2*^{-/-} mice. (a-e) Seed-correlation mapping highlighted convergent reduced connectivity between long-range cortical and subcortical regions and cingulate-prefrontal areas. Red/yellow shows areas with significant correlation with seed regions indicated in red (one-sample t-test, $p < 0.05$ FWE cluster corrected with cluster-defining threshold of $t_{12} > 2.18$, $p < 0.05$). Blue indicates foci of reduced connectivity in *Cntnap2*^{-/-} mutants with respect to control mice (t-test, $p < 0.05$ FWE cluster corrected with cluster-defining threshold of $t_{24} > 2.06$, $p < 0.05$). Rs, retrosplenial cortex; IL, infra-limbic cortex; PrL, prelimbic cortex; Cg, cingulate cortex; Rs, retrosplenial cortex; vHPC, ventral hippocampus; Au/TeA, auditory/temporal association cortices; Pt, parietal cortex.

genotype effect, $F_{1,24} = 5.76$, $p = 0.02$, Fig. 3.8a; retrosplenial-cingulate to prelimbic: two-way repeated-measures ANOVA, genotype effect, $F_{1,24} = 6.82$, $p = 0.02$, Fig. 3.8b).

We then measured social behaviour in adult *Cntnap2*^{-/-} and *Cntnap2*^{+/+} control mice in a male-female interaction test, and correlated the measured social scores with DMN hypoconnectivity measures. Consistent with previous reports (Peñagarikano et al., 2011), behavioural testing revealed significantly impaired social interest (total sniffing, duration: t-test, $t_{24} = 2.29$, $p = 0.03$, Fig. 3.9a; social investigation, duration: t-test, $t_{24} = 2.43$, $p = 0.02$, Fig. 3.9c) and increased non-social behaviour (wall-rearing, frequency: t-test, $t_{24} = 3.09$, $p = 0.01$; Fig. 3.10a) in *Cntnap2*^{-/-} mutants compared to *Cntnap2*^{+/+} control littermates. Hypo-connectivity in key DMN components (retrosplenial-cingulate cortex) was significantly associated with reduced social behaviour (total sniffing, duration: $r = 0.42$, $p = 0.03$, $n = 26$, $R^2 = 0.17$, Fig. 3.9b;

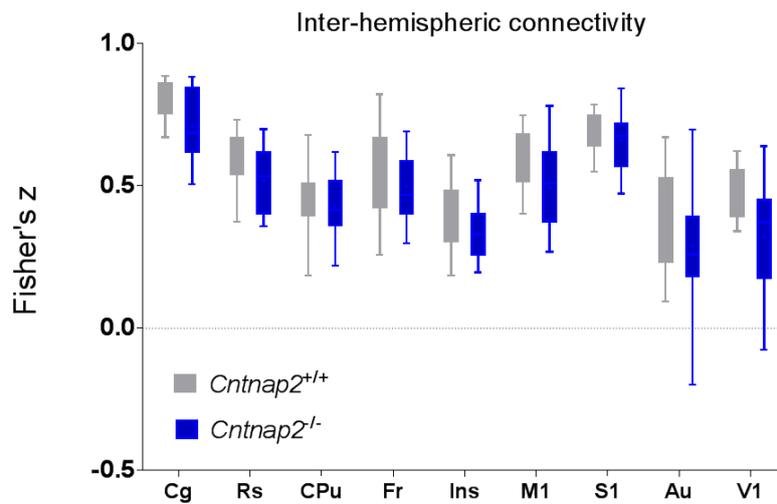


FIGURE 3.6: Largely preserved inter-hemispheric connectivity in *Cntnap2*^{-/-} mutants and control mice. Correlation coefficients were calculated between time courses extracted from VOIs depicted in Fig. 3.1 and the resulting r-scores were transformed to z-scores using Fisher's r-to-z transform. None of these comparisons survived a false discovery rate correction at $q = 0.05$.

social investigation, duration: $r = 0.40$, $p = 0.04$, $n = 26$, $R^2 = 0.16$, Fig. 3.9d) and increased non-social behaviour (wall rearing, frequency: $r = -0.45$, $p = 0.02$, $n = 26$, $R^2 = 0.21$; Fig. 3.10b). These findings highlight a correlation between fronto-posterior connectivity and social behaviour, suggesting that impaired functional couplings produced by mutations in *Cntnap2* could reverberate to affect complex behavioural traits such as sociability and social exploration.

3.3.4 Macroscale cortico-cortical white matter connectivity is preserved in *Cntnap2*^{-/-} mice

To probe a role of macroscale anatomical connectivity alterations on the observed functional decoupling in *Cntnap2*^{-/-}, we performed tractography analysis of the corpus callosum and cingulum, two major white matter tracts characterised by extensive cortico-cortical antero-posterior extension (Fig. 3.11a). These white matter tracts appeared to be largely typical in mutant and control mice as seen with group-level fibre density maps (Fig. 3.11b); in keeping with this, we did not observe statistically significant differences in the number of streamlines between *Cntnap2*^{-/-} mutants and controls (cingulum: t-test, $t_{21} = 1.25$, $p = 0.23$; corpus callosum: t-test, $t_{21} = 1.21$, $p = 0.24$; Fig. 3.12). These results argue against a contribution of gross macroscale white matter alterations to the observed functional connectivity impairments.

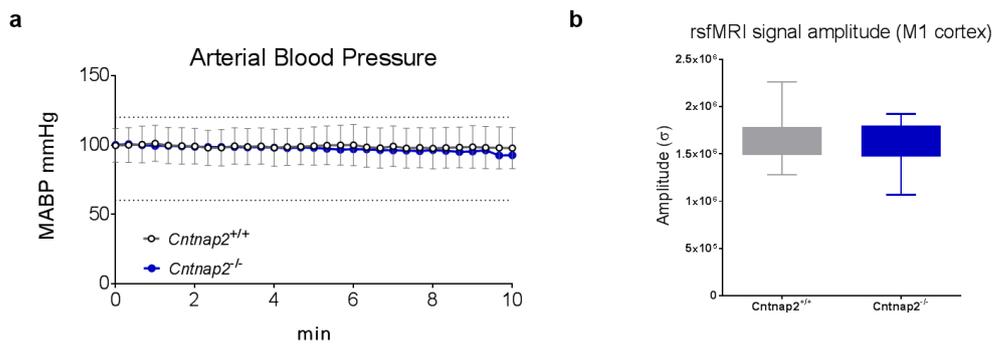


FIGURE 3.7: No genotype-dependent differences in anaesthesia sensitivity were detected as seen with mean arterial blood pressure mapping (t-test, $t_{24} = 0.17$, $p = 0.87$; a) and amplitude of cortical BOLD signal fluctuations in primary motor cortex (t-test, $t_{24} = 0.72$, $p = 0.48$; b). M1, primary motor cortex.

3.3.5 Reduced prefrontal-projecting neuronal clusters in cingulate cortex of *Cntnap2*^{-/-} mice

Although macroscale cortico-cortical connectivity appeared to be normal in *Cntnap2*^{-/-} mutants, the possibility exists that finer-scale miswiring, undetectable by tractography, may contribute to the mapped functional connectivity alterations. To probe this hypothesis, we carried out monosynaptic retrograde tracing of the left prefrontal cortex [prelimbic/anterior cingulate cortex area 1, corresponding to Brodmann area 24Ab, (Vogt and Paxinos, 2014)] and quantified the number of retrogradely labelled cells in representative volumes of interest in mutant and control littermate mice (Fig. 3.13a). The anatomical distribution of retrogradely labelled neurons in both genotypes was in keeping with previously published rodent studies (Hoover and Vertes, 2007) and encompassed several key anatomical substrates considered to be part of the rodent DMN (Gozzi and Schwarz, 2016). Notably, regional quantification of the relative fraction of labelled cells revealed reduced frequency of prefrontal-projecting neurons in the cingulate cortex of *Cntnap2*^{-/-} mutants (Cg: t-test, $t_{10} = 3.90$, $p = 0.003$, FDR-corrected $p = 0.04$; Fig. 3.13b, c). Importantly, no genotype-dependent significant difference in the number of prefrontal projecting neurons was observed in any of the other cortical or subcortical regions examined (Fig. 3.13c).

