What can functional connectivity in mice tell us about Alzheimer’s disease?
An investigation with resting-state fMRI at 9.4T.

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What can functional connectivity in mice tell us about Alzheimer’s disease?
An investigation with resting-state fMRI at 9.4T.

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by

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2014
Nous imitons, horreur! la toupie et la boule
Dans leur valse et leurs bonds; même dans nos sommeils
La Curiosité nous tourmente et nous roule
Comme un Ange cruel qui fouette des soleils.

-Charles Baudelaire, Le Voyage, Les Fleurs du Mal
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>BOLD</td>
<td>Blood oxygenation level dependent contrast</td>
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<td>CT</td>
<td>Computer tomography</td>
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<td>DMN</td>
<td>Default mode network</td>
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<td>DTI</td>
<td>Diffusion tensor imaging</td>
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<td>EPI</td>
<td>Echo planar imaging</td>
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<td>FA</td>
<td>Fractional anisotropy</td>
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<td>FC</td>
<td>Functional connectivity</td>
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<tr>
<td>FDG</td>
<td>Fludeoxyglucose</td>
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<td>FID</td>
<td>Free induction decay</td>
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<tr>
<td>ICA</td>
<td>Independent component analysis</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>RF</td>
<td>Radio frequency</td>
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<tr>
<td>Rs-fMRI</td>
<td>resting-state functional magnetic resonance imaging</td>
</tr>
<tr>
<td>SNR / tSNR</td>
<td>Signal to noise ratio, temporal SNR</td>
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<tr>
<td>SPECT</td>
<td>Single photo emission computer tomography</td>
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<tr>
<td>VBM</td>
<td>Voxel-based morphometry</td>
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Summary

Functional connectivity (FC) refers to the apparent sharing of temporal information between two or more distinct brain regions, and thus allows resolving the functional organization of the brain into interconnected networks. Functional magnetic resonance imaging (fMRI) has become a method of choice to resolve these networks due to the availability of MRI scanners in university centers and in the clinics. In particular, FC derived from fMRI measures performed at rest, hence resting-state fMRI (rs-fMRI), has gained wide-spread interest within the neuroscience research community due to the possibility to address and resolve multiple networks within a short measurement period with good spatial resolution and relative ease. Interest in the method is further explained by the ability of the method to study alteration of brain networks with respect to brain disorders such as autism or Alzheimer’s disease (AD). Of particular interest to AD research, groups at risk of developing the disease already present altered expression of FC several decades prior to disease onset, making this one of the earliest event potentially altered in the disease. The application of the method to rodents, and especially in mice, represents great potential to study altered functional networks in models of neurological disorders, with the purpose to study the phenomenon experimentally to understand the molecular and cellular mechanisms underlying FC.

However, the rs-fMRI method is hampered by several confounding elements, principally the presence of undesirable noise in the signal which may mask or lead to the over interpretation of FC. Noise sources that contaminate the fMRI signal are inevitable during measurements, due to motion, respiration and breathing cycle, as well as instrument instability. Additional problems arise in rodent studies. The application in mice is specifically rendered difficult by the small size of the brain to image, as well as the bias induced by anesthesia, which is used to reduce animal distress and assist in the animal immobilization.

In a first study, we applied and compared several denoising methods commonly used in human rs-fMRI studies. We identified systemic vascular effect as a potent nuisance in the signal. The removal of the vascular contribution from the signal led to improved specificity of the fMRI signal, a necessary condition preliminary to biological interpretations.

In a second study, we addressed the anesthesia bias. In order to identify a regimen suitable for rs-fMRI, we compared 4 anesthetics: isoflurane, medetomidine, propofol, and urethane. We categorized the anesthetics into two groups with regards to their effects on the functional networks of the mouse brain: isoflurane, propofol, and urethane in one group, and medetomidine in the other. While none of the anesthetics was optimal when considered individually, the combination of isoflurane and medetomidine yielded increased FC between regions and highly reproducible functional networks with minimal side effects and a sufficient level of sedation.

We applied FC measures over the life span of ArcAβ transgenic animals, a mouse model of AD. Additionally, we combined the results with measures of fractional anisotropy (FA), a marker of white matter integrity. Unexpectedly, FC and FA both presented significant impairments at an early age in transgenic animals compared to wild-type, prior to the appearance of amyloid plaques in the brain of transgenic the animals. The differences seem to be linked to the improper maturation of
the FC networks in young animals, rather than a reflection of neurodegeneration in older ArcAβ mice.

Finally, we tested the sensitivity of the FC, FA, and volumetric measurements in the context of an intervention studies. We applied a passive anti-Aβ immunization protocol with weekly administration of an antibody over the duration of one month, and measured the differences between baseline and post-treatment values in ArcAβ and wild-type animals. While we did not achieve to lower the peptides concentrations, we put in evidence striking volume changes in young ArcAβ. The presence of hypertrophic nuclei linked to the cholinergic system found in transgenic animals highlight a potential mechanism for functional network dysfunction, associated to neurochemical imbalance, which may result in the altered network maturation as observed in our previous study.

In summary, we approached and circumvented bias in our measurements by establishing a protocol to remove noise sources in our signal and by establishing an optimized anesthesia regimen suitable for rs-fMRI in mice. We identified potent alterations in the functional networks and white matter architecture in the ArcAβ mouse model of cerebral amyloidosis. Alterations in these readouts did not correlate with the amyloid plaque load as concluded from the fact that FC changes preceded plaque deposition. The mechanisms underlying functional alteration in the ArcAβ remain unexplained. The sensitivity and reproducibility of the rs-fMRI methods in mice, especially in the context of models of AD, offer opportunities to conduct further studies to address the biological mechanisms reflected in the FC measurements in both the healthy state, and in models of brain disorders. Advances in our understanding of the phenomenon may provide important knowledge in using FC as a biomarker for AD and other diseases, which can be translated to measures made in humans.
Résumé

La connectivité fonctionnelle est une mesure du partage d’information temporelle dans le signal entre deux ou plusieurs régions distinctes du cerveau, ce qui permet d’interpréter l’organisation du cerveau en réseaux de régions interconnectées. L’imagerie par résonance magnétique fonctionnelle (IRMf) est devenue la méthode préférentielle pour conduire ces études en partie dû à l’omniprésence des scanners dans les centres universitaires et dans les cliniques. En particulier, la connectivité fonctionnelle évaluée au repos par IRMf a suscité l’intérêt de la communauté scientifique en neurosciences, de par sa relative facilité d’application et la possibilité de mesurer l’interaction entre plusieurs réseaux avec une bonne résolution temporelle et spatiale. L’intérêt suscité par la méthode s’explique aussi par la possibilité d’étudier les modifications qui se font dans les réseaux fonctionnels dans le contexte de déficits neurocognitifs, tels que l’autisme ou la maladie d’Alzheimer. Ces études s’avèrent particulièremen interesse dans le cadre de cette dernière : en effet, les groupes de gens présentant des risques élevés de développer la maladie présentent des déficits de connectivités, et ce plusieurs décennies avant l’apparition des premiers symptômes de la maladie. En effet, la connectivité fonctionnelle semble être l’un des premiers éléments affecté par la maladie et mesurable par IRMf. Le transfert, et l’application des méthodes de connectivité fonctionnelle chez les rongeurs, et en particulier chez la souris, a le potentiel de permettre l’étude des changements de l’organisation des réseaux fonctionnels chez des animaux modèles de certains troubles neurocognitifs, dans le but d’étudier le phénomène, et éventuellement de dévoiler les bases moléculaires et cellulaires sous-jacentes aux changements observés.

Cependant, l’IRMf au repos est limitée par certains éléments confondants, notamment la présence indésirable de bruit dans le signal, propre à masquer ou à mener à la surestimation de la connectivité fonctionnelle. La présence de ce bruit dans le signal obtenu par IRMf est inévitable, et est principalement causée par le déplacement de l’objet mesuré dans le scanner, les cycles respiratoires et cardiaques, ainsi que par des imperfections des instruments. Des problèmes s’ajoutent lors de l’application de la méthode chez la souris. Le petit volume du cerveau de la souris, ainsi que le biais induit par les effets d’agents anesthésiants nécessaires à l’immobilisation et à la réduction de la souffrance animale due au stress et aux manipulations, induisent certaines complications.

Lors d’une première étude, nous avons appliqué et testé plusieurs méthodes communément utilisées chez l’humain pour les études IRMf afin d’estimer le bruit d’origine physiologique dans le signal. Nous avons identifié les effets du système vasculaire comme une source de bruit physiologique majeure dans le signal mesuré. La correction des contributions du système vasculaire dans le signal permet d’améliorer la spécificité des résultats, une démarche nécessaire avant l’interprétation biologique des résultats.

individuellement, la combinaison d’isoflurane et de medetomidine augmente la connectivité fonctionnelle, tout en présentant des effets secondaires minimes, et un niveau de sédation suffisant.


Finalement, nous avons testé la sensibilité des mesures de connectivité fonctionnelle, d’anisotropie fractionnelle, et de volumétrie par IRM dans le contexte d’une intervention. Nous avons appliqué un protocole d’immunisation passive anti-Aβ hebdomadaire, sur une durée d’un mois, et mesuré les changements présents entre une session de référence et une session post-intervention chez les souris génétiquement modifiées ArcAβ et les souris typées sauvages. Malheureusement, l’intervention n’a pas suffit à réduire la concentration des peptides visés. L’étude volumétrique des cerveaux à cependant démontré des effets intéressants. La présence de noyaux élargis liés au système cholinergique chez les souris génétiquement modifiées suggère un mécanisme potentiel pour expliquer les altérations des réseaux fonctionnels. Une perte de balance neurochimique associée à l’expression du transgène pourrait mener aux déficits de maturation des réseaux tel qu’observé dans notre étude précédente.

En conclusion, nous avons approché, et circonvenu les biais lors de nos mesures en établissant un protocole pour réduire l’influence des sources de bruit dans notre signal, ainsi qu’en optimisant un régime anesthésiant propre aux mesures IRMf au repos. Nous avons observé des changements très marqués dans la connectivité fonctionnelle ainsi que dans l’architecture des fibres nerveuses chez les souris ArcAβ, un model présentant une amyloïdose cérébrale. Ces changements dans nos mesures ne corrèlent pas avec l’apparition des plaques, ils les précèdent. Les mécanismes sous-jacents aux déficits fonctionnels chez les souris ArcAβ restent insaisissables. La sensibilité et la reproductibilité des mesures IRMf au repos chez la souris, en particulier dans le contexte des modèles de la maladie d’Alzheimer, permet de mener des études pour démasquer les mécanismes biologiques que reflètent les mesures de connectivités fonctionnelles, tant dans les objets sains que chez les modèles de troubles neurocognitifs. Les avancées permettant de comprendre ce phénomène pourront contribuer significativement à la compréhension des premiers changements physiologiques menant à la maladie d’Alzheimer.
Aim of the thesis

The study of functional connectivity has gained sustained interest in the past 15 years in humans to investigate cognitive processes in the healthy and the diseased brain. Studies with resting-state fMRI have demonstrated robust alterations of functional connectivity in patients with Alzheimer’s disease, as well as in groups at risk of developing Alzheimer’s disease. Yet, the biological origins of the functional connectivity changes in Alzheimer’s disease are poorly understood. Experimental studies in human are limited to non-invasive methods, and suffer from large heterogeneity in the disease expression, co-morbidities, latency in developing the disease, and difficulties to establish an early diagnosis in patients. In contrast, transgenic mouse models of the disease benefit from known onset of pathological events, genetic homogeneity, and a large variety of models presenting different aspect of the disease. As such, animal models offer to understand the causal implication of neuronal networks changes, and may help highlight the salient features necessary and sufficient to induce neuronal network changes in pathological conditions. Additionally, it may provide a model to test the effect of disease modifying treatments in pre-clinical studies.

Functional MRI in rodents, especially in mouse, is rendered difficult by the high needs on both spatial and temporal resolution, which implies low signal to noise ratio (SNR) in the images. In traditional, stimulus-evoked, fMRI, stimuli are applied at defined moments during the recording, and can be repeated in several blocks to increase accuracy of the detection using a general linear model approach. Functional connectivity estimated by resting-state fMRI, in contrast, relies on fluctuation in the baseline signal, and therefore is heavily affected by low SNR. Furthermore, resting-state suffers from a model-free approach, implying that the signal observed cannot be readily separated from other noise source contaminants, such as instrumental and physiological noise. Finally, mice fMRI is performed under anesthesia or sedation, which implies a potential effect on the neuronal network structure. Each of these elements needs to be carefully assessed, in order to studying neuronal network in mice with resting-state fMRI.

In view of these points the goal of this PhD thesis was

- to develop methodology to study functional connectivity in mice
- to apply the method to investigate neuronal network changes in a mouse model of Alzheimer’s disease
- to test its potential as a biomarker.
1 Introduction

1.1 Resting-state fMRI

1.1.1 MRI basics and cryogenic coils

Magnetic resonance imaging is a non-invasive and non-ionizing method to image the body in a pre-clinical and clinical setting. The highlights of the method are a very good soft tissue contrast, as opposed to other medical imaging method, and its versatility to resolve a variety of tissue characteristics that complement anatomical readouts. These contrasts include diffusion of water with diffusion weighted imaging, blood flow measurement, and metabolite concentration mapping with chemical shift imaging [1].

The principle behind MRI is that certain nuclei with a net nuclear spin, of which the most abundant in the body are the protons with nuclear spin \( I = 1/2 \), become polarized when exposed to a magnetic field. The spins of \( I = 1/2 \) nuclei in an object will align either parallel or anti parallel to the magnetic field, with a small excess in parallel orientation, and precess around the main axis of the magnet field. The energy difference between the two states is

\[ \Delta E = \gamma_N \cdot h \cdot B_0 \]  

For typical magnetic fields of a few Tesla, transitions between the two states may be induced by irradiation in the radio frequency (RF) domain. The resonance frequency is given by their so-called Larmor frequency \( \omega_0 \)

\[ \omega_0 = \gamma_N \cdot B_0 \]  

with \( B_0 \) the magnetic field strength, and \( \gamma_N \) a constant dependent on the nuclei of choice. The Larmor frequency is dependent on magnetic field, which is important for spatial encoding of images, as will be discussed later in the text.

Hence, nuclei, protons in our case, can be excited from a low energy state to a high energy state by applying RF at the Larmor frequency, causing the macroscopic net magnetization of the protons, which is the vector sum of all nuclear magnetizations, to flip to an angle with respect to the static magnetic field that depends on pulse duration and pulse power. The precession of the transverse component of the net magnetization around \( B_0 \) can be picked up by a receiver coil and constitutes the MRI signal. This non-equilibrium state cannot persist and the system returns to its original state. In addition, the transverse components of the magnetization will become diphase due to inhomogeneities and fluctuations of the magnetic field, which according to [Eq 2] will give rise to slightly different Larmor frequencies. The rate at which the average magnetization returns to its original state aligned along the magnetic field and the rate by which the transverse components of the magnetization become dephased. The so-called longitudinal and transverse relaxation rates are affected by the local tissue properties and constitute the principal source of tissue contrast in MRI.