3.3.6 Preserved microscale white matter organization in *Cntnap2*^{-/-} mice

We next examined the presence of microscale white matter structural abnormalities in control and *Cntnap2*^{-/-} mutants via histological examinations and myelin binding protein (MBP) quantification. In keeping with previous investigations (Poliak et al., 2003; Peñagarikano et al., 2011), we did not observe gross microscale white matter disorganization or morphological changes in mice lacking *Cntnap2* with respect to control littermates (Fig. 3.14a). Similarly, MBP quantification in frontal callosal white matter tracts did not reveal any significant between-group difference (corpus callosum: t-test, $t_8 = 0.84$, $p = 0.42$; forceps minor of the corpus callosum: t-test, $t_8 = 1.06$, $p = 0.32$; Fig. 3.14b).

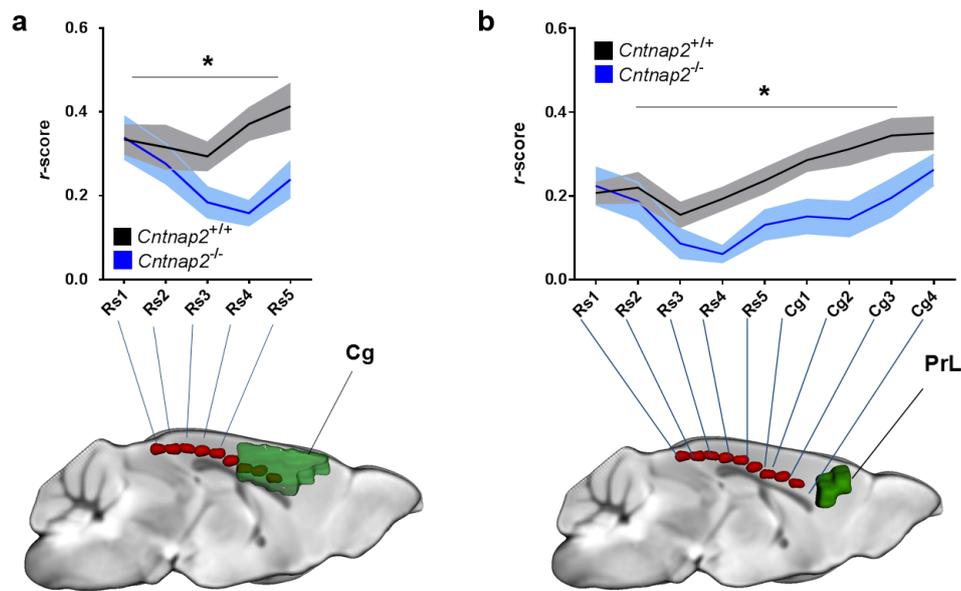


FIGURE 3.8: Fronto-posterior hypoconnectivity in *Cntnap2*^{-/-} mice. (a) Connectivity profile between a series of retrosplenial seeds (Rs, red) and the cingulate cortex (Cg, green) (two-way repeated-measures ANOVA, genotype effect, $F_{1,24} = 5.76$, $p = 0.02$). (b) Connectivity profile between a series of retrosplenial/cingulate seeds (Rs, Cg, red) and the prelimbic cortex (PrL, green) (two-way repeated-measures ANOVA, genotype effect, $F_{1,24} = 6.82$, $p = 0.02$). * $p < 0.05$.

3.4 Discussion

Here we show that homozygous mice lacking *Cntnap2*, a neurexin superfamily member associated with autism, exhibit reduced long-range and local functional connectivity in prefrontal cortical regions and midline functional hubs of the mouse brain, an effect that may involve defective cingulate-prefrontal mesoscale wiring. We also show that reduced fronto-posterior connectivity is associated with impaired social behaviour, revealing a possible link between long-range functional connectivity alterations and mouse behavioural traits recapitulating ASD symptoms. Collectively, these findings suggest that loss-of-function mutations in *Cntnap2* may predispose to neurodevelopmental disorders and autism through dysregulation of macroscale functional network couplings.

Our use of an imaging readout widely employed in human connectivity mapping provides us with the opportunity to cross-compare connectivity findings across species. In this respect, the observation of long-range fronto-posterior hypo-connectivity in *Cntnap2*^{-/-} mice is especially noteworthy, because it is in excellent agreement with the results of a recent human imaging study where an association between common genetic variants in CNTNAP2 and similar long-range frontal hypoconnectivity was described (Scott-Van Zeeland et al., 2010). Our results expand these findings, by revealing a causal contribution of *Cntnap2* loss-of-function mutations to long-range fronto-cortical connectivity impairments. These correspondences also serve as an important proof-of-concept demonstration that ASD-related genetic mutations can lead to comparable macroscale connectivity deficits in humans and lower

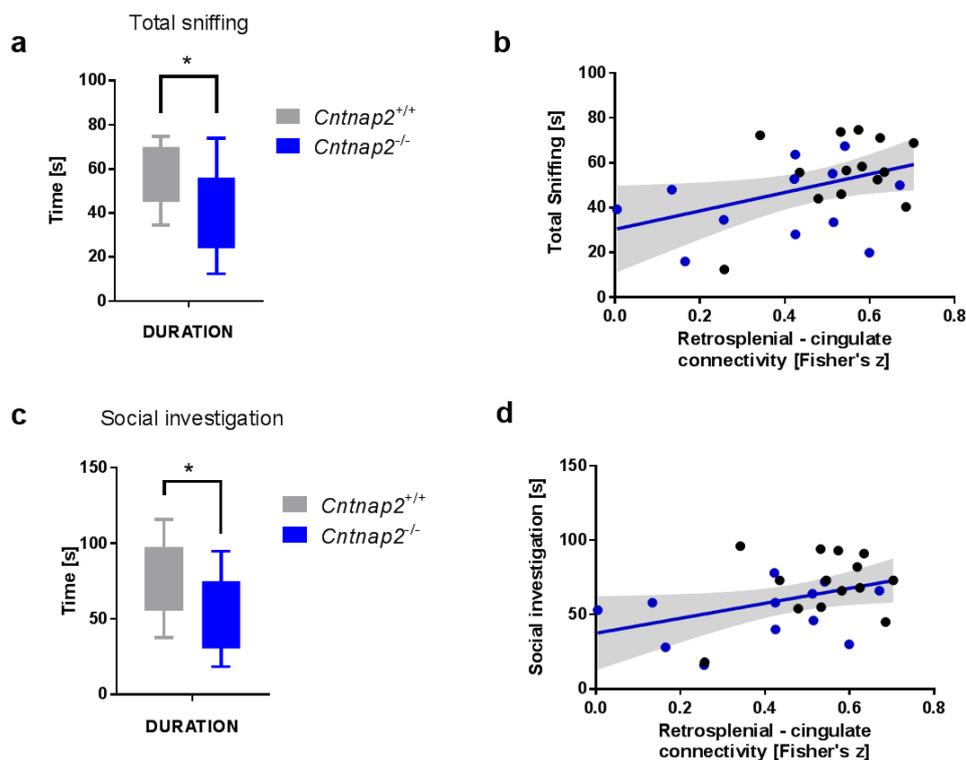


FIGURE 3.9: Fronto-posterior connectivity is correlated with social behaviour. (a) Social behaviour as measured by total sniffing duration (t-test, $t_{24} = 2.29$, $p = 0.03$). (b) Association between retrosplenial-cingulate connectivity (VOI to VOI) and total sniffing duration ($r = 0.42$, $p = 0.03$, $n = 26$, $R^2 = 0.17$). (c) Social behaviour as measured by the duration of social investigation (t-test, $t_{24} = 2.43$, $p = 0.02$). (d) Association between retrosplenial-cingulate connectivity (VOI-to-VOI) and the duration of social investigation ($r = 0.40$, $p = 0.04$, $n = 26$, $R^2 = 0.16$). * $p < 0.05$.

mammal species like the laboratory mouse. The presence of long-range hypoconnectivity in *Cntnap2*^{-/-} mice also adds to the remarkable construct and face validity of this mouse model as an experimental tool for mechanistic and therapeutic investigation of syndromic forms of ASD (Peñagarikano et al., 2011). Specifically, *Cntnap2*^{-/-} mice closely recapitulate major neuropathological features observed in cortical dysplasia-focal epilepsy (CDFE) syndrome, a rare neuronal migration disorder associated with a recessive (suggesting loss of function) mutation in CNTNAP2, and, in nearly two thirds of patients, with autism (Strauss et al., 2006). These include behavioural deficits in the three core domains of ASD (reduced vocal communication, repetitive and restricted behaviours, and abnormal social interactions), hyperactivity and epileptic seizures (both features described in CDFE patients), and reduced GABAergic interneurons, resulting in asynchronous cortical activity as measured with in vivo two-photon calcium imaging (Peñagarikano et al., 2011).