Deriving spatial information in MRI makes use of the relation outlined in [Eq 2]. By rendering the magnetic field dependent on the location by applying magnetic field gradients the Larmor
frequency of the protons will depend on its location within the object. Combining the frequency-encoding principle with slice selective excitation pulses allows exciting slices within a three-dimensional object. Within a selected slice spatially resolved signals using the same encoding principle, via frequency encoding in one direction and phase encoding (which is equivalent to frequency encoding) in the second one. Alternatively full three-dimensional data sets are acquired using one frequency and two phase encoding directions. The signal is acquired and encoded in the frequency domain, referred to as k-space. Reconstruction via multi-dimensional Fourier transformations yields the desired image.

The signal can be collected in several ways, which will also affect the appearance of the image. RF signals originating from the transverse magnetization will take the form of free-induction decay (FID). Typically, this signal is not detected, rather the fact is utilized that the transverse magnetization has a lifetime that is longer than the FID duration. Instead, the signal is refocused either by reversal of a magnetic field gradient direction or by application of a so-called refocusing RF pulse. Both processes might be considered a time-reversal. As a result the dephased magnetization will rephase forming an ‘echo’. The echo amplitude will always be smaller than the original amplitude of the FID due to signal loss caused by stochastic processes. This reduction in amplitude is tissue specific and thus an important contrast mechanism. While the signal intensity upon refocusing with a RF pulse using a spin echo sequence is governed by T2 relaxation, the application of gradient-reversal using a gradient echo sequence will yield T2* contrast.

MRI signal depends on the local proton density, i.e. more proton lead to higher signal intensity, and on relaxation processes that characterize the loss of transverse, i.e. detectable, magnetization. Two types of relaxation processes can affect the signal, constituting complementary contrast mechanisms for MRI. Longitudinal relaxation, the rate at which the local magnetization realigns with the main magnetic orientation, i.e. returns back to its equilibrium orientation, is referred to as T1 relaxation. Transverse relaxation, affecting the magnetization component perpendicular to the main magnetic field, describes the loss of phase coherence of the precessing spins due to local stochastic fluctuations of the field, and is referred to as T2 while T2* also includes static inhomogeneities of the magnetic field due to technical imperfection and tissue-related changes in local magnetic susceptibilities.

Image acquisition can be designed such that image contrast is weighted more towards one of these relaxation rates. T1 contrast in the brain is characterized with dark (low signal intensity) ventricles, grey matter appears grey (medium intensity) and white matter appears bright (high intensity). In comparison, T2 weighted images display bright ventricles, while grey matter appears grey and white matter appears dark. Image contrast is tuned by adapting the repetition time (TR) and the echo time (TE). TR is the time interval between two successive excitations for a given slice. Longer TR values allow more spin magnetization to return to the equilibrium state, while short TR values will induce more T1 contrast, as saturation processes will take place in the tissues characterized by longer T1 values. The echo time (TE) is the time interval between the excitation pulse and the occurrence of the echo. Shorter TE will leave less time for the spins to interact with the local environment and hence produce relatively little dephasing, while longer TE induces T2 and T2* weighted contrast.
Due to the need for phase encoding which require sequential image acquisition (k-space line by k-space line), the encoding methods are time consuming. Yet, for functional MRI, images require to be acquired at a fast temporal resolution. While acceleration method exists to partially acquire the image in the frequency domain, these are generally not sufficient to resolve T2* weighted images at the desired sampling rate for functional imaging. Echo planar imaging (EPI) offers a fast read-out method, by repeated refocusing of the echo from a given excitation until a full k-space is resolved. This allows resolving the mouse brain in a 1 second or less. The downside of such a method is a high susceptibility of the image to inhomogeneous magnetic field, such as induced by high field strength magnets, leading to image distortions. In humans, the frontal cortex and the temporal lobes are severely affected due to neighboring air-tissue interface, while in mice, the olfactory bulb, the midbrain and the cerebellum are most affected.

MRI is characterized by an inherently low signal to noise ratio (SNR) due to small energy $\Delta E$ [Eq 1] involved in the resonance process. As discussed the signal intensity is proportional to the number of nuclei contributing to the signal. This becomes a challenge when imaging small objects, such as the mouse brain, which requires high spatial resolution, of about 3 orders of magnitude higher than typically used in humans. Correspondingly the number of nuclei in a volume element (voxel) is about $10^3$ time lower, which constitutes a challenge in terms of SNR achievable. SNR can be increased by increasing the signal or decreasing the noise level or both. High field magnets yield a greater magnetization, and consequently increase the maximum signal achievable, but lead to more image artifacts due to magnetic field inhomogeneities. The noise figure can be reduced by reducing thermal noise contributions from the detector electronics. Thus the advent of cryogenic coils, receiver coils cooled to 30°K to reduce thermal noise, has led to a significant increase in the SNR by a factor of 2 to 3 [2]. The combined use of both high magnetic field strength and cryogenic detector coil provides the SNR values required for increasing the accuracy of functional MRI measures in mice, a critical prelude to conducting functional connectivity studies.

*Figure 1* | The balloon model, complex mechanism behind the BOLD response show an intricate interplay of cerebral blood volume and flow and tissue O2 consumption rate, from Buxton 2012 [3]. Copyright © 2012, Elsevier. Reproduced with permission
1.1.2 The BOLD effect and hemodynamic response

In 1990 Ogawa [4] reported tubular hypointensities in the rat cortex in gradient echo MRI sequences, but not in corresponding spin echo sequences. The first sequence being susceptible to T2* contrast, while the latter to T2, he uncovered the contrast mechanism in MRI of the paramagnetic effect of deoxygenated blood. Ogawa suggested this could be used to follow hemodynamic response using MRI, using the blood oxygenation level dependant contrast (BOLD). The method has allowed measuring non-invasively the functional response to a stimulus, with a good spatial resolution and whole brain coverage. It gained a very rapid popularity among the neuroscience community, as BOLD fMRI uses an intrinsic contrast, and did not depend on the administration of contrast agent as in MRI bolus tracking, or radioactive compounds, as used with single photon emission computer tomography (SPECT) or positron emission tomography (PET).

The BOLD response to local brain activation reflects a complex effect. Local brain activity is linked to increase glucose and O2 consumption, yet the BOLD response is marked by an increase in the fMRI signal in putatively active regions, denoting an increase in blood oxy-hemoglobin to deoxy-hemoglobin ratio. In fact the response displays a brief drop in signal at the onset of the stimulus, a drop in blood oxygenation to deoxy-hemoglobin ratio, followed by a delayed increase in signal and plateau, before the signal returns to baseline values, or present a brief undershoot. The shape of the response is considered to originate from an imbalance between local O2 consumption rate, response in cerebral blood volume and flow, as well as to mechanical resistance in the capillaries, causing the typical BOLD response shape (Figure 1)[3, 5].

The BOLD signal has been considered to reflect neuronal activity, and has been root in most fMRI experiments. Yet the underlying molecular, cellular, and biophysical mechanisms remain elusive. Early experiments comparing BOLD fMRI experiments on vision stimulus in humans with similar experiment in monkeys during local electrophysiological measures observed a correlation pattern between neuron spiking response in the visual cortex of monkeys and BOLD response in humans [6]. Later, a combined BOLD and electrophysiological experiment has demonstrated the BOLD pattern is actually better explained by the local field potential in monkeys [7-8], which is considered to reflect the local synaptic activity, i.e. the input and integration of the neuronal impulse, rather than spiking activity, reflecting the local output.

With the growing interest of BOLD measurement at rest, a need became apparent to confirm a link between electrophysiological and BOLD signal fluctuations. Networks observed with resting-state fMRI were corroborated with both electroencephalography and magnetoencephalography [9], indicating an electrophysiological basis underlying resting-state fMRI signal. Local electrophysiological recordings have also been performed in monkeys [10] and rats [11-13]. Electrophysiological recordings in monkeys revealed local correlation between BOLD signal fluctuation at rest and local field potentials, but also revealed a low but consistent correlation between global BOLD signal across the whole cortex and local electrophysiological recordings. In rats, it was found that the correlation between electrophysiological and BOLD signals was retained in the anesthetized state using propofol [11], medetomidine [12], or isoflurane [13].

Overall, evidence suggests that rs-fMRI measures aspects linked to electrophysiology, despite discordance with regard to the relevant frequency bands implied in animal studies. The details are
not clear, especially regarding the observation of an underlying global BOLD signal correlation with local electrophysiological recording [10], or the origin of the signal fluctuation themselves. However, while a neuronal origin is assumed during resting-state fMRI, the absence of a model, as in stimulus evoked fMRI, renders the control of the spurious noise difficult. As such, the signal observed cannot be solely interpreted as of neuronal origin. Additionally, the varying modulation of neuronal signal by an altered vasculature response in the disease state as well as in under different anesthetic regimens may add further complexity in the results interpretation. This remains a major pitfall of the method, which will be addressed in the following points.

1.1.3 Brief history and theory behind resting-state

In 1995, Biswal et al. [14] performed a standard finger tapping experiment with both hand during fMRI recording, which revealed bilateral activation in the motor cortex. The insight was to investigate the correlative effects of the signal during baseline sessions preceding the finger tapping from one motor region. The signal from the left motor cortex correlated with the signal from the right motor cortex, revealing that the two structures shared aspects of the BOLD signal in the absence of task, referred as functionally connected (Figure 2). The ease of the approach, the measurements can be done in less than 5 minutes, the exploratory nature of the results, and the fact that no paradigm or stimulus is required for the studies, implies that clinical patients with difficulties to follow instructions can be investigated as well.

Figure 2 | Results from Biswal’s seminal paper on functional connectivity at rest shows spatial activation map to finger tapping (left) and cross-correlation of signal fluctuation with respect to a seed in the right motor cortex during the baseline (right). From Biswal et al [14]. Copyright © 1995 Wiley-Liss, Inc., A Wiley Company. Reproduced with permission

Rapidly, resting-state fMRI was used to describe a set of about a dozen functional networks, brain regions correlating with one-another in their temporal signal, which can be robustly observed across subjects and studies [15]. These networks follow the delineation of known brain and cortical structures, and appear to reflect both sensory and cognitive processes (Figure 3). In human, the sensory processes include the somatosensory motor network, which include S1, S2, M1 and M2,
two to three networks in the visual cortex, and one network in the auditory cortex. Sensory networks seem to include both primary and secondary cortical structures, and are organized bilaterally. Higher order cognitive networks include the left and right attention networks, which include the lateral posterior cingulate, middle frontal and orbital, superior parietal, middle temporal cortex, and the default mode network (DMN) [16], which gathers the posterior cingulate cortex, the precuneus, the medial frontal lobes and the medial temporal lobes. The latter has gained considerable attention by the research community. It has been linked to inner voice, self reference, and consciousness [17]. It is the most robust network detectable in human at rest, the most metabolically active in absence of a task [18], and seems to be anti-correlated with task related regions, i.e. the signal amplitude is reduced in the DMN when it increases in task related regions [19].

In addition to the topological organization, which follow known anatomical structures and anti-correlative organization between DMN and task driven regions, additional properties of the signal may be derived from its temporal structure. Correlation between brain structures in a given network is best described in the signal by the fluctuations in the 0.01Hz to 0.15Hz frequency range. The nature of this frequency band is elusive. Current hypothesis suggest the hemodynamic response, as detected by BOLD fMRI, applies a low frequency transformation to the high frequency neuronal activity, leading to the temporal profile observed. In addition to be spatially coherent only in given frequency band, the resting-state fMRI signal typically follows a 1/f frequency distribution, i.e. lower frequencies have higher power than higher frequencies, whereas random noise, known as
Gaussian or white noise, follows a flat power distribution across all frequency ranges. This property allows, to some extent, differentiating biologically relevant signal from random, system-induced, noise, although the exact nature of the low frequency fluctuations have not been pinpointed.

Networks are organized in what seem to be biological relevant structures, which are distinct for each sensory systems, and higher order cognitive systems. Evidence confirms that networks are reflecting cognitive aspects in both healthy and disease condition. For instance, higher connectivity within the DMN, a central network linked to inner voice and mentation [17], showed higher correlation values in schizophrenic patients showing positive symptoms [20]. Evidence also suggest the networks observed in resting-state fMRI experiments have electrophysiological origins as similar networks were recorded with both electroencephalography and magnetoencephalography [9] and overlap with the activation patterns of task evoked fMRI [21]. Further, the BOLD signal was also correlated with local electrophysiological recordings in the monkey [10], and in the rat [22]. This positions the functional networks, as devised by rs-fMRI experiments, in an intermediary position between local neuronal activity and cognition, and may as such offer to bridge the two entities.

1.1.4 The different resting-state read-outs

Stimulus-evoked fMRI has been used for many years, and its method of analysis has been rather invariant. A hypothesis-driven approach is taken, a model is created to explain the response of certain regions according to a given stimuli design and timing. This model is compared across all brain voxels within a general linear model framework, which returns statistical interpretations for each voxel of the brain, at the individual level in the form of a statistical map of activation. The difficulties of the method lie in designing an appropriate model to explain the observed signal, and to correct for the false positives induced by the multiple statistical comparisons performed.

Resting-state fMRI data analysis is a model-free approach. Spontaneous fluctuations in the signal are compared across to brain to generate functional connectivity maps. The term of functional connectivity encloses a large variety of methods, principally focusing on linear correlations, as opposed to effective connectivity, which aims at identifying causality. Functional maps can be devised is several fashions. Seed-based analysis was the first method applied in by Biswal in his 1995 paper [14]. A region-of-interest (ROI) is selected; the corresponding time series is extracted and compared, usually by linear correlation, with the time series of all other voxels in the brain image. The result is a map of the brain displaying the absolute or relative strength of the correlation with this seed region. The interpretation is rather straightforward; regions displaying stronger correlations are interpreted as having higher functional coupling with the seed region. The method is however very dependent on the pre-processing done on the data, in the form of linear regression of noise terms in the temporal signal. The signal observed can be decomposed into the linear addition of several factors, which include a signal of neuronal origin, physiological nuisance factors, such as changes in blood pressure, motion or ventilation of the animal, and scanner induced noise. These will be detailed in section 1.1.5. Not removing the contribution of systemic nuisance factors by appropriate pre-processing would result in inflated correlation measures, or false positive correlations to regions unrelated to the seed region. In contrast, it is possible to diagnose bad nuisance correction from correlation maps, as cortical regions exhibiting correlations...
with ventricles or muscle are likely to do so via systemic effect, motion or vascular for instance, rather than neuronal interactions. A further bias of seed-based analysis is introduced by the operator-interactive positioning the seed, which will inherently involve some variability and, in turn, affect the results and render them difficult to compare with other results in the literature. Yet, the simplicity of the method, as well as the ease of interpretation of seed-based analysis renders it a highly praised method of analysis.