The observation of defective mesoscale axonal wiring in the cingulate cortex corroborates the presence of selective prefrontal dysregulation in *Cntnap2*^{-/-} mutants, and serves as a possible neuroanatomical correlate for some of the prefrontal functional connectivity impairments mapped with rsfMRI. Regional differences in GABAergic interneuron density, and developmental processes related to circuit and

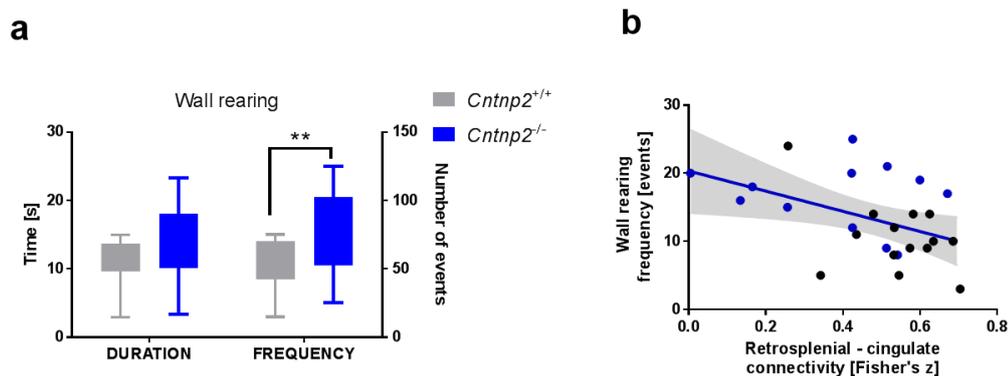


FIGURE 3.10: Increased non-social behaviour in *Cntnap2*^{-/-} mutants compared to control littermates. (a) Non-social behaviour as measured by the duration and frequency of rearing up against the wall of the home-cage (frequency: t-test, $t_{24} = 3.09$, $p = 0.01$). (b) An inverse association between non-social behaviour and connectivity between retrosplenial and cingulate cortices (wall rearing, frequency: $r = -0.45$, $p = 0.02$, $n = 26$, $R^2 = 0.21$). * $p < 0.05$, ** $p < 0.01$.

network refinements (Zhan et al., 2014; Riccomagno and Kolodkin, 2015) are, however, likely to play a role in the observed functional desynchronization as well, given the established contribution of GABAergic oscillatory rhythms in mediating large-scale functional synchrony (Gonzalez-Burgos and Lewis, 2008) and the recent evidence of altered spine density and increased spine eliminations in *Cntnap2*^{-/-} mice (Gdalyahu et al., 2015). The relative contribution of anatomical versus neurophysiological mechanisms in determining the observed desynchronization remains however undetermined, and interventional studies entailing the regional manipulation of excitatory/inhibitory ratio or inactivation of projection-specific pathways may be required to disambiguate this issue.

Recent human studies described possible microstructural white matter alterations in carriers of CNTNAP2 mutations as assessed with water diffusion anisotropy. Specifically, gender-dependent reductions in fractional anisotropy (FA) in the inferior fronto-occipital fasciculus or anterior thalamic radiation have been described by Tan et al. (2010). Similarly, Clemm von Hohenberg et al. (2013) described an interaction between a single genotype (rs2710126) and FA, in which homozygotes for the risk allele showed reduced FA values in uncinate fasciculus.

These preliminary results suggest the presence of possible white matter microstructural alterations as a result of CNTNAP2 gene mutations. However, anisotropic water diffusion reflects multiples biophysical contributions that prevent an unequivocal microstructural interpretation of these findings. For example, reduced FA could be the result of reduced neuronal packing, myelination, axonal diameter, neuronal integrity and maturation, as well as regional differences in gray matter fraction (Beaulieu, 2009). To investigate potential microstructural white matter disruption at a more detailed level than permitted by diffusion MRI, we carried out a histological assessment of white matter fibres using MBP immunofluorescence. We did not observe any gross white matter microstructural abnormality in *Cntnap2*^{-/-} mutants in terms of fibre orientation, packing or organization. Moreover, MBP quantification did not reveal any significant genotype-dependent differences. Together with

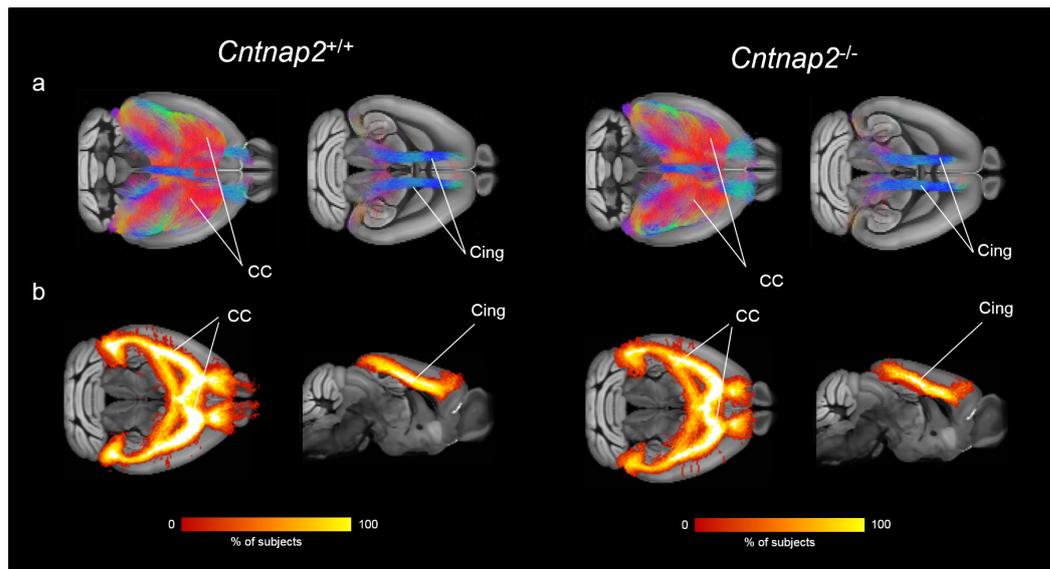


FIGURE 3.11: Preserved cortico-cortical white matter organization in *Cntnap2*^{-/-} mutants. (a) Corpus callosum and cingulum tracts virtually dissected in two representative subjects (*Cntnap2*^{+/+} left, *Cntnap2*^{-/-} right), (b) Fractional group fibre density maps for corpus callosum and cingulum tracts (*Cntnap2*^{+/+} left, *Cntnap2*^{-/-}, right).

previous electron microscopy investigations, where normal myelin thickness was reported in *Cntnap2*^{-/-} mutants (Poliak et al., 2003), these findings argue against the presence of gross microscale white matter alterations in these mutants. While this finding appears to be in contrast with human investigations of CNTNAP2 polymorphisms and suggestive of possible species-specific divergence, additional research is required to more thoroughly investigate the presence of white matter microstructural aberrancies in *Cntnap2*^{-/-} mutants. It should also be noted that CNTNAP2 polymorphisms studies in humans are typically correlative and involve small patient samples, which make them more prone to confounding factors related to heterogeneity in clinical samples, and individual adaptive differences in microstructural parameters (Scholz et al., 2009).

The observation of hypoconnectivity in prefrontal hub regions of the DMN (Liska, Galbusera, et al., 2015) is suggestive of a deficient “maturation” of this functional network (supekar2010), and is in keeping with the hypothesis of a key role of this region as a mediator of deficits in global perception and its cognitive representations in ASD patients (Martínez-Sanchis, 2014). The notion that “underconnectivity” may preferentially affect complex cognitive and social functions and their high order cortical substrates rather than low-level sensory and perceptual tasks has recently found some theoretical support (Kana, Libero, and Moore, 2011). Within this framework, heteromodal integrative hubs like the anterior cingulate and prefrontal cortex, as well as retrosplenial regions would serve as major points of vulnerability for the stability of distributed functional network couplings. rsfMRI mapping in additional mouse lines harbouring ASD-related genetic mutations will be instrumental in assessing whether the observed alterations represent a generalizable endophenotype that may converge across mutations and genetic etiologies, or are the specific consequence of *Cntnap2* mutations. It is, however, interesting to note that so far hypoconnectivity appears to be predominant in mouse imaging studies of ASD: reduced connectivity in several brain regions including the prefrontal cortex and the

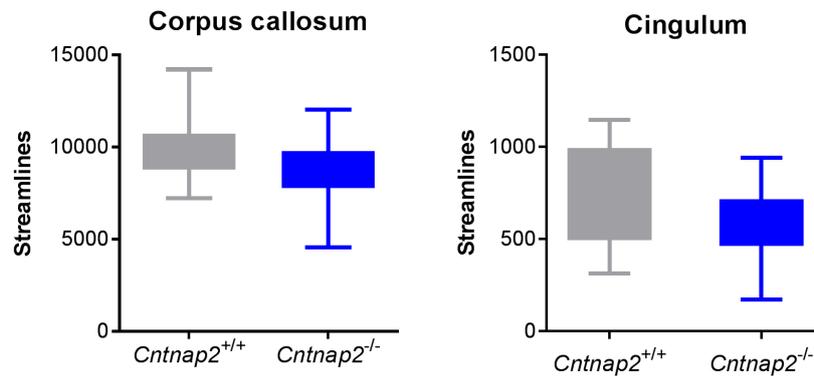


FIGURE 3.12: White-matter tractography-based streamline counts. Numbers of streamlines in corpus callosum and cingulum showed no significant differences between the *Cntnap2*^{-/-} and control littermates (cingulum: t-test, $t_{21} = 1.25$, $p = 0.23$; corpus callosum: t-test, $t_{21} = 1.21$, $p = 0.24$).

DMN has been observed in the BTBR model of idiopathic autism (Sforazzini, Bertero, et al., 2016), and in mice characterised by reduced synaptic pruning (Zhan et al., 2014), a pathological trait associated with autism (Tang et al., 2014). Reduced connectivity between motor sensory regions and a general reduction in primary visual cortex connectivity were also recently described in a mouse model of fragile X syndrome (haberl2015). Although preliminary, these initial mouse findings are consistent with and somehow support the “under-connectivity theory” of autism, according to which reduced functional connectivity, at least in the adult brain (Uddin, Supekar, and Menon, 2013), may emerge as a dominant feature of ASD in the face of heterogeneous etiopathological pathways (Di Martino et al., 2014; Uddin, Supekar, and Menon, 2013).

In contrast with our imaging results, human rsfMRI mapping in CNTNAP2 common variant carriers revealed increased, instead of decreased, local connectivity in lateral prefrontal regions (scott-van2010). The reason behind this discrepancy is not clear, although several important experimental factors, including methodological, species- and/or age-related differences may contribute to this inconsistency. For example, local connectivity was found to be increased in human lateral prefrontal areas, a region that does not have a clear cyto-architectural correlate in rodents (Vogt and Paxinos, 2014). Moreover, our study was performed in adult male subjects, while human mapping was carried out in pre-pubertal subjects (mean age 12 years old), a discrepancy that could account for the differences in local connectivity alterations. Indeed, a dramatic reorganization of large-scale functional brain networks occurs during childhood and late adolescence in humans, involving developmental shifts from short-range to long-range connectivity, notably within fronto-insular and cortico-subcortical networks [reviewed by (Ernst et al., 2015)]. Moreover, an age-related dichotomy has been suggested in ASD-related connectivity aberrancies, with generally reduced intrinsic functional connectivity in adolescents and adults with autism compared with age-matched controls, and increased functional connectivity in younger children with the disorder (Uddin, Supekar, and Menon, 2013). A similar age-related shift could therefore possibly explain the discrepant direction

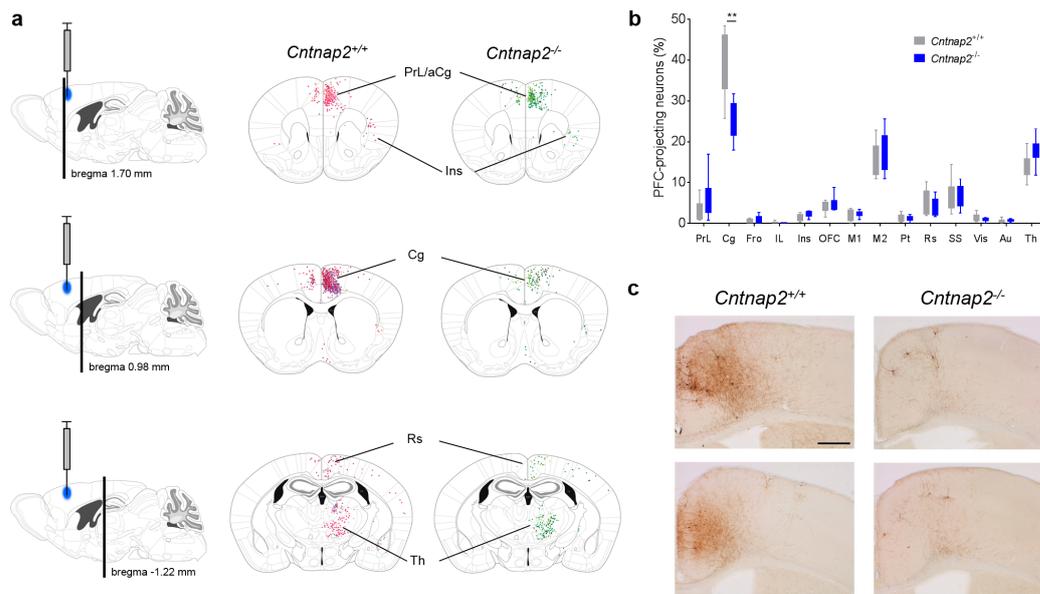


FIGURE 3.13: Reduced frequency of cingulate-prefrontal projecting neurons in *Cntnap2^{-/-}* mice. (a) Locations of retrogradely labelled cells superimposed on the corresponding Paxinos Atlas coronal tables. Injection location is indicated in blue on the sagittal tables. (b) Regional quantification of the relative regional number (frequency) of retrogradely labelled cells (t-test; Cg: $t_{10} = 3.90$, $p = 0.003$, FDR-corrected $p = 0.04$). (c) Enlarged view of the distribution of retrogradely labelled cells in a coronal section of the cingulate region (bregma 0.98 mm) in two representative *Cntnap2^{+/+}* and two *Cntnap2^{-/-}* subjects. The scale bar indicates 250 μm . * $p < 0.05$, ** $p < 0.01$.

of local connectivity observed in our study with respect to the finding reported by Scott-Van Zeeland et al. (2010) in human pre-adolescent subjects. Longitudinal investigations of connectivity in rodent genetic models of autism are highly warranted to enable empirical testing of the developmental trajectory of ASD-related connectivity aberrancies across development and network maturation (Liska and Gozzi, 2016). Finally, differences in the nature of the investigated mutations should also not be neglected, as the functional consequences of the genetic variants imaged by Scott-Van Zeeland et al. (2010) are unclear, and the possibility that not all the imaged genetic variants are loss-of-function cannot be ruled out.

We also note here that our study specifically addressed male mice only, owing to the greater ASD incidence in this gender (Lai, Lombardo, Auyeung, et al., 2015). While this choice has the advantage of reducing within-group variation and subsequent increase in statistical power of our measurements, this should be considered a limitation of our study, as it does not permit to assess whether our findings can be generalized to female carriers of *Cntnap2* loss-of-function mutations.

In conclusion, we document that the absence of *Cntnap2* leads to functional connectivity reductions and defective mesoscale wiring in prefrontal functional hubs of the mouse brain, an effect associated with impaired social behaviour. These findings suggest that loss-of-function mutations in *Cntnap2* may predispose to neurodevelopmental disorders and autism through selective dysregulation of connectivity in integrative prefrontal areas, and provide a translational model for investigating connective perturbations in syndromic ASD forms.