Independent component analysis (ICA) based tools are another widely used method to derive information from data from resting-state fMRI scans [23-24]. The method is a multi-variate method to explore the data, and reduce it into a set of temporally coherent components, that are a set of maps displaying regions that exhibit similar temporal patterns. Each component is represented by both a vector, reflecting the temporal pattern, and a spatial map, representing the occurrence of this temporal pattern. The algorithm aims at decomposing the signal into orthogonal, independent component. The user bias is limited to selecting the number of desired component required to explain a dataset, which typically varies between 10 and 40. Artifacts, such as motion, or vascular change often appear spatially and temporally separated from the other components, either in the brain boundaries for motion, or in the ventricles and arteries for vascular effects. Difficulties reside in identifying components into known networks or artifacts, as well as performing group analysis, as different components, and thus functional networks, may not be represented identically into each animal or subject. Grouping methods which performs ICA algorithms on whole groups of images rather than individual animals exists and are more robust than single-level analysis, but the interpretations of the results is however rendered slightly more complicated, as the underlying algorithms are complex. The method is however robust, and does not necessitate a priori knowledge on the data or regions of interest.

Network analysis, or graph analysis, is the third most used method to derive information from resting-state fMRI data and infer functional connectivity [25]. Graph analysis starts with defining correlative values between ensembles of brain regions, usually derived from an anatomical atlas. The correlative values between each pair of regions are compared to a threshold with values exceeding the threshold value defining a functional link, referred to as edge, between regions, referred to as nodes. Edges and nodes can be represented visually in the form of matrices, or in the form of maps, both being visual representation of the networks. Graphs can be further analyzed with mathematical tools from the field of graph theory, which can derive the mean path length, the average length of connections, or the small-world properties of a network, i.e. how small clusters of locally inter-connected regions send few edges to other clusters. Such measures can be used to assess the efficiency of network, and have been shown to be altered in several brain disorders [25].

In addition to deriving functional connectivity maps, i.e. maps which indicate to what extent sets of regions are functionally coupled, other methods that do not infer on linear connectivity offer different metrics to complement the analysis of rs-fMRI data. As mentioned in section 1.1.3, the resting state signal used to derived functional connectivity is bounded in a frequency range of 0.01Hz to 0.15Hz. Amplitude of low frequency fluctuation measures the power of frequency bands within the 0.01Hz to 0.15Hz range and allows constructing a map of frequency distribution in the
brain; this has been used as a marker in some cases, such as a readout for chronic pain [26]. However, while being a useful tool for visualizing and inspecting the data without prior assumptions, the method is hampered by difficulties in interpretation. While frequency power comparison has been widely used and studied in electroencephalography, the meaning to different frequency power in the rs-fMRI signal remains speculative.

The methods described above assume that connectivity follows a linear scheme; however, there is no evidence that is in fact the case. Some methods, such as synchronization likelihood [25] or comparative approximate entropy [27], aim to overcome limitations imposed by assuming linearity by highlighting the non-linear interactions involved in the temporal signal of region pairs. These methods are however computationally intense, and the algorithms are often difficult to be understood by non-experts, while the benefits of such methods over linear methods such as seed-based analysis are not obvious, and interpretation of the results remain speculative at this stage.

Finally, methods have been developed to infer not only correlation between regions but also a causality link, i.e. a directed relationship between regions. This is the case, for the Granger causality method, which compares signal from one region with the delayed signal in another regions and returns a probability of causality. While this method is attractive, it appears difficult to apply in fMRI signal, as regions are thought to interact rapidly together, while technical limitations in fMRI impose a temporal resolution of 1 to 4 seconds, possibly not sufficient to capture the rapid directed interaction between regions. Yet, the method is attractive in the sense that correlation between A and B does not necessarily imply that they are actually structurally connected, but could be both coupled to a region C. While a simple functional correlation analysis would give the illusion of actual connection, a causality analysis might indicate that the coupling between regions A and B is relayed via a third region C.

The large range of methods available to study and derive information from rs-fMRI data may render comparisons between studies difficult. Additionally, the interpretations may not always identical for different analysis methods, but may depend on the approach chosen. Considering the use of different methods in the same study may combine the benefits from the different approaches, which may lead to a more consistent and robust interpretation. When interpreting the data, it remains essential to consider the bias of each approach and understand the limitations.

1.1.5 The bias of noise in resting-state fMRI
Functional MRI aims at measuring the hemodynamic response, typically the BOLD response, linked to neuronal response. Yet, this measure is contaminated by several noise sources, which can dramatically affect the results and the interpretation of a study, in particular in the case of resting-state fMRI. Resting-state data analysis is usually performed using a model-free exploratory approach as compared to the model-based hypothesis-driven stimulus-evoked fMRI. In this sense, the operator bias is reduce, i.e. the confounding error introduced by the operator when designing and selecting the model. However, due to the inherent impossibility to predict the true signal components in a resting-state data set, it is difficult to segregate meaningful information in the signal from confounding noise sources [28].
In stimulus-evoked experiments, the observed signal $Y$ can be denoted as the weighted sum of a set explanatory vectors $X$ with weighting factors $\beta$. Apart from the true stimulus evoked contribution the vectors capture the contributions of systemic effects such as overall motion, respiration and cardiac cycle. The system is complemented by an error term $\varepsilon$, hence

$$[\text{Eq 3}] \quad Y = \beta_1 X_1 + \beta_2 X_2 + \ldots \beta_n X_n + \varepsilon$$

Under the assumption of normality, a proper model should lead to an error term $\varepsilon$, which comprises only random normally distributed residuals. A model can be considered valid when the residuals of an analysis follow such a distribution [29]. Several tools allow testing the validity of the assumptions based on residual analysis, such as SPMd [30], a plug-in diagnostic tool for the fMRI data analysis software SPM. In this way, nuisance factors may be contained and eventually removed from the analysis. In fMRI measures, the major noise sources are of three kinds: 1) overall object motions during measurement, 2) cyclic contribution arising e.g. from respiratory and cardiac effects, and 3) scanner induced factors [28-29].

To minimize overall motion, animals are restrained during measures by means of ear and incisor bars, and human patients are asked not to move during scanning sessions. Despite these considerations, small displacements of the object inadvertently occur during acquisition, resulting in marked effects in the data. In stimulus-evoked fMRI, such motion effects add variance to the signal, which will mask the response and thus reduce statistical inference [29, 31]. In the case of resting-state fMRI, object motion adds spurious correlations between regions, as motion effect will affect all voxels in the image and lead to shared variance. As part of the pre-processing pipeline for fMRI data, motion vectors are estimated during the temporal realignment of the images, a method aimed to reposition the images series and thus correct for the images motion. An algorithm is used to estimate the displacement of the object, the brain, over time using a 6 parameter estimation, 3 for translations (along x, y, and z coordinates) and 3 for rotations (yawl, roll, and pitch) [32]. While motion correction may perfectly realign the object in the image series, residual correlation with the motion parameters persist in the voxel time series, leading to spurious correlations in resting-state analysis, requiring further temporal correction [31] by means of linear regressions. In rats, motion of the object and its residual effect on the voxel time series was shown to represent 22% and 12% of the total signal variance in the temporal time series [33], thus representing the major source of physiological noise.

Cycle related nuisance induced by heart beats and respiration represent another source of physiologically related noise in the fMRI data set [34]. Breathing occurs in human at about 12-18 beats per minute (bpm), or 0.2Hz and 0.3Hz, the heart rate lies between 60 to 100 bpm, or 1Hz to 1.6Hz. These frequencies are higher than the resting-state frequency domain of interest of 0.01Hz to 0.15Hz. In theory, band pass filtering would remove the contribution of both cycles in the data. However, data points are sampled discretely rather than continuously, which implies a maximum frequency that can be sampled, referred to as the Nyquist frequency, which is given by

$$[\text{Eq 4}] \quad f_{\text{Nyquist}} = \frac{1}{2} f_{\text{sampling}}$$
with $f_{\text{sampling}}$ being the sampling frequency (Nyquist-Shannon theorem). Higher frequencies than the Nyquist frequency will aliased, i.e. folded into the frequency domain band-limited by $f_{\text{Nyquist}}$. For instance, if a brain is sampled every 3 seconds, which is a common value for human rs-fMRI studies, then the sampling frequency is 0.333Hz, and the corresponding Nyquist frequency is 0.166Hz. That implies that only frequencies of up to 0.166Hz will be fully sampled in the signal, while higher frequencies will be present in the signal in an aliased form, which will not be affected by band pass filtering. This effect is more striking in rodents, as the breathing rate in mice during the studies is either set at a 90 bpm with a mechanical ventilator, or nearing this value in free-breathing anesthetized animals, and the cardiac cycle approximates 300-400 bpm. This implies frequencies in the range of 1.5Hz and 6.6Hz respectively. To resolve the frequencies necessary to remove the nuisance of noise in mice by means of band pass filtering, it would be necessary to have a temporal resolution of 70 ms, which is exceeding the currently achievable limits of current MRI systems.

Methods have been devised to overcome this limitation, using either data-independent approaches, independent measures for cardiac and respiratory cycles, or data-dependent approaches for modeling the noise sources from the images directly. Both breathing and cardiac cycles can be recorded with external MR compatible hardware, such as breathing belts or ECG electrodes. Yet, the signal from these devices cannot be readily used to yield nuisance vectors to be regressed from the rs-fMRI signal. The sampling rate of fMRI scans is in the order of 1s to 3s (1Hz and 0.33Hz) while it is of the order of 0.01s to 0.001s (100Hz and 1000Hz) for ECG and respiratory recordings preventing the immediate correspondence of the two signals. Consequently, methods of signal modeling and data reduction, such as RETROICOR [34], are used to render breathing and cardiac signals comparable to the fMRI signal, allowing for signal correction using linear regression.

Alternatively, data-driven methods can be applied to estimate the noise sources from respiration and cardiac cycles from the fMRI data itself. Regions such as white matter and ventricles do not show coherent and relevant signals of neuronal origin. Consequently, signals within these regions are dominated by contributions from non-neuronal, physiological noise sources, and can therefore be used to derive nuisance vectors using data reduction schemes such as principal component analysis methods, as used in e.g. CORSICA [35]. Estimations in rat resting-state fMRI suggested, however, that contributions from both cardiac and respiration effects were minimal, accounting for only 1% and 5% of the variance in the signal [33].

The third noise source, induced by the MRI spectrometer, becomes manifest in two ways, signal drift over time, which is reflected as a low frequency component in the rs-fMRI signal, and random noise measured as SNR. Scanner induced noise depends on several variables, including the magnetic field strength, type and configuration of the coil, images parameters, image acceleration scheme, and system stability. Signal drift can be removed from the signal by means of a high or band pass filter. Random noise in the signal is related to measurement error, independent and evenly distributed, and in that sense included in the error term $\varepsilon$ in equation [Eq 3]. It is present as an unpredictable random error, which differs with each voxel. While the magnitude of random noise can be estimated, it cannot be differentiated from the remaining signal. Functional MR image inherently have low SNR, due to the high demands in temporal resolution. In the case of stimulus
evoked fMRI, the SNR contributions is of minor importance as additional stimulation blocks can be added to the paradigm design to render the statistical inference more robust. In resting-state fMRI experiment, on the other hand, SNR plays a more intricate role, as the random fluctuations induced by the system will add to the BOLD fluctuations of interest in the measured signal, and will thus be indistinguishable from the signal of interest. This result in a lower apparent correlation between regions displaying synchronization, depending on the level of noise compared to the amplitude of the BOLD fluctuation. SNR can be increased by data averaging, as the error terms will differ with each image, leading to a better estimate of the true signal. However, this approach is time consuming, as SNR improves with the square root of time, and thus is not appropriate for fMRI, which demands for high temporal resolution. Alternatively, SNR can be increased by decreasing the image resolution, i.e. larger voxels resulting in higher signal, while noise remains constant. This is, however, is also not a desirable approach for fMRI, which aims at resolving small structures in the brain. Higher magnetic field strength increases MR signal due to a higher degree of polarization of the nuclear spins. Although higher field strength leads to higher SNR, magnetic field inhomogeneities also increase with field strength, leading to more severe image distortions, especially for gradient-echo EPI images used for fMRI, which are highly susceptible to such effects. The advent of cryogenic coils presents an interesting alternative to using higher field strength [2]. Cooling the RF coil liquid allows reducing the thermal noise by a factor of two for coils corresponding to the dimension of a mouse head without compromising the image spatial or temporal resolution, or causing additional image artifacts as higher magnetic field strength.

Global signal regression is an alternative to estimating each noise source individually [36]. A large portion of the rs-fMRI studies use it as a mean to correct for undesired noise, under the assumption that noise signals will distribute throughout the brain, while local neuronal activity will not, and thus will not be apparent in the global signal. While it is an easy method to apply, the use of such a method is a matter of debate, as global signal regression might artificially induce the reported anti-correlation in the fMRI signal between task-related regions and rest-related regions [19, 37]. Additionally, combined electrophysiological and fMRI recordings have suggested a global BOLD correlation across the brain with local neuronal activity [10], in contrast to the assumption used in global signal regression strategy that neuronal related BOLD response is locally confined.

While several approaches exist to estimate and remove nuisance terms from the rs-fMRI signal, methods to qualitatively assess the effectiveness of such corrections are limited if not lacking. The principal method has been to measure the effect of nuisance vector regression on the temporal variance of the rs-fMRI time series [33], as the total variance can be considered an addition of the variance of different noise sources and neuronal contribution. While it is easy to measure variance, the remaining variance following a nuisance vector regression may not be exempt of further contaminations. In contrast, visual inspection of the functional correlation maps offers some insights. Signal from grey matter should not correlate with non-grey matter regions, such as ventricles, white matter or muscle, to be considered as of neuronal origin. Correlation involving
tissue boundaries is an indicator of a motion effect. The very nature of the resting-state measures, which is inherently a model-free approach, implies that the differentiation of signal with respect to noise is difficult, and requires particular caution in the analysis and interpretation of the results.

1.1.6 The bias of anesthetics

It is common practice in animal experiment to use anesthetics in order to help restrain animals, and to reduce animal distress. While both monkeys and rats have been trained to remain still while undertaking awake fMRI measures [38-41], mice, on the other hand, have been less resilient to an awake protocol [42]. More specifically, isoflurane [43], medetomidine [44-45], and ketamine [46], were used in earlier rs-fMRI studies. A comparison of these studies reveal that while different anesthetics appeared to yield similar functional network organization, medetomidine induced a different effect, as cortical structures appears as unilateral networks, compared to bilateral structures observed both under isoflurane and ketamine regimes. Anesthetic use constitutes a severe limitation to in vivo neuroscience experiments, as the brain is directly being affected by the anesthetic, which imposes a bias to be considered during experimentation. In addition to effects on neurotransmitter systems, anesthetics also have a systemic effect on the animal physiology, on heart rate, blood pressure, and vasoreactivity. In particular, anesthetic may affect neurovascular coupling, which is the intrinsic mechanism underlying BOLD fMRI signals.