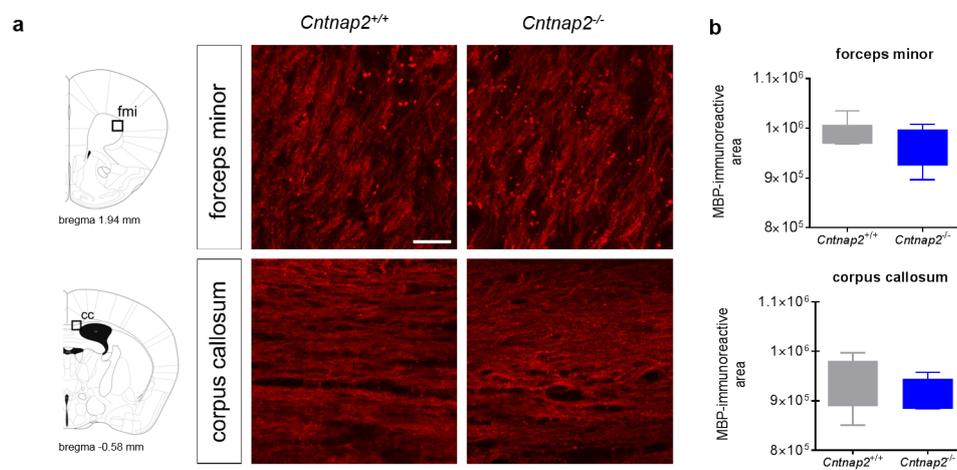


FIGURE 3.14: Histological and immunohistochemical analysis of white matter. **(a)** Representative images of anterior callosal regions characterized by parallel or transversal fibre extension with respect to the imaging plane (corpus callosum and forceps minor of the corpus callosum, respectively). No apparent difference in fibre organization or MBP stained regions was observed between genotypes. **(b)** MBP-immunoreactive area averaged from three random image fields per region and animal ($n = 5$, each group; corpus callosum: t-test, $t_8 = 0.84$, $p = 0.42$; forceps minor of the corpus callosum: t-test, $t_8 = 1.06$, $p = 0.32$).

Chapter 4

Mouse imaging and the autism connectivity chaos

This chapter has been published as:

Adam Liska and Alessandro Gozzi (2016). “Can Mouse Imaging Studies Bring Order to Autism Connectivity Chaos?” In: *Frontiers in Neuroscience* 10, p. 484. DOI: [10.3389/fnins.2016.00484](https://doi.org/10.3389/fnins.2016.00484)

4.1 The connectivity theory of autism: open questions and controversies

Autism is a heterogeneous syndrome characterised by core behavioural features including deficits in social communication and interaction, as well as restricted, repetitive patterns of behaviour and interests (Association, 2013). Although a primary and unitary aetiology for autism spectrum disorder (ASD) has not been identified, its high heritability has been consistently documented, revealing a contribution of complex and highly heterogeneous genetic mutations (Geschwind, 2009; Geschwind and State, 2015; Sanders et al., 2015). Remarkably, although previously identified mutations, genetic syndromes and de novo copy number variations (CNVs) account for about 10–20 % of ASD cases, none of these single known genetic causes accounts for more than 1–2 % of cases [reviewed in (Abrahams and Geschwind, 2008)]. The phenotypic expression (i.e., “penetrance”) of these genetic components is also highly variable, ranging from fully penetrant point mutations to polygenic forms with multiple gene–gene and gene–environment interactions. Remarkable variability exists also in the extent of cognitive and behavioural abnormalities presented by affected individuals (Lai, Lombardo, and Baron-Cohen, 2014; Chang et al., 2015; Georgiades et al., 2013), making heterogeneity a dominant theme for this group of disorders.

The advent of non-invasive brain imaging raised hopes that such clinical heterogeneity could be narrowed down to a small number of identifiable “imaging endophenotypes” that could help ASD diagnosis, patient stratification, and possibly provide clues as to the elusive aetiology of this group of disorders. Unfortunately, the results of imaging studies have proven overall as variable as the clinical manifestations of ASD (Ecker, Bookheimer, and D. G. M. Murphy, 2015; Stanfield et al., 2008). A notable exception to this scenario was the initial observation of reduced connectivity between brain regions in ASD patients, a finding first reported by Horowitz and colleagues (Horowitz et al., 1988) using PET, and later corroborated by task-based (Just, Cherkassky, et al., 2004) and resting-state fMRI (rsfMRI) studies (Assaf et al., 2010; Cherkassky et al., 2006; Kennedy and Courchesne, 2008), which

revealed impaired long-range synchronization in spontaneous brain activity. Together with evidence of reduced white matter connectivity detected with MRI [reviewed in (Evdokia Anagnostou and Taylor, 2011)], these observations form the basis of the so called “under-connectivity theory of autism” (Evdokia Anagnostou and Taylor, 2011; Just, Keller, et al., 2012), according to which deficient long-range communication between brain regions may underlie ASD symptoms and pathophysiology. However, recent imaging studies have strongly challenged this view, highlighting a much more heterogeneous picture [see (Vasa, Mostofsky, and Ewen, 2016) for a recent review]. For example, rsfMRI mapping in a large cohort of patients has revealed the presence of concomitant hypo- and hyper-connectivity (Di Martino et al., 2014), although a clear prevalence of hypo-connected regions was apparent. Similarly, widespread hyper-connectivity during childhood has also been recently described (Keown et al., 2013; Supekar et al., 2013; Uddin, Supekar, and Menon, 2013), suggesting a possible neurodevelopmental origin for these alterations. More recently, the hypothesis that such conflicting findings could reflect greater inter-subject variability in ASD patients than in neurotypical controls (i.e. idiosyncratic connectivity) has been proposed (Hahamy, Behrmann, and Malach, 2015). A putative confounding contribution of ASD-related motion and its effect on functional connectivity readouts is also the subject of an open controversy in the imaging community (Deen and Pelphrey, 2012; Power, Schlaggar, and Petersen, 2015; Power, Barnes, et al., 2012; Pardoe, Kucharsky Hiess, and Kuzniecky, 2016).

Collectively, the extensive literature published to date points at the presence of major functional connectivity alterations in ASD populations, although the identified regional patterns vary considerably across studies and patient cohorts (Ameis and Catani, 2015; Bernhardt et al., 2016; Ecker and D. Murphy, 2014; Ecker, Bookheimer, and D. G. M. Murphy, 2015; Kana, Libero, and Moore, 2011; Müller, 2014; Vasa, Mostofsky, and Ewen, 2016). Despite this rapidly accumulating evidence, many fundamental questions as to the origin and significance of connective alterations in ASD remain unanswered. For one, the neurophysiological underpinnings of these connective aberrancies are largely unknown, and a causal etiopathological contribution of specific genetic variants to impaired connectivity in ASD remains to be firmly established. More broadly, it is unclear whether these abnormalities are a causative or epiphenomenal consequence of the disease, and whether their heterogeneous expression reflects cohort effects, different genetic aetiologies or neurodevelopmental trajectories. The exact relationship between connectivity alterations and the severity of ASD manifestation remains also obscure, with the vast majority of the human neuroimaging literature being focused on high functioning ASD cohorts (Vissers, X Cohen, and Geurts, 2012).

A deeper understanding of the origin and significance of these phenomena is greatly complicated by our very limited understanding of the neurobiological foundations of macro-scale neuroimaging readouts commonly employed in ASD research, such as white matter microstructural parameters [e.g. fractional anisotropy (Owen et al., 2014)], or the elusive functional couplings underlying rsfMRI-based functional connectivity. This has left us with a major explanatory gap between mechanistic models of brain function at the cellular and microcircuit level, and the emergence of macroscale functional activity in health and pathological states such as those that are observed in autism. As a result, we are currently unable to properly interpret and back-translate clinical evidence of aberrant connectivity into interpretable neurophysiological events/models that can help understand, diagnose or treat these disorders. It is also becoming apparent that a full disambiguation of the multifactorial and complex determinants of aberrant functional connectivity in ASD can

only be obtained through the combined use of refined clinical imaging methods and multimodal-multiscale investigational approaches that currently can only be applied in experimental animal models.

4.2 Bridging the gap: functional connectivity mapping in mouse autism models

The identification of several high-confidence ASD-risk genes involved in syndromic forms of autism (Sanders et al., 2015) has been paralleled by the generation of mouse lines recapitulating human mutations. Despite predictable limitations in reliably modelling the full phenotypic spectrum of a complex (and possibly only human) developmental disorder like ASD, mouse models can be harnessed to understand how genetic alterations translate into relevant changes in cells and circuits, and ultimately to identify points of convergence for molecular pathways, cells, circuits, and systems that may result in a deeper understanding of the pathophysiology of ASD and related behavioural deficits (Arguello and Gogos, 2012; Nelson and Valakh, 2015; Vasa, Mostofsky, and Ewen, 2016). For example, molecular investigations in ASD mouse models have been instrumental in the identification of a limited set of molecular pathways to which ASD-involved genes seem to converge, including, among others, synaptogenesis, synaptic function, and neuronal translational regulation [reviewed in (de la Torre-Ubieta et al., 2016)]. This effort has been accompanied by the development of ASD-relevant behavioural phenotyping assays, primarily targeted at social, communication and repetitive behaviours (Wöhr and Scattoni, 2013; Kas et al., 2014; Silverman, Tolu, et al., 2010; Homberg et al., 2016). Interestingly, many – but not all – models showed autism-like traits, with manifestations ranging from repetitive behaviours to reduced social communication (ultrasonic vocalizations) and social interest [reviewed in (Ellegood and Crawley, 2015)]. However, despite the widespread application and high face validity of ASD behavioural phenotyping, the significance and translational relevance of mouse behavioural alterations to human ASD remain debated (Wöhr and Scattoni, 2013), and should be extrapolated with caution.

Recent advances in mouse rsfMRI mapping [reviewed in (Gozzi and Schwarz, 2016)] offer the opportunity of extending mouse modelling of ASD to the investigation of the neurobiological underpinnings and etiopathological significance of ASD-related connectivity aberrations. Specifically, improvements in MRI imaging hardware, together with tighter control of physiological and motion artefacts (Ferrari et al., 2012; Weber et al., 2006) have led to robust and reproducible identification of homotopic rsfMRI networks covering known cortical and subcortical systems in the mouse by several research groups (Mechling et al., 2014; Nasrallah, Tay, and Chuang, 2014; Sforazzini, Schwarz, et al., 2014; Shah et al., 2016; Zerbi et al., 2015). Interestingly, distributed networks encompassing heteromodal prefrontal and posterior cortical regions have also been identified (Sforazzini, Schwarz, et al., 2014; Shah et al., 2016; Zerbi et al., 2015), leading to the suggestive hypothesis of the presence of evolutionary precursors of the human salience network and default mode network (DMN) in this species [reviewed in (Gozzi and Schwarz, 2016)]. This notion is empirically corroborated by the recent observation that cytoarchitecturally-homologue regions such as anterior cingulate and retrosplenial cortices (Vogt and Paxinos, 2014) similarly serve as connectivity hubs in humans and mice (Cole, Pathak, and Schneider, 2010; Liska, Galbusera, et al., 2015; Tomasi and Volkow, 2011). Moreover, the application of rsfMRI to the mouse brain comes with several important advantages, including the possibility to use quantitative imaging modalities for an objective

endo-phenotypic characterization of ASD-related pathology complementary to behavioural assays, and to validate its readouts with invasive techniques that are off limits for human research, including local field potentials (LFPs) coherence mappings (Zhan et al., 2014), local injection of neuronal tracers (Sforazzini, Bertero, et al., 2016), as well as an ever-increasing array of histopathological, stereological or immunohistochemical post-mortem analyses.

Collectively, these correspondences strongly support the use of rsfMRI as a means to bridge research of functional connectivity aberrancies in autism across species (man vs. mouse) and levels of inquiry (from cellular- and microscale to meso- and macroscale, Fig. 4.1), along two main investigational routes. First, rsfMRI can be used to establish causal (rather than associative) etiopathological contributions between specific ASD-associated genetic variants and macroscale connectivity, thus complementing analogous clinical research efforts using imaging genetics (Rudie et al., 2012; Scott-Van Zeeland et al., 2010). One notable experimental advantage of mouse imaging with respect to current human imaging genetic approaches is the possibility of mapping and comparing the effect of multiple mutations (via the use of different autism mouse models) under rigorously controlled experimental conditions, thus reducing the confounding contribution of experimental variables that can be only minimally controlled in human research, such as genetic and environmental variability, age (Uddin, Supekar, and Menon, 2013), ASD-related motion, and group differences in cognitive states (Vasa, Mostofsky, and Ewen, 2016). The main goal of this line of investigation is to assess whether seemingly unrelated ASD-risk mutations do converge on a limited number of distinct functional connectivity endophenotypes. An elegant demonstration of this approach has been recently described using morpho-anatomical MRI. Brain-volumetric phenotypes of 26 ASD mouse models using structural MRI methods exhibited clustering into three main groups, each with a distinct set of concomitant changes in size across different brain regions (Ellegood, E. Anagnostou, et al., 2015). Such reduction of morpho-anatomical heterogeneity is not surprising, given the wide (and sometimes opposing) stream of pathophysiological alterations observed in syndromic forms of autism, which range from basic molecular or synaptic mechanism such as protein synthesis (Geschwind and Levitt, 2007; Auerbach, Osterweil, and Bear, 2011) up to homeostatic regulations of excitatory and inhibitory neurotransmission (Nelson and Valakh, 2015). Analogous analyses with regards to functional connectivity phenotypes should be possible in the future to associate basic pathophysiological traits with macroscale connectional aberrancies.

A second main line of investigation is the combined use of mouse rsfMRI and multiscale neurobiological techniques to obtain a mechanistic description of ASD-related phenotypes and pathophysiological pathways leading to aberrant functional connectivity. This research can include, but is not limited to, a deeper investigation of syndromic ASD mutations associated with specific pathological traits [e.g. Tuberous Sclerosis 2 as a key mediator of impaired autophagy and increased synaptic density (Tang et al., 2014)], and can possibly be extended to investigate risk factors that have been also more loosely implicated in autism. This research effort may generate crucial mechanistic information that can be used to back-translate clinical evidence of aberrant connectivity into interpretable neurophysiological events/models that can help understand, diagnose or treat these disorders. A brief description of initial steps towards these two main goals is reported in the next two sections.

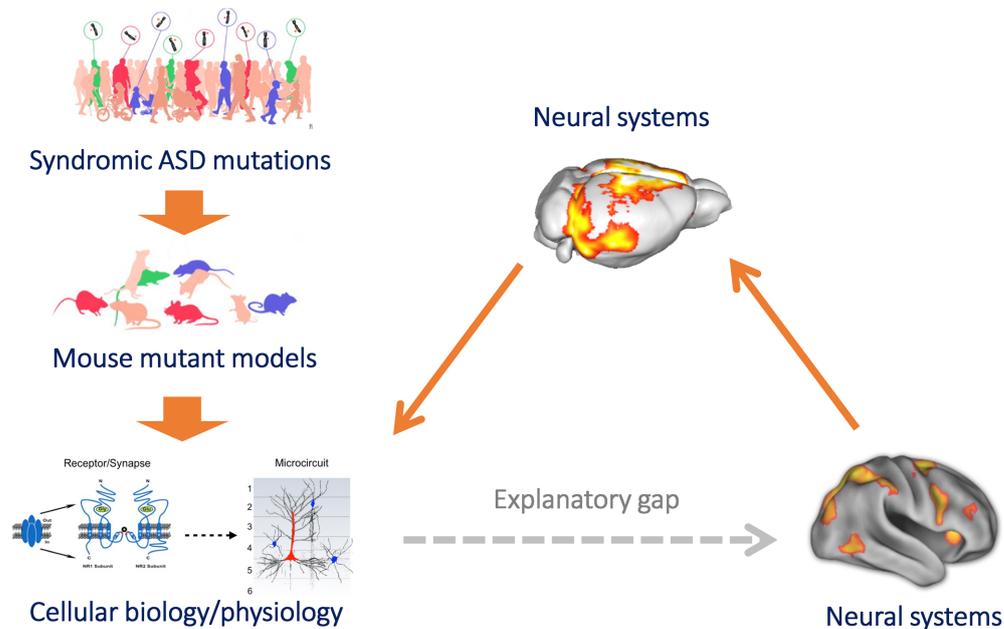


FIGURE 4.1: Mouse imaging can bridge the gap between microscale models of brain function, and clinical research of macroscale functional connectivity. Mouse models provide a powerful reductive platform that can be employed to link etiological determinants of ASD, such as syndromic mutation or neurodevelopmental traits, to basic molecular and cellular signatures of pathology (left, top to down). However, until recently we have been unable to use this approach to study the neurobiological underpinnings of macroscale functional connectivity, owing to difficulty in translating models of brain function across levels of inquiry. This results in a major explanatory gap between clinical research (heavily relying on macroscale neuroimaging measures of brain function, such as rsfMRI) and preclinical neurobiological investigation in rodent models (bottom, right). The implementation of functional connectivity mapping via rsfMRI in the mouse (right) can bridge this gap, by permitting to causally relate connective changes with basic molecular or cellular processes, and by permitting a direct translation of these findings from and to humans owing to the shared biophysical principle underlying these measurements [adapted from (Arguello and Gogos, 2012; Anticevic, Cole, et al., 2013)].