Study in humans use anesthetic to gain insights into the modulation of neuronal networks under constrained consciousness [47] or to understand the effects of commonly used anesthetics [48-54]. The motives are therefore different to those used in animals, yet the insights can be transferred. Sedation and light anesthesia have been shown to dampen the correlative strength within networks linked to higher order cognition, such as the default mode network [47, 55], and the salience network. However, and surprisingly, networks linked to sensory processing, such as the visual networks and the somatosensory network were reported unaffected by sedation, sleep or anesthesia, or were, in some cases, reported to increase in correlative strength [53]. These reports in humans are consistent with the current views, that higher order cognition, such as consciousness is first lost during sedation, while other more basic processes persist. This enhances the possibility that sedation or light anesthesia in animals produce valid results, at least within the simpler networks in the brain.

In rats, measures in the awake state were compared to measures in the anesthetized state. Strong similarities in networks have been reported between isoflurane anesthesia and wakefulness [39], although anesthesia seemed to dampen the strength of functional correlations. On the other hand, electrophysiological measures performed in parallel with fMRI in rats under anesthesia revealed that correlation between the local BOLD fluctuations and the electrophysiological recordings are retained with several anesthetics, such as propofol [11], medetomidine [56] and isoflurane [13]. These results in rats suggest aspects of functional connectivity can be retained during the anesthesia.

Discrepancies in the effect of various anesthetics highlight the need for identifying a proper anesthetic regime in small animal studies. Such a regimen should maximize the similarities of functional connectivity patterns known in the awake rat [40], monkeys [57], and human [15], while minimizing side effects, and being non-lethal. However, studying anesthesia effects in mice
also offers an opportunity to gain insights into the effect of different anesthetics and their effects on the brain as a whole in a controlled environment.

### 1.2 Alzheimer’s disease

Alzheimer’s disease (AD) is progressive neurodegenerative disease, the most common form of dementia in the elderly, which affects cognition in the elder population. In 2012, it was affecting 24 million worldwide [58], of which 113’000 in Switzerland and current trends suggest this will increase further in the coming decades. Today, there is no cure to the disease. Treatments to temporally alleviate the cognitive symptoms exist, but have a mild effect. The cost of care for the patients is currently a heavy burden to the medical systems in developed countries. It has been estimated by the Swiss Alzheimer association to reach 7 billion CHF in Switzerland. As the median age of the population in developed countries is set to increase, the impact of the disease is estimated to gain considerable proportion of the health budget in these countries.

The disease was first characterized by Alois Alzheimer in 1907, in a patient aged 51, presenting memory loss and troubled emotions [59]. Following autopsy, abnormal structures in the brain were put in evidence, neurofibrillary tangles and senile plaque structures, which have become the key characteristic in Alzheimer’s patients (Figure 4).

![Figure 4](image)

**Figure 4** | Histopathological features present in Alzheimer’s disease show amyloid plaques (A, C, D), neurofibrillary tangles (B, C, E), and lewy body inclusion (E), a feature present in some cases of AD. From Serrano-Pozo et al. [61]. Copyright © 2010, Cold Spring Harbor Perspectives in Biology by Cold Spring Harbor Laboratory Press. Reproduced with permission

The senile plaques, also referred as amyloid plaques, come in distinct forms. They are found in the brain parenchyma, or surrounding the vascular tissues, as cerebral amyloid angiopathy. The parenchymal plaques themselves are present either as diffuse or dense core plaques. Dense
plaques bind differently dyes such as thioflavin and Congo red compared to diffuse plaques, while Aβ targeted anti-bodies allow visualizing both types. Aβ protein, a natural cleavage product of the amyloid precursor protein (APP) by the β- and γ-secretase complex, has been identified to be enriched in these structures [60]. Aβ peptide can be cleaved differently, as to produce different species; the most common comprise 40 and 42 amino acids (Aβ40 and Aβ42). Due to extra hydrophobic amino acids, the Aβ42 form is more prone to aggregation and is found enriched in amyloid plaques [61]. Plaques are a central aspect of the pathology, and a necessary condition to confirm the AD diagnostic.

Neurofibrillary tangles are the second major histopathological feature of AD, described in Alois Alzheimer’s initial report [59]. They consist of filamentous inclusions within cells, or retaining the shape of lost cells. The major constituent of these filaments are microtubule-associated Tau proteins, in misfolded and hyperphosphorylated state [61]. Tangles are distributed differently from amyloid plaques, starting in the medial temporal lobes, and limbic regions, before reaching associative cortical areas.

In addition to plaques and tangles, AD involves neuroinflammation, in the form of reactive glia, and neuronal death and synaptic loss, leading to severe bilateral brain atrophy, starting in the medial temporal lobes [61].

The major risk factor for developing the disease is age. In North America, the incidence per age group doubles every 5 years, from 0.17% at 65, to 2.92% at 85 [62]. In addition to being linked to age, epidemiological studies have revealed a gender effect in the disease distribution, women representing 68% of the cases [62]. Risk factors for the disease overlap with those of cardiovascular disease, including obesity, type II diabetes [63], smoking, and traumatic brain injury [58]. On the other hand, higher education, and the overall “cognitive reserves”, as well as Mediterranean diet and physical exercise have been linked to a reduced risk [58]. Earlier large genome wide association studies have highlighted an allele encoding for the APO protein, APO ε4, as a risk factor in the disease [64]. APO proteins are involved in the transport of lipids, suggesting a metabolic cause underlying the disease. Later studies and meta-analysis have highlighted several other risk alleles and single nucleotide polymorphism in genes involved in the metabolism and in the immune system [65-66].

Despite decades of research, AD continues to elude the scientists. The etiology is unknown, and while several treatments have been proposed, including antibodies directed at amyloid plaques, and antioxidants, none have been demonstrated to significantly alter the disease progression. The need for disease modifying agents is an international priority, considering the progression of the aging population worldwide. Current views to treating the disease consider that mild to severe AD might be too late to effectively rescue the cognitive losses. The priorities are to better understand the early events leading to AD, and finding sensitive methods to diagnose the disease in its early stages, while monitoring the effects of potential treatments during clinical trials.

1.2.1 The different hypothesis of the disease

Alzheimer’s disease is a complex brain disorder and the pathological elements leading to the disease are poorly understood. A large number of hypothesis have been formulated, based on
epidemiological risk factors, genome wide association studies, molecular and cellular in vitro experiments, experiments on animal models, neuroimaging, and histopathological findings in humans. While not all hypotheses can be discussed within the scope of this introduction, the selected elements are relevant with respect to the work presented, as synaptic activity, axonal integrity, inflammation, and vascular reactivity effects are observable with the MRI applications described in this work. Other important elements of AD pathology include mitochondrial dysfunction, oxidative stress and loss of calcium homeostasis, will not be discussed during the frame of this work, but are amply discussed in selected reviews [67-69].

1.2.1.1  **Aβ oligomers and amyloid cascade hypothesis**

The Aβ peptide, a cleavage product of the APP, is considered to play the preponderant role in Alzheimer’s disease pathology. The peptide was found enriched in senile plaques [60], linking it to the pathology. Further evidence of the preponderant role of Aβ linkage to AD is the presence of mutated genes encoding for the APP protein [70-71], as well as proteases involved in APP processing in familial early-onset AD, and the occurrence of triplicated APP gene in Down syndrome, leading to senile plaques in mid life [72]. Research has focused on the role of Aβ peptides, which have been shown to have a high propensity to aggregate into multimers, oligomers, proto-fibrils, fibrils, and amyloid plaques [67]. While earlier versions of the amyloid cascade hypothesis place the amyloid plaques in a central role in the AD pathology [73-74], more recent revisions consider smaller forms of the peptide, oligomers, as a core feature leading to cognitive deficits [75]. Oligomers have been shown to affects synapses in several ways, such as affecting long term potentiation (LTP) [76-77], leading to abnormal dendritic spines [78], and potentially causing epileptically burst-like periods in transgenic mice over-expressing human APP (hAPP) [79]. In addition to actions at the synaptic level, Aβ peptides and plaques have been linked to a plethora of effects, such as disrupting axon guidance [80] and loss of calcium homeostasis [81]. Studies in humans with positron emitting tomography (PET) revealed a significant proportion of cognitive healthy human to carry amyloid plaques [82], while some cases of atypical cases of Alzheimer’s disease are negative for amyloid PET scans. In addition, amyloid plaques burden has been loosely linked with cognitive deficit and early AD apparition [83]. Animal models involving the transgenic hAPP gene do present amyloid plaques and mild cognitive deficits, but usually do not present element of neuroinflammation or cell death. Decreasing Aβ levels with antibody targeted at the peptide restores LTP [77] and memory impairments in transgenic mice, however early clinical trials of Aβ immunization have shown minimal effect despite the removal of amyloid plaques from the brain parenchyma [84]. While Aβ retains a preponderant role in the various hypothesis of AD, it appears that the presence of the peptide itself, especially in its aggregated form as amyloid plaque, might not be sufficient to elicit AD.

1.2.1.2  **Axonal dysfunction and Tau hypothesis**

Neurofibrillary tangles are another histopathological core feature of AD pathology. Tau proteins, a microtubule binding protein, have been associated with AD in the form of hyperphosphorylated protein, Tau multimers inclusions and in tangles [85]. Often considered a by-product of AD pathology, Tau has been linked to other neurodegenerative disease, including frontotemporal lobar degeneration, sporadic corticobasal degeneration, and parkinsonism linked to chromosome 17, making it a potential causal element of neurodegeneration [86]. The occurrence of neurofibrillary
tangles has been shown to better correlate with cognitive decline than plaques [87], while abnormal Tau has been demonstrated to be also present in asymptomatic subjects [88]. In hAPP transgenic mice, lower Tau levels are linked to reduced behavioral impairments [89], prevented neurotoxicity and behavioral deficits in the absence of Tau [90], but did not affect the level of amyloid plaques in mice [89]. It has been demonstrated that axonal stabilizing agents, such as taxol and Epothelione D have the potential to restore behavioral deficits in Tau transgenic mice, supporting Tau dependent axonal failure to partake a key role of AD pathology [91-92].

1.2.1.3 The neuroinflammation hypothesis
Axonal failure has been linked to neuroinflammation, another histopathological pillar of AD pathology [93]. Reactive glia was shown to correlate with neurofibrillary tangles levels but not with amyloid burden [94]. Recent genome wide association studies have linked sporadic AD occurrence with proteins involved in the inflammation process [65-66]. Chronic inflammation is linked to an increase in APP production in hAPP transgenic mice [95], suggesting a potential causal relationship between inflammation and Aβ pathology. While positive for amyloid plaque PET scans is a feature in some cognitively healthy human subjects, neuroinflammation was only present in subjects with AD [96], which is corroborated by anti-correlation between cognitive levels and microglia activation in AD patients [97-98]. Epidemiological studies have linked anti-inflammatory drugs, non-steroid and steroid, usage to decreased incidence of the disease [99], however, clinical trials have so far failed to show a benefit of anti-inflammatory treatment in mild AD.

1.2.1.4 The vascular hypothesis
Cerebrovascular related dementias enclose several diseases with vascular origin causing cognitive impairments, such as subcortical ischemic vascular disease and post-stroke dementia. This category represents the second most common forms of dementia in the population. However, vascular dementia strongly overlaps with cases of AD, highlighting the vascular implication in the disease [100]. Cerebral amyloid angiopathy, the amyloid deposition of mostly Aβ peptide around the vasculature, is present in over 80% of the AD cases [101], and in some cases is sufficient to induce dementia, in the absence of parenchymal amyloid plaques or tangles [102]. Furthermore, several risk factors for AD such as high blood cholesterol [103], type II diabetes [63], or atherosclerosis [104] have vascular implication, while approaches decreasing the risk of cardiovascular diseases such as physical exercise and diet are linked to reduced risk of AD [58]. Altered cerebrovascular structure may act in AD pathology in several ways, such as inducing neuroinflammation [105], altering neurovascular coupling, reducing blood flow, thus neuronal access to nutrient and oxygen, or blood brain barrier function and impact the clearance of Aβ peptide [106]. It remains one central hypothesis underlying the pathological cascade in AD.

1.2.2 Biomedical imaging to study AD
The brain is a difficult organ to access by conventional methods in medicine. The skull forms a difficult barrier to penetrate, and invasive procedures should be avoided due to the fragile structure of the brain and the dire consequences of complications. Biomedical imaging tools offer to perform measures on the human brain in a minimally invasive approach. Imaging methods can be classified into three categories: structural, molecular/metabolic, and functional, though the methods are typically used in combination. Functional imaging in AD will be treated of the following sub-section.
Structural imaging aims at resolving the brain with high soft-tissue contrast in order to distinguish several structures within an object. Computer tomography (CT) is an X-ray based method for structural imaging. It has the advantage of being fast and having a high spatial resolution, but uses ionizing radiation, which can induce tissue damage, and yields poor tissue contrast, which is based on the attenuation of x-rays upon the passage through tissue. Novel CT methods that exploit phase information hold promise of overcoming this limitation. Magnetic resonance imaging, on the other hand, is slower in acquiring images, but uses non-ionizing radiofrequencies, and provides very good tissue contrast. In fact, the different contrast mechanisms of MRI allow resolving a large variety of different features, such as gray and white matter, fiber orientation, cerebrospinal fluid, cerebral vessels and is therefore sensitive for detecting abnormalities within these structures. Structural imaging is a core feature in modern medicine, and is used to diagnose a large variety of brain disorders. Recommendations for diagnosing AD suggest structural imaging in patients to exclude other causes of dementia such as bleedings or tumors. Moreover, being a non-invasive method, structural imaging can be performed repeatedly, therefore gaining insight into the disease progression.

In addition to observing gross changes of structures in the brain, imaging has been used to derive complementary information such as local brain volume changes, which can be used to estimate atrophy rates. In AD, both gray and white matter is subject to atrophy [107]. Atrophy could be detected before the onset of dementia [108], and was observed in healthy subject converting to mild cognitive impairment (MCI), or from MCI converting to AD [109]. Additionally, the measure appears to correlate well with cognitive decline [110]. The rate of brain atrophy in patients with AD was 2.37% per year compared to 0.41% in healthy controls [111]. Extrapolating atrophy and atrophy rates in MCI and AD patients hint toward an early onset of brain structure alteration, preceding cognitive decline by several years. The reliability of the method suggests that measuring rates of atrophy may be a significant marker to use in clinical trials, and may allow reducing the group sizes in trial [111]. However, the pitfall of volumetric measures resides in the absence of specificity in the measures, which render volume changes difficult to attribute to specific biological mechanisms.

Diffusion tensor imaging (DTI) allows resolving several parameters linked to structural integrity of the tissues. The method resolves the anisotropy of local water diffusion, which is captured in the form of diffusion tensor that is determined by measuring water diffusion in several directions. Water diffusion is anisotropic along nerve bundles in the brain, i.e. higher along the fiber than across, while it is more isotropic in gray matter. Fractional anisotropy of water diffusion can be used as a marker for white matter integrity. Water diffusion is altered in the majority of the white matter in AD [112], however, evidence of changes in DTI scans in preclinical AD is low, suggesting that the method may lack sensitivity, or that the changes in gross white matter structures are secondary in the disease.

While structural imaging allows investigating changes in the brain, the shortcoming of the method resides in the absence of specificity. Analogous to atrophy measures the changes in diffusivity of water are difficult to attribute directly to a specific biological process. Molecular imaging, on the other hand allows overcoming this by adding molecular and cellular information to the biomedical
images. Positron emission tomography (PET) is a powerful method to collect molecular information. Radioisotopes, such as fluorine-18 or carbon-11, emit positrons, which upon annihilation electrons generated two $\gamma$-photons that travel in opposite directions, the recording of which can be traced to the spatial position of their emission. Labeling ligands with such radioisotopes allows studying receptors, and can be used to gain relative receptor concentration and availability. Fluorodeoxyglucose, [$F$-$18$]FDG, is such a labeled molecule, which serves as indicator of glucose consumption in tissues. Metabolism is markedly reduced in AD, and FDG-PET is a good biomarker for cognitive decline and progression into AD [113]. In addition to studying metabolism, PET ligand have been developed to bind to amyloid plaques, such as the [C-$11$]Pittsburg Compound-B [114], [F-$18$]florbetaben [115], [F-$18$]flutemetamol [116], and [F-$18$]florbetapir [117]. Other elements of the pathology can be investigated with PET ligands, such as Tau pathology [118], neuroinflammation [98], or altered levels of nicotinic receptors [119]. While PET offers a large palette of possibilities to image different ligand-receptors interactions, PET remains an expensive method, and scanners are not available in most clinics.

MRI systems offer more limited possibilities in term of molecular imaging. The use of intrinsic contrast is usually unspecific to molecular events, and MR compatible ligand-bound contrast agents have a large molecular weight and are thus mostly confined to the blood pool. Spectroscopy stands out, as a MRI-based molecular imaging method to measure the local tissue concentration of the major metabolites. Brain alteration in AD lead to metabolite imbalances, notably reduced N- acetyl-aspartate in the medial temporal lobes, a marker for neuronal density [120], and increased myo-inositol and creatine [121], markers for glial cell and energy metabolism respectively.

Biomedical imaging allows making observation in vivo that were previously only available in post mortem analysis. Combining structural read-out with molecular information and the possibility to perform measures on the same individuals allow for a more detailed understanding of AD pathology progression. However, every method suffers from limitations, such as lack of specificity in structural imaging, or spatial resolution in molecular imaging. Furthermore, the functional implications of the changes are not captured by such methods. As such, functional imaging allows putting the molecular and structural information in a context, as well as allowing to bridge information at the macroscopic level derived from structural or molecular imaging, with behavioral and cognitive measures.

1.2.3 fMRI and functional connectivity changes in Alzheimer’s disease

Functional imaging has become a very common approach to image the brain response to stimuli or task, as well as during rest. Early studies have shown deficits in memory encoding and visual encoding, as represented with reduced functional response to stimuli in mild to moderate cases of AD [122-123]. Patients at risk with developing the disease, such as patients with mild cognitive impairments and genetic risks, also showed altered fMRI responses to different tasks, principally in the medial temporal lobes [124-128], hinting toward early impaired synaptic response decades before disease onset.

Stimulus or task-evoked fMRI record the hemodynamic response to a given stimulus in spatially resolved manner. Recent approaches have aimed at studying the integration of information between regions, during task or rest, under the common term of functional connectivity. Studying
the brain at rest offers several advantages over stimulus-evoked fMRI, especially with regards to studying AD. The measurement is rapid, 300 image repetitions or less are sufficient to establish correlations between brain regions. Resting-state data sets can be recorded within 5 to 10 minutes, while stimulus evoked fMRI experiment may last longer, depending on the number and complexity of stimulation blocks. Observing the brain at rest does not require a priori hypothesis compared to stimulus-evoked, which tackles a specific task-related system per paradigm. Additionally, studying the brain at rest does not require the patient to understand a stimulation paradigm, which may be preventive in moderate to severe cases of AD. Finally, resting-state measures do not require any additional equipment, such as those involved in stimulation paradigm, which makes the comparison of studies between centers less complicated.

Resting-state functional connectivity in AD reveals reduction in connectivity within the default mode network [129-131], similarly to stimulus-evoked fMRI, and consistent with the finding of correspondence between stimulus-evoked and resting-state fMRI structural organization [21]. Furthermore, deficits in groups at risk with developing the disease were also observed with rs-fMRI, including MCI patients [23], in plaque positive cognitively healthy controls [132-133], and in APO ε4 carriers [24, 134].

Interestingly, medial temporal lobe hyperactivity was observed in young people at risk of developing the disease with both resting-state [24] and stimulus-evoked fMRI [135-137]. These findings were established despite reduced hippocampal volume, which has lead to suggest this as a compensatory mechanism to neuronal losses [137], and a necessary element to proper memory encoding [138].

Resting-state studies have revealed the macro-structural organization of the human brain into networks, leading to the hypothesis of the default mode network [16], a set of interconnected regions enclosing the medial temporal lobes, precuneus, posterior cingulate cortex and medial prefrontal cortex. The default mode network was found to be central in the patterns of functional disruptions observed with fMRI, but also with FDG-PET for glucose metabolism, which is thought to reflect local synaptic activity. PET and MRI studies reveal an overlap of regions implied in atrophy, functional organization, plaque deposition, and energy metabolism [139]. These observations place AD related macro-structural and molecular changes in a context centered on the default mode network regions (Figure 5).

Functional imaging was shown to be an early marker of the pathology, along with brain atrophy and reduced metabolism. While structural MRI methods are well established, commonly used in the clinic, functional MRI suffers from poor reproducibility at the individual level, and difficulties to perform comparison between studies, as method of data correction and analysis differ markedly between studies. Another pitfall of fMRI measures with regard to AD is its lack of specificity as a biomarker. Altered connectivity in the default mode network is also a feature observed in several psychiatric disorders of the brain, including depression, schizophrenia, and epilepsy [140], in this regard, fMRI may rather be integrated as a complementary measure, combined with other biomarkers, and as a measure of treatment efficacy during clinical trials.
Several questions underlie the functional changes occurring in AD. The primordial question relates to the temporal ordering of events, such as brain atrophy, amyloid deposition, tangles formation, and synaptic or neuronal dysfunction [141]. Clinical trials failure to observe treatment efficacy in mild to moderate cases of AD was interpreted as the difficulty to restore function at advanced pathological stages. Consequently early diagnostics and classification into preclinical AD has become a crucial element in drug development. Current models consider that insults to the brain occurs several years prior to dementia diagnostic, Aβ deposition into plaque may precede the appearance of clinical symptoms by a decade, while tangles may appear later in the disease progression [141]. Under these considerations, it was proposed that synaptic aberration and network dysfunction, as observed with FDG-PET and fMRI, might be the earliest occurring and measurable events at preclinical stages of AD, potentially occurring even prior to amyloid plaques deposition (Figure 6) [142].

Analogous to structural readouts, changes in functional connectivity lack specificity for the pathology. The basis underlying functional changes would need to be addressed, both to better understand fMRI as a potential the biomarker, but also to bring insights into the early development of the pathology. The use of experimental animals may allow bridging the gap between fMRI changes and molecular principles.
Figure 6 | Hypothetical development dynamics of biomarkers during the Alzheimer’s disease progression suggest synaptic dysfunction as the earliest event serving as a biomarker in the pathological cascade. From Sperling et al. [142]. Copyright © 2011, Elsevier. Reproduced with permission

1.2.4 Animal models of AD
Transgenic mice are the most common animal model of AD. They usually over express mutated human genes encoding for proteins related to the disease, principally derived from early onset familial AD, such as APP, and presenilin I and II, which are elements of the γ-secretase complex. These mice usually exhibit dense core amyloid plaques similar to human plaques, positive for thioflavin and congo red stains. Along with plaques, the mice display cognitive deficits, usually occurring prior to amyloid plaques [143]. Additionally, some animals expressing hAPP with specific mutations tend to induce marked vascular pathology [144-145]. Single APP, or double APP/presenilin transgenic animals only mimic some aspect of the disease, but do not display typical features such as neuronal cell death, the formation of neurofibrillary tangles, or marked inflammation [146]. In that sense, it has been suggested that they model the preclinical stage of the pathology, and more effectively the familial disease, which might therefore not directly related to late onset sporadic AD.

Different transgenic mice have been developed that model other aspects of the disease, e.g. by adding mutated genes encoding for Tau protein leading to neurofibrillary tangles along with amyloid plaques in 3xTg mouse model [147]. Other transgenic mice with different allele of the gene encoding for the APO protein allow studying the molecular pathway linked to sporadic AD. These models allow complementing the previous models, and thus offer a wide approach of phenotypes to study.

Models of the disease have already been used to study functional implication of Aβ over-expression in mice and rats. Studies in mice reveal late impairments for the functional response to sensory stimulation [148], as well as to vascular reactivity in mice with amyloid related vascular pathology
However, joint fMRI and electrophysiological recordings in rats that received brain infusion of Aβ peptide suggested the major impairments were neuronal, rather due to neurovascular coupling impairments [151]. Initial studies involving functional connectivity in APP/PS1 mouse model revealed hyper-connectivity in young transgenic animals, while older animals showed marked functional decrease in the sensory motor and cingulate cortex [152]. The regions involved with the most marked functional changes in connectivity were also most prone to develop increased amyloid plaques, suggesting a relationship between functional changes and amyloid pathology.

While the models are incomplete representations of the disease, they allow the experimental study of specific aspects of the disease. The key advantage of using models is the early appearance of the features of interest and the homogenous expression of the phenomenon, which allows for smaller group size than human studies to power studies. Additional, transgenic animals are marked with the absence of other co-morbidities as typically found in humans, including obesity or diabetes, which might complicate the analysis. Finally, the large selection of models and the possibility to develop specific transgenic animals allows studying specific interactions in laboratory conditions.

Developing MRI \textit{in vivo} methods in mice potentially allow bridging the observations made in humans, to mice, and ultimately understanding the molecular and cellular dynamics underlying the changes.


2 Noise modeling in resting-state fMRI in mice improves specificity of results in seed-based analysis

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Abstract
Non-white noise contributions in blood oxygenation level dependent (BOLD) functional magnetic resonance imaging (fMRI) lead to artifacts that may obscure the result from commonly adopted analysis such as seed-based correlation. They are typically not normally distributed and hence invalidate a basic assumption of parametric statistical models, which in return, would impose bias on statistical analysis. Sources of non-white noise include low frequency drift, residual movement effects, and respiration induced noise. Today, various methods to model the non-white noise are available for the analysis of human fMRI data, however these tools have not been implemented for mouse fMRI. In this study we applied the nuisance variable regression model for analyzing mouse fMRI data and first investigated the spatial distribution of the noise sources, the result of which was similar to human fMRI. Physiological sources are estimated from data-dependent and data-independent methods, with RETROICOR and CORSICA, global signal, and vascular signal estimation. Seed-based analysis and temporal variance analysis are used to compare the different noise estimates. Vascular contribution, as estimated from the major arteries and veins sites, is shown to represent a major confounding element in the measure. The regression of the vascular signal improves the seed-based analysis specificity. Our results highlight noise terms, and demonstrate the effectiveness of their removal, a necessary element to the application of resting-state fMRI in mice.
2.1 Introduction

Resting-state functional magnetic resonance imaging (rs-fMRI) has evolved as a potent method in neuroscience to investigate brain function. While stimulus evoked fMRI aims to study the hemodynamic response to a stimulus of known intensity and timing, resting-state fMRI compares the signal fluctuations during rest between two or more distinct regions under the assumption that the fluctuation in the signal originate predominantly from neuronal activity [1]. The application of fMRI to the mouse brain is attractive with regards to the many genetically engineered mouse lines available that allow studying mechanistic aspects underlying the neuronal signal and neurovascular coupling under normal and pathological conditions. Accurate and reproducible rodent fMRI experiments puts a high demand on signal-to-noise (SNR) ratio and therefore require high field dedicated MRI systems in combination with sensitive radio frequency detection devices [2] to obtain precise measures. However, the signal measured during fMRI recording contains not only signal of neuronal origin, but also contaminants from several noise sources. Notably, the impact of physiological noise increases with higher magnetic field strength [3-4]. As a consequence, accurate physiological modeling becomes critical when analyzing rs-fMRI data. Hardware imperfections, animal movement and physiological processes such as respiration and cardiac motion are common sources of non-white noise in the fMRI signal, i.e. noise that does not follow a normal random distribution [5-6]. These non-white noise contributions may lead to significant artifacts resulting in an over-estimation of functional connectivity and unspecific correlations between uncoupled regions. For instance, physiological noise sources were shown to contribute to more than 40% of the BOLD fMRI signal in rat rs-fMRI and accounting for them led to a significant improvement of the specificity of the results obtained with spatial independent component analysis (ICA) [7].

Several approaches exist to estimate nuisance variable, either independent from the image data, using external recordings such as breathing balloon or cardiac cycle recorder, or data dependent methods based on the intrinsic signal. Regions such as the ventricles and the white matter are not expected to contain coherent signals of neuronal origin. Consequently, such regions are considered enriched in noise and can be used to estimate nuisances.

Data-independent methods based on extra-recordings pose the problem of proper data conversion: the signal recorded from devices such as breathing balloons needs to be converted to match the sampling frequency of fMRI signal, as devices usually sample the signal in the order of 1000 Hz, while the fMRI data are sampled at an order of 0.25 to 1 Hz. This achieved using specific models for breathing and cardiac cycle as described by the RETROICOR method [8].

Data-dependent methods do not face the problem of conversion, however may inadvertently enclose aspects of the neuronal signal during the estimation, which would cause partial removal of fluctuations of interest from the signal. Global signal correction is a common data-dependent method to estimate and remove the physiological noise by computing the mean time series of all brain voxel, under the assumption that the global signal reflects the noise sources, and is orthogonal to the local functional signal of the brain [9-10]. However, global signal correction was shown to introduce negative correlation and to alter the functional connectivity maps [11]. In addition, the assumption that the global signal is orthogonal to the specific brain response is being questioned, as combined electrophysiological and rs-fMRI recordings have suggested a global brain
correlation with local neuronal signal in awake monkeys [12]. Global neuronal coherence present in the signal across large portion of the brain may thus influence the global signal estimate, leading to the removal of signals of neuronal origin. Other data-dependent methods exist, from simpler methods such as extracting the signal from regions of interests (ROI) in the ventricles or white matter, to more elaborate methods based data reduction schemes including independent or principal component analysis, such as CORSICA [13].