4.3 Functional connectivity mapping in genetic models of autism

An outstanding question in ASD connectivity studies is whether genetic mutations associated with syndromic forms of autism are sufficient to produce aberrant macroscale functional connectivity. Initial mouse rsfMRI studies seem to corroborate this hypothesis. Specifically, Haberl and colleagues have recently investigated functional and structural connectivity in the *Fmr1*^{-/y} model of fragile X syndrome (FXS) (Budimirovic and Kaufmann, 2011) and described connective aberrations in sensory networks (Haberl et al., 2015). These included reduced structural integrity of the corpus callosum and an increase in local connectivity of the primary visual cortex, as probed by viral tracers, an effect accompanied by reduced rsfMRI coupling between visual and other neighbouring sensory cortical regions. The authors suggested that the observed decoupling could explain sensory processing defects that are often observed in FSX patients (Boyd et al., 2010).

In another recent study, homozygous mice lacking the ASD-risk gene *CNTNAP2*

(Peñagarikano et al., 2011) exhibited reduced long-range and local functional connectivity in cingulate and prefrontal regions (Liska, Bertero, et al., 2017), two key heteromodal areas of the mouse brain previously characterised as functional connectivity hubs, owing to their rich connectivity with other brain areas (Liska, Galbusera, et al., 2015). Interestingly, impaired antero-posterior prefrontal connectivity between components of the mouse DMN was associated with reduced social investigation, a behavioural measure regarded as a core “autism trait” in mice (Wöhr and Scattoni, 2013). This finding recapitulates analogous imaging results obtained in human carriers of CNTNAP2 gene polymorphisms (Scott-Van Zeeland et al., 2010), hence providing a first example of the translational value of this approach. This finding is consistent with the presence of impaired GABAergic neurotransmission in these animals (Peñagarikano et al., 2011), a trait that could result in aberrant oscillatory rhythms. It is interesting to note that analogous prefrontal hypo-connectivity has been observed using rsfMRI in BTBR mice, an idiopathic model of autism characterised by agenesis of the corpus callosum and by analogous excitatory/inhibitory imbalances (Sforazzini, Bertero, et al., 2016).

rsfMRI mapping has also been recently carried out in a mouse model of human 15q13.3 microdeletion, a CNV associated with schizophrenia, intellectual disability and ASD (Shinawi et al., 2009). Compared to wild-type mice, 15q13.3 mice showed widespread patterns of hyper-connectivity along the hippocampal-prefrontal axis, a network commonly affected in schizophrenic patients (Natalia Gass et al., 2016). Notably, Gass and colleagues also showed that aberrant functional connectivity could be acutely rescued by pharmacological stimulation of nicotinic acetylcholine alpha 7 receptors, in keeping with a contribution of this mechanism to the development of schizophrenia-related phenotypes in these mice (Natalia Gass et al., 2016). Although the phenotypic traits of this mouse line appear to be more closely related to schizophrenia rather than to ASD (Fejgin et al., 2014), the results of this study are important as they show that CNVs and genetic alterations with partial penetrance to ASD could produce divergent connective phenotypes (e.g. hyper- and hypo-connectivity), suggesting a plausible contribution of genetic heterogeneity to some of the discrepant imaging findings in humans. Importantly, these initial mouse studies argue against an artefactual (e.g. motion-driven) origin of connectivity aberrations reported in human ASD research, because the use of light sedation in mice along with artificial ventilation allows for the acquisition of virtually motion-free images.

4.4 Neurobiological pathways leading to aberrant functional connectivity

A few recent studies have provided important mechanistic investigations of ASD-relevant phenotypes associated with aberrant functional connectivity. In the first of such studies, Zhan and colleagues (Zhan et al., 2014) investigated whether deficits in synaptic pruning, a putative pathophysiological determinant of autism (Hutsler and H. Zhang, 2010), result in impaired connectivity alterations. To probe this hypothesis, the authors measured rsfMRI connectivity in Cx3cr1KO mice, a mouse line characterised by microglia-dependent synaptic pruning deficits as a result of deficient neuronal-microglia signalling (Paolicelli et al., 2011). Synaptic pruning deficits in Cx3cr1KO were found to be associated with long-range functional connectivity impairments, a finding corroborated by LFPs coherence recordings in freely-behaving

animals. Interestingly, the authors also showed that impaired pruning was associated with core mouse “autism traits”, and that long-range fronto-hippocampal connectivity was a good predictor of social behaviour. This study is of special importance, as it was the first to suggest a role for dysfunctional synaptic maturation in shaping long-range functional synchronization, and to postulate a contribution of immune system mediators to this cascade. Empirical evidence in support of this hypothesis comes from another recent study (Kim et al., 2016), where analogous phenotypes were observed in mice characterised by defective autophagy in microglia, including increased synaptic density, impaired social activity, and a trend for impaired connectivity between posterior-sensory and prefrontal regions. Similarly, Filiano and colleagues (Filiano et al., 2016) recently showed that deficiency in interferon- γ , a key immune signalling protein, is associated with social deficits and frontal rsfMRI hyper-connectivity in SCID mice, thus corroborating a putative mechanistic link between immune dysfunction, impaired social behaviour and functional connectivity. Although promising and mechanistically relevant, these initial results should be extrapolated to autism research with great caution, as a pathophysiological contribution of immune and microglia deficits to ASD has yet to be unambiguously demonstrated (Estes and McAllister, 2015). They, however, powerfully illustrate how the combined use of rsfMRI, mouse genetics and state-of-the-art neuro-biological approaches can elucidate pathways leading to aberrant functional connectivity, an approach that can be extended to investigate the role of multiple ASD-relevant pathophysiological factors, including syndromic genetic mutations.

4.5 Limitations and future perspectives

Like any other experimental approach, mouse rsfMRI is accompanied by limitations that should be taken into account when the approach is used to investigate the basis of connectivity alterations in ASD. First and foremost, as mouse rsfMRI experiments normally employ sedation to minimize stress and motion of animals during scans, the contribution of possible genotype-dependent differences in sensitivity to anaesthesia (Petrinovic et al., 2016) should be controlled. The fact that to date only a minority of studies (Sforazzini, Bertero, et al., 2016; Zhan et al., 2014) have reported genotype-dependent measures of anaesthesia sensitivity is a factor for concern, as differences in anaesthesia depth/sensitivity can affect connectivity strength and distribution of the imaged networks (Nasrallah, Tay, and Chuang, 2014). The impact of anaesthesia per se as a putative modifier of intrinsic connectional architecture appears to be less of an issue, as a large body of human and rodent research shows that, under light controlled sedation, the regional patterns of functional correlation seem to be largely preserved [reviewed in (Gozzi and Schwarz, 2016)]. As pointed out in previous work, a rigorous control of motion and physiological state is also of paramount importance to obtain reliable network mapping (Gozzi and Schwarz, 2016; Jonckers, Shah, et al., 2015). It should also be mentioned that, although the field is still lacking in standardised protocols and methods that would facilitate comparison of experimental results across studies and sites, this issue is receiving increased attention and collaborative efforts are underway to address it.

The initial studies described here represent only the first step toward a greater understanding of the origin and underpinnings of connectional alterations in ASD. Future investigations are required to describe commonalities and differences between brain functional networks in the mouse and human from multiple points of view, including topology (Sporns and Betzel, 2016; van den Heuvel, Bullmore,

and Sporns, 2016), biological underpinnings (van den Heuvel, Scholtens, et al., 2016; Richiardi et al., 2015; Wang et al., 2015), and functional equivalence (Li et al., 2015). Similarly, studies of additional genetic aetiologies associated with ASDs, covering heterogeneous pathophysiological pathways, are crucial to achieve a deeper understanding of whether the connectional signatures are mutation specific, or can be regarded as a generalizable phenomenon. When coupled to analogous clinical efforts aimed at identification of connectional aberrancies in genetically homogeneous populations [e.g. 16p11.2 deletion (Owen et al., 2014; Simons Vip Consortium, 2012)], the method can also be used to investigate the cellular and physiological basis of clinically-relevant neuroimaging readouts, and, via a comparison between human and mouse imaging findings, to obtain an assessment of the translational and construct validity of mouse models of ASD. The developmental trajectory of these alterations could in principle also be investigated in mouse models, although critical limitations in the accuracy of physiological control in young mice and pups exist.