Motion during scanning session is another confounding element to consider. Despite temporal realignment of the image series in space, traces of the motion effect usually persist in the individual voxel time series [14], which can be accounted for by linear regression. It appears that the motion effect can leave a further impact the signal in the form of residual effects due to spin history, i.e. the imprint effect of previous magnetizations on the spins, which is affected by the displacement of the spins due to motion [14].

Models of low frequency system noise [15], motion effects [14], and aliased physiological noise [8] are well-established for human fMRI, however, data correction methods have not been applied mouse fMRI data, neither for stimulus evoked fMRI [16-18] nor for rs-fMRI [19]. In particular, modeling of physiological noise contributions using data-independent RETROICOR [8] and data-based CORSICA [13] have not yet been described in rodent fMRI.

The objectives of this study are to apply different nuisance estimation model of physiological noise in the analysis of resting-state fMRI data in mice and to identify an appropriate model to reduce confounding variables in the mouse resting-state fMRI signal. We use variance analysis of the signal and correlation analysis to a seed in the S1 cortex in different models of nuisance estimation as an approach to study the impact of different source of noise in the signal.

2.2 Methods

2.2.1.1 Movement effects

Changes in head motion can significantly contribute to the changes in fMRI signal intensity. Rigid body realignment is commonly used to remove motion effects, but this algorithm does not take into account the effect of movements on the signal intensity that is imprinted in the fMRI time series data, referred as the spin history effect. The observed signal of the \(i^{th}\) scan is proportional to the transverse magnetization generated, which depends on the efficiency of the radiofrequency excitation and the longitudinal magnetization at \(i-1\), which itself is dependent on the number of spins excited in the previous volume and longitudinal relaxation. The observed signal can be expressed as [14]

\[
Y_i = Y_i^* + N_m(m_i, m_{i-1}, ...)
\]

where \(m\) represents the six parameters of the rigid body transformation (3 translations, 3 rotations). \(Y_i^*\) is the signal orthogonal to the noise term \(N_m(m_i, m_{i-1}, ...)\) from residual motion, which is a function of the parameters of the current scan \(m_i\) and the previous scans \(m_{i-1}, m_{i-2}, ...\). First order motion consists of 6 vectors representing the translation along the 3 axes and 3 rotations.
Expanding the noise function and considering terms up to second order (cross terms not included) adds another 18 regressors for the movement \([5]\).

### 2.2.1.2 Determination of respiration-induced noise based on RETROICOR

The RETROICOR method is commonly used to predict aliased respiration noise. The periodic noise is modeled by a superposition of the aliased frequency and its higher harmonics \([8]\)

\[
N_R[i] = \sum_{j=1}^{P_R} (a_{R,j} \cos(j \varphi_R(t_i))) + b_{R,j} \sin(j \varphi_R(t_i))
\]

with \(\varphi_R(t_i)\) being the phase in the respiration cycle at time \(t_i\) and \(P_R\) is the order of the Fourier series.

In the present study, we did not model the cardiac cycle due to technical difficulties to take electrocardiogram measures because of MRI gradient induced currents in the electrodes during fMRI recording.

### 2.2.1.3 Determination of respiration-induced noise based on ICA

CORSICA \([13]\) is a method based on independent component analysis (ICA) to model the respiration induced noise. Briefly, spatial ICA is first conducted to separate the independent components. Then a k-means algorithm was applied to cluster the ventricle area, for which physiological fluctuations are known to be prevalent. Characteristic noise signals are obtained by averaging the time series of the voxels in each cluster. Lastly, stepwise regression is performed to select the component that can optimally explain the characteristic noise signals across the whole brain.

### 2.2.1.4 Global signal and vascular noise regression

A mask enclosing the whole brain, or the major arterial and venous vessels was used to extract the mean time series from the image to obtain global signal or a set vascular noise regressors respectively.

### 2.2.2 Animal preparation

All animal experiments in this study have been performed in strict adherence to the Swiss law of Animal Protection and were approve by the Zurich cantonal veterinary office. A group of 18 mice with C57/B6 background have been used in this study. Animals were housed in standard mouse caging, with 12h:12h light and dark cycles with food and water provided ad libitum. Mice were anesthetized with 3% isoflurane in a 1:4 oxygen to air gas mix. After induction of anesthesia, the mice were transferred to a custom build animal cradle equipped with a face mask, warm water bed and ears bar for head restraining. The isoflurane level was reduced to 1.5% for the rest of the experiment. Body temperature was recorded with a rectal thermometer and water temperature was adjusted to 36 ± 0.5 °C. The breathing cycle was recorded with an air-cushion positioned under the animal belly. Sampling of the breathing was synchronized with each volume acquired. Animals were imaged each on two separated sessions providing a total of 36 rs-fMRI recordings.

### 2.2.3 MRI and fMRI data acquisition

A BioSpec 94/30 animal MRI system (Bruker BioSpin GmbH. Karlsruhe, Germany) equipped with a 9.4 T magnet with a horizontal bore of 30 cm diameter, a BGA12S gradient system capable of a maximum gradient strength of 400 mT/m with a 80 µs rise time, a 2 x 2 cryogenic phased-array...
receive coil and a linear volume resonator coil for excitation have been used. Tripilot scans were used for accurate positioning of the animal head inside the magnet. Prior to fMRI, anatomical images have been recorded using a multi-slice rapid acquisition with relaxation enhancement (RARE) sequence with repetition time TR = 2500 ms, echo time TE = 11 ms, effective echo time T\text{Eeff} = 33 ms, pulse angle FA = 90°, number of experiments NEX = 1, matrix dimension MD = 180 x 180, pixel dimensions V = 111 x 97 µm², slice thickness ST = 500 µm, inter-slice distance ISD = 200 µm, and number of slices NSI = 12. For fMRI studies gradient echo echo-planar imaging (EPI) has been used with TR = 1500 ms, TE = 9.3 ms, FA = 50°, number of repetition NR = 500, NEX = 1, MD = 90 x 70, V = 250 x 220 µm², ST = 500 µm, ISD = 200 µm, NSI = 12.

2.2.4 Data analysis
Data pre-processing and statistical analysis were performed using SPM05 (FIL Methods Group, London, UK) with the spmmouse plug-in (Wolfson Brain Imaging Centre, Cambridge, UK). Images were realigned using 6 rigid body parameters, were corrected for slice timing and normalized to an in-house EPI template of the mouse brain. A linear regression was performed at each voxel with different nuisance models, and the residuals of the regression were used as the denoised signal. Seed time series were extracted from the left sensory S1 region using a ROI template.

Figure 1 | A Reference template EPI image. The distance indicated is relative to bregma. B Color coded temporal variance of the unprocessed fMRI images. High signal variance is concentrated in regions co-localizing with major blood vessels including the anterior cerebral artery, in surrounding muscular tissues, and in the brain boundaries.

2.3 Results
2.3.1 Spatial distribution of the variance
We performed a variance analysis of the signal in the unprocessed images time series (Figure 1 B). The map reveals highest variance in temporal signal near tissue interfaces displaying high differences in signal intensity, in particular the brain boundaries, in the surrounding muscle tissues, in the ventricles, and in regions associated with major arteries and veins, including the anterior, pericalosal, and the posterior cerebral arteries.
Figure 2 | A Unprocessed signal trace from the somatosensory S1 cortical seed in one animal. B Breathing signal recorded from a breathing balloon positioned under the animal belly during MRI recordings. C & D 1 of 4 slice specific RETROICOR vectors estimated with the algorithm. Summed absolute correlation between the RETROICOR vectors and the fMRI signal reveal weak correlation between the estimated vectors and the signal in the ventricles and brain boundaries. E & F CORSICA vector trace and its correlation map reveal widespread correlations, especially with the signal in the ventricles. G & H Global signal vector and correlation to the fMRI signal reveal strong correlations to the boundaries, but also to the cortical ribbon. I & J 1 of 8 estimated vascular vectors based on regions co-localizing with cerebral and extra-cerebral major blood vessels and the summed correlation of the vectors to the fMRI signal. Strong correlations are found to co-localize with major
vessels, and brain boundaries. \( \mathbf{K} \) & \( \mathbf{L} \) 3 axis translation vectors of 6 motion parameters describing the estimated animal displacement during the measure, and their summed correlation to the fMRI signal highlighting brain boundary regions and muscles.

### 2.3.2 Noise spatial distribution

We estimated physiological noise in the data using data-dependent and data-independent methods. Figure 2 A and B shows a typical signal trace from the S1 sensory cortex and a sample of the breathing signal recorded with a breathing balloon positioned under the animal belly. The RETROICOR vectors estimated by modeling the breathing signal (Figure 2 C, D) showed very weak correlation between the vectors and the fMRI time series, mostly confined in the ventricles and in the brain boundaries, as opposed to CORSICA (Figure 2 E, F), global signal estimate (Figure 2 G, H), and motion parameters (Figure 2 K, L) which all show marked correlations with the ventricles, brain boundaries, and surrounding tissues. Finally, signal estimated from the major vessel regions shows correlations principally localized in the vessels and ventricles, and to a lesser extend at the brain boundaries (Figure 2 I, J).

![Figure 3](image)

**Figure 3 |** Color coded seed-based correlation maps of the somatosensory S1 cortical seed, shown in red on the template image, for unprocessed images and data processed with CORSICA, RETROICOR, global signal, or vascular signal regression. CORSICA and RETROICOR regression led to only marginal differences to the unprocessed condition, notably with increased correlation in the lower brain boundaries with CORSICA. Global signal and vascular regression show reduced cortical correlations compared to the unprocessed data, and reduced correlation to the cingulate cortex and the posterior parietal cortex. Patterns of anti-correlation, represented in blue, become apparent with global signal regression, notably in the brain boundaries.

### 2.3.3 Noise model effect on S1 seed analysis

Seed based analysis was conducted on data regressed with different noise estimates (Figure 3). The S1 seed correlation map in unprocessed signal showed correlation across the whole cortical ribbon to the seed, with some residual correlation in the ventricles and in the surrounding muscles. Correlation maps accounting for respiration using either CORSICA or RETROICOR did not yield
substantial differences with respect to the unprocessed data. The correlation between the S1 seed and the contra lateral S1 region was 0.27 for CORSICA and 0.27 for RETROICOR, compared to 0.25 in the unprocessed data (Figure 4 B), while the correlations to the cingulate cortex remained unaffected by both CORSIA and RETROICOR compared to unprocessed condition (Figure 4 C). In contrast, correlation within cortical regions appeared more confined when applying global signal or vascular regression, and yielded reduced values for the correlation seed to the contra lateral S1 regions, 0.19 and 0.22, respectively. Both conditions also showed reduced S1 seed correlations to the cingulate cortex (Figure 3) with correlation values of 0.04 and 0.05 for the global signal and vascular regression condition compared to 0.1 in the processed data (Figure 4 C). Vascular regression was combined with either motion parameters (6 vectors) or residual motion effect (24 vectors) to assess the cumulative effect of different parameters. Vascular regression removal accounted for a decrease of 12.3% in the S1 contra lateral correlation to the seed compared to uncorrected data, and 49.2% in the cingulate. Motion parameters and residual motion effect in turn accounted 6.2% and 3.6% of the correlation in the contra lateral cortex, and 7.1% and 1.8% respectively within the cingulate cortex (Figure 5 A, B).

**Figure 4** | A Reference template image showing the position of the contra lateral cortex ROI (orange), and the cingulate cortex ROI (green). B Mean correlation in the contra lateral cortex to the S1 cortex seed in RETROICOR (blue), CORSICA (red), global signal (green), and vascular signal (violet) regression condition. The unprocessed data reference is displayed as a black line. Global signal and vascular signal regression led to a reduction of correlation in the contra lateral cortex with respect to the seed. Both CORSICA and RETROICOR lead to marginal increases in correlation to the seed. C Mean correlation in the cingulate cortex to the S1 seed revealed no effect of either RETROICOR or CORSICA compared to the unprocessed data. However, both global signal and vascular signal regression indicate a reduction
of correlation between the seed and the voxels in the cingulate cortex. D & E Signal variance changes in the contra lateral cortex and cingulate ROI following regression. RETROICOR, CORSICA, and global signal regression had only minor effects on the signal variance in both sensory and cingulate cortex. Vascular signal regression had the most marked effect on both regions. Error bars represent +/- 1 SEM.

2.3.4 Noise regression impact on signal variance

The impact of noise regression was assessed by measuring the residual signal variance following noise removal. The mean variance in the unprocessed data was 33.7 in the S1 cortex, and 40.2 in the cingulate cortex. The variance was reduced by 6% in S1 and 5.2% in the cingulate using CORSICA, 6.9% and 4.6% with RETROICOR, and 3% and 3.3% with global signal regression. Vascular signal regression had the most impact in the variance, with a 16.9% reduction in the S1 cortex, and 23.1% in the cingulate (Figure 4 C, D). Following vascular signal regression, motion parameters and residual motion explained 5.6% and 3.2% of the total variance in the S1 cortex, and 6.5% and 3.5% in the cingulate cortex respectively. Instrumental noise was estimated by computing the mean temporal variance in voxel outside the object in the image. Following linear regression of vascular signal, motion parameters and residual motion, instrumental noise accounted for 55.2% of the total variance in the S1 cortex, and 62.4% in the cingulate cortex, leaving 18.8% of unexplained variance in S1, and 4.3% in the cingulate (Figure 5 C, D).

**Figure 5** | A & B Combined effect of vascular, motion parameters, and residual motion effect on mean correlation between the seed and the contra lateral (A) and the cingulate cortex (B). Vascular signal regression led to a decrease by 12.3% of the S1-to-S1 correlation, and by 49.2% of S1-to-cingulate. Adding motion parameters and the residual motion effects accounted for a further reduction by 9.8% and 8.9% for the S1-to-S1 and S1-to-cingulate correlations,
respectively. C & D Variance in the unprocessed signal explained by vascular, motion parameters, residual motion effects, and measurement noise in the sensory cortex (C) and in the cingulate cortex (D). Vascular signals account for 16.9% and 23.1% of the variance in the S1 and cingulate cortex, respectively, while contribution from motion parameters and motion residuals accounted for 8.8% and 10%. Random noise accounted for 55.2% of the variance in the S1 cortex, and 62.4% in the cingulate. The variance unaccounted for in the analysis was 18.8% in the sensory cortex, and merely 4.3% in the cingulate cortex.

2.4 Discussion

Reliable detection of the small fluctuations in the signal intensity observed in resting-state fMRI in mice puts high demands on sensitivity. This challenge has been met by using a cryogenic receiver coils which allows minimizing the noise contributions from the instruments. Apart from measurement noise, which is addressed with higher magnetic field strength and dedicated radiofrequency coils, physiological noise represent a major contribution to signal fluctuations in fMRI studies [2, 4], on which functional connectivity estimation depend. In evaluating connectivity within the central nervous system it therefore becomes important to account for contributions due to non-specific physiological processes such as motion, respiration and cardiac cycle.

We have applied nuisance variable regression models to estimate the contributions of non-white noise in mouse rs-fMRI data. This included two models for predicting the impact physiological confounds: data-independent RETROICOR [8] or data-driven CORSICA [13], which to our knowledge have not yet been applied to rodent fMRI. We completed the comparison by including vascular signal regression and global signal regression to the analysis, the latter being one of the most common methods to account for noise sources in human fMRI studies, albeit it has been reported that the method may induce artificial negative correlation [11].

Methods to assess noise removal in resting-state fMRI are lacking due to the unpredictability of the underlying local neuronal processes. Metrics exist to assess the effectiveness of nuisance modeling in stimulus-evoked fMRI based on parametric statistics [5, 20]. Principally, residuals of a given model are expected to display white noise properties such as absence of autocorrelation between the residuals, constant mean and variance over time. In return, models can be designed to maximize these properties, thus providing a strong framework to model noise. In resting-state analysis, however, there is no direct approach to model the signal of neuronal origin other than from the data itself with ROI analysis, yet the signal estimated from the ROI may include undesirable nuisance, as well as the signal of interest. This aspect precludes a residual approach to estimating accurate modeling, and poses a limitation to the analysis of nuisance variables in resting-state fMRI data. We have thus opted for seed-base correlation maps specificity as a metric for noise removal, especially given the prior knowledge that task related brain regions such as the sensory cortex should present minimal correlation to the cingulate cortex in humans and rodents [21-22]. Further, we used analysis of signal variance, a metric of noise removal by linear regression and an indicator of portions of the signal, which are explained by different element of the models.
In effect, the RETROICOR vectors did not display significant correlations with the fMRI signal despite the use of slice specific regression accounting for the timing of each slice with respect to the breathing when computing the RETROICOR vectors. The method did not have a marked effect on both the correlation maps using somatosensory S1 seed, and on the variance in the signal. Similarly, CORSICA did not display a major impact on the correlations or the variance despite strong correlations between the CORSICA derived nuisance vector and the signal. When analyzing the mouse rs-fMRI data it becomes apparent that accounting for physiological noise contributions using either RETROICOR or CORSICA methods do not result in substantial benefits, similarly to observations in rats, where respiration cycle only contributed to 1% of the signal variance [7].

Global signal displayed very strong correlations across the whole brain and markedly reduced the correlation between ipsi and contra lateral S1 regions (S1-to-S1) despite minimal impact of the signal temporal variance within the sensory cortex, in particular in comparison to vascular signal regression, which reduced this correlation to a lesser extent, but accounted for a larger portion of the variance. Systemic vascular signal is expected to be orthogonal to the neuronal signal, i.e. there should be no correlation between signal in large blood vessels and neuronal signal, while the global signal may enclose an unknown amount of neuronal contributions. We therefore interpret the reduction of S1-to-S1 correlation differently for vascular and global signal regression: the first is essentially linked to the vascular contribution, while the second includes portions of the neuronal signal in addition to other systemic effect, which might explain the larger reduction of the S1-to-S1 correlation in this case due to the partial removal of neuronal information in the signal. When accounting for vascular effect, motion parameters, residual motion effects and intrinsic SNR, the unexplained portion of the signal was 18% in the sensory cortex, and considerably less in the cingulate cortex (4.3%). These values are similar to what has been reported for rats at 11.7T [7] and may be accountable, in part, to signals of neuronal origin.

The low levels of unexplained variance attributable to neuronal signal highlights the vulnerability of resting-state fMRI application in mice, and the high demand on SNR that the method requires. Residual correlations in the S1-to-S1 pair following nuisance correction were reduced to 77% of their original value following the removal of nuisance, illustrating the potential overestimation engendered by insufficient noise models. Furthermore, in our study, the unexplained variance, which is left to potentially reflect neuronal origin, in the cingulate cortex was reduced to 4.3% of its original value, highlighting a marked vascular effect in this region, with marginal neuronal influence. This was despite the cingulate cortex putative central role in the rodent default mode network, a network linked to inner thought processing in humans and central to higher order cognition [23], which includes the cingulate cortex, the hippocampus, and the medial frontal cortex both in humans and potentially also in rodents [23-25]. The low residual variance following nuisance regression in the cingulate poses limitations to the interpretation of the involvement of the cingulate cortex in reports of default mode network activity in previous studies in rats and mice [24-27]. However, the residual variance in the cingulate attributable to neuronal process may have been dampened by the effect of anesthesia, which has been observed to affect connectivity within the default mode network in human in a dose dependent manner [28-29].
Our study highlights vascular contribution as a major element affecting the BOLD signal at a systemic level. There are several mechanisms that can explain the marked effect of vascular signal regression: changes in arterial CO₂, blood pressure, or vasomotion [6, 30]. Using several vessels, both arteries and veins, as regions of interests for the regression analysis, it is difficult to distinguish signal contribution by one mechanism from the other. Each of them may impact the BOLD signal by affecting the cerebral blood flow, either by altering vasodilatation in response to CO₂, auto regulation mechanisms to compensate for blood pressure changes, or to the low frequency pulsing constriction induced by vasomotion [30]. Results in mice are in accordance with observations in humans, suggesting that blood pressure explains up to 60% of the changes in cerebral blood flow, and CO₂ fluctuations for 17% [31]. These observations suggest that vascular nuisance is a significant element to be considered in the interpretation of rs-fMRI data, especially in mice, and potentially a better alternative to global signal regression, or models such as RETROICOR or CORSICA.

Our approach to nuisance modeling in fMRI is however limited, as the methods to assess noise contribution are constrained by the model-free approach of resting-state fMRI. While seed-based analysis can identify spurious correlation, for instance between S1 and ventricles, it is more limited to distinguish genuine from spurious correlations between two grey matter regions. Variance analysis is equally limited, as the residual variance unexplained by the model, may be either due to further nuisance effect absent in the model, or by signal of neuronal origin, and thus of interest, without the possibility to readily distinguish one from the other. Improved handling of the animal leading to reduced physiological noise, such as mechanical ventilation combined with complete muscle relaxation may reduce effects of motion, respiration, and potentially stabilize the animal physiology such that systemic vascular fluctuations are also reduced. Improved anesthesia regimen may also contribute to improving the stability of the animals, leading to more reproducible results. Such improvements are expected to significantly reduce the noise contribution to mouse resting-state fMRI, while mitigating the need for complex noise reduction schemes in the data processing.

In conclusion, we have evaluated the effect of including various models of non-white noise for the analysis of rs-fMRI data in mice and used residual analysis to select an optimal model for removing noise within a seed-based analysis and variance analysis context. We have shown that correcting for systemic vascular effects, motion and its residual effect accounted for the majority of non-white noise sources. Proper modeling decreased the number of spurious significant correlations in the signal. The two approaches for modeling aliased physiological noise, RETROICOR and CORSICA, did not yield measurable benefits both with regard to the seed-based (confinement of regions correlated with seed) and the residual analysis. Correct modeling of the non-white noise contribution in rodent resting-state fMRI data is critical in order to reveal subtle differences caused by pathological conditions.


3 Optimization of anesthesia protocol for resting-state fMRI in mice based on differential effects of anesthetics on functional connectivity patterns

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* These authors contributed equally to this work.

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Abstract

Resting state-fMRI (rs-fMRI) in mice offers to study the mechanisms underlying functional connectivity (FC), as well as the alterations taking place in murine models of neurological diseases. Mouse fMRI experiments are typically carried out under anesthesia to minimize animal movement and potential distress during examination. Yet, anesthesia inevitably affects functional connectivity patterns. Such effects have to be understood for proper interpretation of data. We have compared the influence of four commonly used anesthetics on rs-fMRI and also evaluated the effect of different doses. Data acquired under isoflurane, propofol, and urethane presented similar patterns when accounting for anesthesia depth. FC maps displayed bilateral correlation with respect to cortical seeds, but no inter-hemispheric striatal connectivity. In contrast, for medetomidine we detected bilateral striatal connectivity, but compromised inter-hemispheric cortical correlations. The spatiotemporal patterns of the rs-fMRI signal could be rationalized in the context of anesthesia depth and pharmacodynamic effects of the anesthetics. Our results bridge the different studies from the burgeoning field of mouse rs-fMRI and offer a framework for understanding the mechanisms of anesthetics on FC. Utilizing this information we suggest the combined use of medetomidine and isoflurane representing the two proposed classes of anesthetics. In fact, the combination of low doses of the two anesthetics retained strong correlations both within cortical and subcortical structures, without the potential seizure-inducing effects of medetomidine, rendering this regimen an attractive anesthesia for rs-fMRI in mice.
3.1 Introduction

Resting state functional magnetic resonance imaging (rs-fMRI) yields information on the functional connectivity (FC) of spatially distinct regions of the brain and thus insight into its overall organization. The relative ease of data acquisition, the identification of distinct networks within the human brain, and the demonstration that alterations in FC could be associated with neurological disorders has generated widespread interest in rs-fMRI [1-2]. Experimental studies in monkeys [3], rats [4-5] and recently also in mice [6-10] allow addressing questions regarding mechanisms underlying the fluctuations that constitute the rs-fMRI signal and offer an experimental approach for studying models of human disease.

While rs-fMRI measurements in sedated or anesthetized humans are of interest for evaluating FC features related to consciousness [11] as well as for understanding the effects of anesthetics [12-13], the use of anesthesia in rodent fMRI studies constitutes a practical need to keep the animal restrained during experiments. Controlled anesthetic conditions offer potential advantages with respect to the use of awake animals as contributions arising from motion, irregular breathing and heart rhythm as well as from changes of stress-related physiological parameters will affect FC analysis to a level that might not be fully accounted for by post-processing procedures. Correspondingly, imaging of awake animals remains a matter of debate as these might experience non-negligible stress despite time-consuming acclimation training.

Yet, anesthesia will inevitably affect the fMRI response. We have previously shown that different anesthetics affect the cerebral hemodynamic fMRI responses in a drug-specific manner and that these effects could be largely explained by the known effects of the anesthetics on animal physiology. Furthermore, our results revealed that independent of the anesthetic used fMRI responses to electrical hindpaw stimulation in mice were influenced by stimulus-induced cardiovascular changes. The observed systemic physiological changes indicated an arousal response even when applying innocuous stimuli, which might mask specific fMRI signals associated to the stimulus [14]. Hence, studying the processing of peripheral input in mice using fMRI techniques constitutes a major challenge. In view of these results, analysis of BOLD signal fluctuations during resting state becomes attractive as it should be largely devoid of changes in the arousal state of the mouse during the course of the experiment. Nevertheless, effects of anesthesia on neuronal activity, systemic physiological parameters and on the cerebral vasculature will influence the measured rs-fMRI signal and understanding these effects is essential for the interpretation of the results.

Recent studies in mice investigating the effects of specific anesthetic regimens on FC data derived from rs-fMRI revealed anesthesia specific patterns, but lack coherence [6-10]. Hence, comparative studies using several anesthetics with differential effects on neural and vascular properties should help identifying commonalities as well as anesthesia-specific signatures within FC patterns and might provide insight into mechanisms underlying the FC observed. Furthermore, in order to minimize potential confounds arising from anesthesia it is important to identify an anesthetic regimen for rs-fMRI studies in small animals that ensures maximum resemblance of FC compared to that observed in awake animals and humans.
In this study, we compared the effects of isoflurane, medetomidine, propofol, urethane and a combination of medetomidine and isoflurane on spontaneous low frequency fluctuations of blood oxygenation level-dependent (BOLD) fMRI signal during resting state. Using seed-based analysis, frequency power analysis, and approximate entropy approach to characterize the differences in FC we propose a categorization of anesthetics based on phenomenological characteristics of the FC patterns. Our comparison identifies a combination of low-dose medetomidine and isoflurane as a suitable anesthesia for rs-fMRI measurements in mice, as the application of this protocol seems to recover both the cortical and subcortical functional topology of FC networks.

3.2 Material and methods

3.2.1 Animals, preparation and anesthesia

The experiments were performed in compliance with the Swiss law of animal protection. Female C57BL/6 mice (Janvier, Le Genest-St Isle, France) between 10 and 15 weeks old, weighing between 20 and 23 g were studied. Animal numbers are indicated in Table 1. All mice were initially anesthetized with isoflurane in a 20% O₂ / 80% air mixture: 3.5% for induction, 2% for endotracheal intubation and during set-up on the animal bed. Throughout the course of the experiment the animals were connected to a small animal ventilator (CWE, Ardmore, USA) and mechanically ventilated with a 20% O₂ / 80% air mixture at a rate of 80 breaths/min, with a respiration cycle of 25% inhalation, 75% exhalation and an inspiration volume of 1.8 ml/min. The head was placed with the animal’s incisors secured over a bite bar and fixated by ear bars, ophthalmic ointment was applied to the eyes, and a rectal temperature probe was inserted to keep the animal at 36.5 ± 0.5 °C by means of a warm-water circuit integrated into the animal holder (Bruker Biospin GmbH, Ettlingen, Germany). The tail vein was cannulated for intravenous (i.v.) administration of anesthetics and the neuromuscular blocking agent pancuronium bromide (Sigma-Aldrich, Steinheim, Germany).

Following the animal preparation anesthesia was continued according to the following scheme (Table 1):

**Isoflurane**: During measurements isoflurane (Abbott, Cham, Switzerland) was maintained at 1%. In a set of experiments a higher dose of 1.5% was used.

**Medetomidine**: A bolus of 0.1 mg/kg medetomidine (Domitor, medetomidine hydrochloride; Pfizer Pharmaceuticals, Sandwich, UK) was injected i.v. and isoflurane discontinued 5 min afterwards. Continuous infusion of medetomidine was started 10 min after bolus injection at a dose of 0.2 mg/kg/h to maintain the sedation level. In a set of experiments half dose of medetomidine, i.e. 0.05 mg/kg for the bolus and 0.1 mg/kg/h for the infusion was used.

**Propofol**: A bolus of 30 mg/kg propofol (2,6-Diisopropylphenol, 97%; Sigma-Aldrich, Steinheim, Germany) was injected i.v. and isoflurane discontinued 5 min afterwards. Continuous infusion of propofol was started 10 min after bolus injection at a dose of 120 mg/kg/h during the first 30 min, and then - due to possible development of pharmacodynamic tolerance - increased to 150 mg/kg/h to maintain the sedation level. In a set of experiments a higher dose of 45 mg/kg for the bolus and 187 mg/kg/h during first 30 min of infusion, and then 225 mg/kg/h were used.
Urethane: Urethane (20%; Sigma-Aldrich, Steinheim, Germany) was injected intraperitoneally (i.p.) at a dose of 1.5 g/kg (2 x 0.75 g/kg separated by 5 min) before positioning the mouse on the animal holder. In a set of experiments a lower dose of 1.2 g/kg (2 x 0.6 g/kg separated by 5 min) was used.