Much of mouse ASD modelling has been so far primarily addressed at monogenic ASD syndromes, which represent approximately 10 % of ASDs (Silverman, Tolu, et al., 2010; Nelson and Valakh, 2015). The recapitulation, in mice, of high-confidence genetic aetiologies associated with ASD offers the opportunity to probe specific hypotheses about circuit dysfunction and ASD pathology that can be directly extrapolated to homologous clinical populations [e.g. 16p11.2 microdeletion (Owen et al., 2014; Simons Vip Consortium, 2012)]. An important limitation of current ASD translational research is its inability to reliably model “idiopathic” autism, which is the most frequent diagnostic label for ASD-related behavioural manifestations. Attempts to use forward genetic approaches in inbred mouse lines exhibiting ASD-like behaviours without a specific genetic determinant have been proposed, with the inbred BTBR mouse line probably being the most notable example in the field (Silverman, Tolu, et al., 2010; Gogolla et al., 2014; Squillace et al., 2014). Translational relevance of neuro-behavioural findings obtained by comparing genetically homogeneous inbred lines like asocial BTBR and “normosocial” B6 mice is, however, debated (Dodero et al., 2013; Squillace et al., 2014). Nevertheless, novel neuromolecular approaches and the use of induced pluripotent stem cells (iPSCs) from patients have begun to reveal common downstream neurobiological pathways in idiopathic forms of autism characterised by shared neuroanatomical features [e.g. macrocephaly (Marchetto et al., 2016; Nicolini et al., 2015)]. Controlled manipulation of such signalling and molecular pathways in animal models is a foreseeable strategy that can be employed to expand our translational framework to the investigation of macroscale brain network aberrancies in idiopathic forms of ASD.

Finally, studies in which connectivity alterations are pharmacologically or genetically rescued may help clarify the relevance of functional connectional alterations to ASD pathology and its behavioural manifestations. Specifically, if connectivity alterations are an underlying cause of observed behavioural deficits, then behavioural phenotypic “rescue” should be accompanied by normalised patterns of brain functional connectivity in the brain. This research could indicate whether connectivity alterations are necessary for the expression of ASD-related behaviours in mice, or are instead an epiphenomenal manifestation of underlying pathophysiology, thus providing an empirical assessment of the pathophysiological relevance of connectivity aberrancies in ASD. “Rescue” studies may also help identify putative endophenotypes (complementary to behaviour) that could serve as measurable readouts for early clinical translation and evaluation of novel ASD treatments in genetically defined autism syndromes (Smucny, Wylie, and Tregellas, 2014).

In conclusion, functional imaging of the mouse has now reached a turning point

such that accurate modelling and investigation of ASD-connectivity aberrations is currently possible, via the use of readouts amenable to direct translation to human research (i.e., rsfMRI). Despite caveats, in the next few years the approach is poised to offer breakthroughs in our understanding of the pathogenesis of ASD-related connectivity aberrancies, possibly bringing some order to the intricate and often contradictory body of research detailing connectional alterations in patient populations.

Chapter 5

Conclusions

Resting-state functional Magnetic Resonance Imaging (rsfMRI) methods have been extensively used to explore the intrinsic organization of the human brain. In keeping with the common conceptualisation of many brain disorders as instances of neuronal miswiring, rsfMRI methods are being increasingly applied to study functional connectivity alterations associated with disease, with the goal of improving diagnoses and providing a deeper understanding of the underlying pathophysiology. However, despite intensive human research, many research questions relating connectivity to brain disorders remain open. As we have argued in the preceding chapters, this situation may stem from the fact that there is an explanatory gap between “macroscopic” connectivity studies performed in humans and “microscopic” research in animals, which has made it difficult to translate models of brain function across these different levels of enquiry.

5.1 Overview of the results

The research presented in this work focused on bridging the explanatory gap by causally relating connective changes with basic molecular or cellular processes through the application of rsfMRI to the mouse brain. The main advantage of this method is the potential to directly translate findings to and from humans owing to the shared biophysical principle underlying rsfMRI measurements in both species. We describe the intrinsic functional organization of the mouse brain at the macroscale and show that there exist at least six distinct functional modules related to known functional partitions of the brain, including a potential rodent homologue of the default-mode network (DMN). We next focused on describing functional connectivity in homozygous mice lacking the gene *Cntnap2*, a mutation which is strongly associated to autism. *Cntnap2* knock-out mice exhibited reduced long-range and local functional connectivity within prefrontal and midline brain regions. Moreover, long-range rsfMRI connectivity impairments strongly affected the fronto-posterior components of the mouse default-mode network, and this effect was associated with reduced social investigation, a core “autism trait” in mice. While macroscale cortico-cortical white-matter organization appeared to be preserved in these animals, viral tracing revealed reduced frequency of prefrontal-projecting neural clusters in the cingulate cortex of *Cntnap2* KO mutants, suggesting a possible contribution of defective mesoscale axonal wiring to the observed functional and consequently behavioural impairments. Collectively, the results described in this thesis highlight the presence of evolutionarily-conserved networks and functional hubs in the mouse brain. Moreover, they also reveal a key contribution of ASD-associated gene CNTNAP2 in modulating macroscale functional connectivity, and suggest that homozygous loss-of-function mutations in this gene may predispose to neurodevelopmental

disorders and autism through a selective dysregulation of connectivity in integrative prefrontal areas.

5.2 Limitations

A limitation of the studies presented in this thesis is that all mouse imaging has been performed under light anaesthesia, while majority of human imaging is performed in the awake state. The main motivation for this methodological choice is that it facilitates rigorous control of motion and physiological state, which are both very important for reliable connectivity mapping with fMRI. As discussed in previous chapters, light anaesthesia does not seem to affect the intrinsic functional architecture of the brain (Gozzi and Schwarz, 2016). Nevertheless, genotype-specific effects of individual anaesthetics cannot be in general ruled out. In the CNTNAP2 study, we have attempted to mitigate such confounds by comparing mean arterial blood pressure and amplitude of cortical BOLD signal fluctuations, two measures which had been previously shown to correlate with anaesthesia depth, across the two experimental groups. The impact of future studies could be greatly increased by moving towards awake mouse rs-fMRI; however, awake rs-fMRI in this animal presents specific problems and it is currently developed by several research groups.

Another limitation of the presented studies is that they were performed on adult mice, while the results of human neuroimaging highlighted diverse connectional disruptions at different points during the lifespan of individuals with ASD (Ecker, Bookheimer, and D. G. M. Murphy, 2015). Longitudinal investigations of connectivity in rodent genetic models of autism are therefore highly warranted in order to study whether connectivity aberrancies in these models also follow similar developmental trajectories.

5.3 Future directions

There are several areas in which we can expect important contributions from pre-clinical rsfMRI in the near future. Cell-type specific manipulations through chemo- and optogenetics make it possible to establish causal links between the activity of neuronal subpopulations and large-scale brain activity and behaviour (Deisseroth, Etkin, and Malenka, 2015; B. L. Roth, 2016). The application range of these techniques is wide and – when coupled with fMRI (Giorgi et al., 2017; Grayson et al., 2016) – they provide us with means to move beyond the description of genetic models of autism to testing specific hypotheses about the neural drivers of macroscale functional connectivity and their potential aberrations in autism. As an example, an intriguing result across a large number of human studies investigating functional connectivity disruptions in brain disorders points at the preferential disruption of network hubs in brain disease (Crossley, Mechelli, Scott, et al., 2014). The conservation of functional hubs in the human and mouse (Chapter 2) along with the observation that functional hubs may also be points of vulnerability in mouse models of ASD (Chapter 3) pave the way to further investigations into the role of functional hubs in orchestrating the dynamics of brain function. By inhibiting or exciting physiologically distinct neuronal populations in hub and peripheral regions, altering therefore the balance between excitatory and inhibitory neurons crucial to both local and long-range cortical computations (Anticevic and Murray, 2017; Krystal et al., 2017), we could study whether the hub regions indeed represent entry-points for network breakdown. Such studies could also shed light on the effects of inhibitory

neuronal dysfunction and increased excitatory/inhibitory ratio observed in individuals with ASD (Marín, 2012).

Multimodal investigations linking gene expression, structural connectivity and functional connectivity represent another interesting line of research enabled by advances in rodent rs-fMRI. Recent experiments show that gene expression patterns exhibit strong correlation with functionally-coupled resting state networks of the human cortex (Richiardi et al., 2015; Konopka, 2017; Wang et al., 2015). However, the regulatory program leading to the establishment of network-specific transcriptional signatures remains undefined. Uncovering such a program using high-resolution gene expression data available for the mouse brain would enable us to investigate – through enrichment analyses – the potential link between aberrations in autism-risk genes and connectivity disruptions within specific functional networks.

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