Medetomidine and isoflurane: A bolus of 0.05 mg/kg medetomidine (Domitor, medetomidine hydrochloride; Pfizer Pharmaceuticals, Sandwich, UK) was injected i.v. and, 5 min afterwards, isoflurane turned down to and kept at 0.5%. Continuous infusion of medetomidine was started 10 min after bolus injection at a dose of 0.1 mg/kg/h to maintain the sedation level.

Each animal received an injection of pancuronium bromide alone or in combination with the i.v. administered anesthetic dissolved in saline at an equal bolus volume of 3 ml/kg followed by a continuous infusion of 3 ml/kg/h. Pancuronium bromide doses were 0.5 mg/kg for the bolus and 0.5 mg/kg/h for the continuous infusion.

Animal preparation, anesthesia protocols and the conditions during resting state measurements were identical for the fMRI experiments and for the assessment of systemic physiological parameters. Approximately 20 min were used for animal preparation, and further 20 min for preparatory MRI scans. Subsequently, rs-fMRI data were acquired within a period of 6 min. After the experiments, time for recovery from anesthesia and pancuronium bromide administration was provided for all the animals, except the urethane-anesthetized ones, which were euthanized immediately after the experiment.

<table>
<thead>
<tr>
<th>Anesthetic regimen</th>
<th>Abbreviation</th>
<th>Animal numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoflurane 1%</td>
<td>Iso1</td>
<td>11 5</td>
</tr>
<tr>
<td>Isoflurane 1.5%</td>
<td>Iso1.5</td>
<td>4 2</td>
</tr>
<tr>
<td>Medetomidine 0.1 mg/kg bolus / 0.2 mg/kg/h infusion (i.v.)</td>
<td>Med0.1</td>
<td>13 4</td>
</tr>
<tr>
<td>Medetomidine 0.05 mg/kg bolus / 0.1 mg/kg/h infusion (i.v.)</td>
<td>Med0.05</td>
<td>6 3</td>
</tr>
<tr>
<td>Propofol 30 mg/kg bolus / 120-150 mg/kg/h infusion (i.v.)</td>
<td>Pro30</td>
<td>6 4</td>
</tr>
<tr>
<td>Propofol 45 mg/kg bolus / 187-225 mg/kg/h infusion (i.v.)</td>
<td>Pro45</td>
<td>0 1</td>
</tr>
<tr>
<td>Urethane 1.5 g/kg (i.p.)</td>
<td>Ure1.5</td>
<td>13 3</td>
</tr>
<tr>
<td>Urethane 1.2 g/kg (i.p.)</td>
<td>Ure1.2</td>
<td>2 2</td>
</tr>
<tr>
<td>Medetomidine 0.05 mg/kg bolus / 0.1 mg/kg/h infusion (i.v.) + Isoflurane 0.5%</td>
<td>Med/Iso</td>
<td>8 3</td>
</tr>
</tbody>
</table>

Table 1 | Anesthetic regimens, abbreviations, and animal numbers for the rsfMRI experiment and the systemic physiological parameter measurement using MouseOx.

3.2.2 fMRI
MRI experiments were conducted using a Bruker Biospec 94/30 small animal MR system (Bruker BioSpin MRI, Ettlingen, Germany) operating at 400 MHz (9.4 T). A four-element receive-only cryogenic phased array coil (Bruker BioSpin AG, Fällanden, Switzerland) was used in combination with a linearly polarized room temperature volume resonator for transmission. For anatomical
orientation, images in the sagittal and horizontal direction allowed exact positioning of 12 adjacent coronal slices of 0.5 mm slice thickness. The first slice was placed 2 mm rostrally of bregma according to a stereotaxic mouse brain atlas (Paxinos, 2004). An anatomical reference scan was acquired using a spin echo (Turbo-RARE) sequence: field of view (FOV) = 20 x 20 mm², matrix dimension (MD) = 200 x 200, repetition time (TR) = 3500 ms, echo time (TE) = 13 ms, effective echo time (TE_{eff}) = 39 ms, RARE factor = 8, number of averages (NA) = 2. Before running the fMRI imaging sequence, local field homogeneity has been optimized in the area of interest using previously acquired field maps. BOLD and CBV fMRI experimental data were acquired using a gradient-echo echo-planar imaging (GE-EPI) sequence: FOV = 23.7 x 14 mm², MD = 90 x 60, yielding an in-plane voxel dimension of 263 x 233 µm, flip angle (FA) = 90°, TR = 1000 ms, TE = 10 ms, NA = 1, yielding a temporal resolution of 1 s, with interleaved acquisition of slices.

3.2.3 Measurement of systemic physiological parameters

For physiological parameter measurement, the animal’s left hindpaw was shaved and a fiberoptic pulse oximeter (MouseOx, STARR Life Science, Oakmont, USA) fixed to the flank in order to record heart rate (in beats per minute, bpm), pulse distention (in µm), and O₂ saturation (in %).

<table>
<thead>
<tr>
<th>Anesthesia</th>
<th>Heart rate [bpm]</th>
<th>Pulse distention [µm]</th>
<th>O₂ saturation [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso1</td>
<td>515.6 ± 22.3</td>
<td>12.8 ± 0.3</td>
<td>95.8 ± 1.0</td>
</tr>
<tr>
<td>Iso1.5</td>
<td>555.5 ± 33.5</td>
<td>14.8 ± 0.3</td>
<td>97.5 ± 0.4</td>
</tr>
<tr>
<td>Med0.1</td>
<td>335.0 ± 36.9</td>
<td>21.6 ± 7.1</td>
<td>95.8 ± 1.0</td>
</tr>
<tr>
<td>Med0.05</td>
<td>364.2 ± 14.0</td>
<td>16.2 ± 2.4</td>
<td>97.0 ± 0.2</td>
</tr>
<tr>
<td>Pro30</td>
<td>563.4 ± 29.5</td>
<td>44.2 ± 10.1</td>
<td>97.1 ± 1.1</td>
</tr>
<tr>
<td>Pro45</td>
<td>507.0</td>
<td>31.3</td>
<td>97.9</td>
</tr>
<tr>
<td>Ure1.5</td>
<td>640.2 ± 10.5</td>
<td>10.1 ± 0.5</td>
<td>95.6 ± 0.4</td>
</tr>
<tr>
<td>Ure1.2</td>
<td>647.4 ± 22.3</td>
<td>9.9 ± 0.1</td>
<td>96.9 ± 0.6</td>
</tr>
<tr>
<td>Med/Iso</td>
<td>384.9 ± 3.8</td>
<td>29.4 ± 4.5</td>
<td>96.7 ± 0.5</td>
</tr>
</tbody>
</table>

Table 2 | Systemic physiological parameters for the different anesthetic regimens. Heart rate (in beats per minute, bpm), pulse distention (in µm) and O₂ saturation (in %) have been recorded in bench-top experiments. All data are given as mean across animals ± standard error of the mean (SEM).

3.2.4 Data analysis

The data of this study are publicly available on central.xnat.org repository in Analyze 7.5 format (Project ID: fMRI_ane_mouse).

Pre-processing was performed using SPM05 (Wellcome Trust Centre for Neuroimaging, London, UK) for MATLAB © (The MathWorks, Natick, USA). The first 10 time points were dismissed to account for the T1 relaxation effect. Temporal images were realigned with 6 rigid body parameters, slice-timing corrected, and normalized to an in-house EPI template. Global signal regression (GSR) and 6 motion vectors were regressed from the time series to correct for systemic noise. Alternatively, un-regressed (UR) images were used. The time series were furthered band-pass
filtered between 0.01 Hz and 0.3 Hz using the REST toolbox (State Key Laboratory of Cognitive Neuroscience and Learning, Beijing, China) for MATLAB. A large band-pass filter was used following observations of high frequency fluctuations in the data acquired under medetomidine anesthesia during exploratory analysis.

In a preliminary step we used independent component analysis (Medical image analysis lab, Albuquerque, USA) in order to explore the data. Seed-based analysis was then performed using 2 x 3 voxels regions as seed. Pearson’s correlation with regard to the seed was computed in MATLAB. Correlations are shown as mean at each voxel per group. The results are presented as color-coded overlap on an EPI template image, with a threshold set to R ≥ 0.1 for correlations and R ≤ -0.1 for anti-correlations, corresponding to a p-value of p ≤ 0.05. Frequency analysis was computed in MATLAB with Fourier transformation using the signal extracted from the seed.

Approximate entropy (ApEn) was computed in MATLAB on the z-score corrected time series extracted from the seed as previously described [15-16]. The calculation of ApEn involves two parameters, the window length m, and the tolerance range r. ApEn can be understood as the likelihood that similar patterns of observations of length m will not be followed by additional similar observations, defined within the tolerance range r. We used the parameters r = 0.25, and m = 1 to 5, where values of m ≤ 2 yielded marginal differences between groups and m > 3 resulted in larger deviations within groups. We used a value m = 3, as a heuristic choice to balance sensitivity to detect differences between groups and specificity within groups. Lower ApEn values indicate a predictable system, i.e. highly repeated patterns, and higher ApEn indicates a more chaotic system.

Baseline CBV values were quantified as previously described [14].

The systemic physiological parameter values for each animal were calculated by averaging the data points acquired over a period of 15 min starting from 45 min after induction of anesthesia up to one hour. These mean values of the animals per anesthetic regimen were then averaged.

Statistics were performed in R (R Foundation for Statistical Computing, Vienna, Austria). Correlation values were corrected with Fisher’s z correction to account for the boundary effect. An ANOVA served to test the anesthetic effect between groups, followed by a t-test to probe each pair of anesthetic regimens. Assumption of a normal distribution within the data could not be made for the ApEn value; consequently, a non-parametric Kruskal-Wallis test was performed to assess the anesthetic effect, and a Wilcoxon test was performed between each pair of anesthetic regimens. The hypotheses regarding the presence of a true difference between groups were not rejected at p-values below 0.05, and referred to as significant difference between the groups compared in the text. The significance level is shown in figures as follows: p ≤ 0.05 *, 0.01 **, and 0.001 ***.

3.3 Results

GE-EPI images presented minimal susceptibility-related signal dephasing and geometrical distortions, which facilitated accurate registration to a template, a prerequisite for proper analysis of rs-fMRI data (Suppl. Fig. S1).
Figure 1 | Seed-based analysis of global signal regressed (GSR) data for the different anesthetic regimens. Images display regions with correlation coefficients exceeding the threshold value for seeds in the anterior, medial, and posterior parietal cortex (ctx; A, B, C), the dorsal and ventral striatum (D, E), the limbic system (F), the cingulate cortex (G), and the thalamus (H) for Iso1, Med0.1, Pro30, and Ure1.5, respectively. (A, B, C) Analysis of the Iso1 and the Pro30 group revealed above threshold contralateral correlations to the cortical seeds, which was not the case for Med0.1 and Ure1.5. (D, E, F) Data acquired under Med0.1 display intra- and inter-hemispheric correlation in subcortical areas with respect to the seed in the dorsal and ventral striatum, and the limbic system. Weak bilateral limbic FC was also observed for Ure1.5. (G) Correlation to the seed in the cingulate cortex was found confined to that region for all four anesthetic regimens with presence of anti-correlation with the parietal cortex in the case of Pro30. (H) No significant correlation was detected when placing the seed voxel in the thalamic region. Distances relative to bregma in mm are indicated in the lower left corner of the images. Values for correlation coefficients to the seed are color-coded for each voxel, blue for negative correlation (-0.5 \leq R \leq -0.1) and yellow for positive correlation (0.1 \leq R \leq 0.5).

3.3.1 Seed-based analysis under global signal regression

Seed-based analysis of GSR data was performed using regions highlighted in an independent component analysis, and included 3 parietal regions (the frontal, medial and posterior parietal cortex) in addition to components in the cingulate cortex, ventral and dorsal striatum, and limbic areas, which regroup partial amygdala, insula, and piriform cortex (Suppl. Fig. S2). The analysis revealed intra- and inter-hemispheric correlation with a delineation of anatomical structures. Bilateral correlations with respect to the seed in the anterior parietal cortex were observed for all anesthesia regimens (Fig. 1A). However, only data acquired under Iso1 and Pro30 showed intra-
and inter-hemispheric correlation to the medial parietal cortex seed, while the posterior parietal cortex seed only yielded ipsi- and contralateral correlation under Iso1 (Fig. 1B, C). In contrast, only data acquired under Med0.1 presented above threshold bilateral correlation to the ventral and dorsal striatum seed (Fig. 1D, E). Finally, ipsi- and contralateral correlations within the limbic system were observed in both the Med0.1 and the Ure1.5 group (Fig. 1F). Seed-based analysis using a seed in the cingulate cortex revealed correlations that were principally confined within the cingulate for all regimens (Fig. 1G) with the notable presence of anti-correlation in the parietal cortex when Pro30 had been used. In GSR data, no correlation above threshold has been observed between the cortex and the thalamic seed irrespective of the anesthetic regimen (Fig. 1H).

**Figure 2 |** Seed-based analysis of un-regressed (UR) data for the different anesthetic regimens. Images display regions with correlation coefficients exceeding the threshold value for seeds in the anterior parietal cortex (ctx; A), and the thalamus (B) for Iso1, Med0.1, Pro30, and Ure1.5, respectively. (A) Analysis of the Iso1 and the Ure1.5 group revealed widespread FC across the cortex with respect to the seed in left anterior parietal cortex. For Med0.1 and Pro30 delineated cortical structures were found, as well as anti-correlation to the signal extracted from the cingulate cortex in the case of Pro30. (B) Correlations between cortical regions and the seed in the thalamus were detected for Iso1, and to a lesser extent for Ure1.5. For Med0.1 the correlations were found confined within the thalamic region, and the Pro30 group showed widespread correlations across most brain structures as well as to surrounding muscle tissue. Distances relative to bregma in mm are indicated in the lower left corner of the images. Values for correlation coefficients to the seed are color-coded for each voxel, blue for negative correlation (-0.5 ≤ R ≤ -0.1) and yellow for positive correlation (0.1 ≤ R ≤ 0.5).

### 3.3.2 Seed-based analysis of un-regressed data

Performing seed-based analysis of UR data revealed correlations to extended bilateral cortical regions with respect to the cortical seeds under Iso1 and Ure1.5, while data acquired under Med0.1 and Pro30 showed correlations to better delineated regions, similar to the results found under GSR. The Pro30 group presented anti-correlation with the cingulate cortex as well as both correlation and anti-correlation with the surrounding muscle tissue with respect to the anterior parietal cortex seed (Fig. 2A). A seed positioned in the dorsal thalamus revealed correlations with the sensory cortex in data acquired under Iso1, also present in data acquired when using Pro30 and to a lesser
extent in the Ure1.5 group. For Med0.1 no correlations exceeding the threshold value were found between the cortical regions and the thalamic seed (Fig. 2B).

Figure 3 | Seed-based analysis of global signal regressed (GSR) data for the two different doses of medetomidine and Med/Iso. Images display regions with correlation coefficients exceeding the threshold value for seeds in the anterior and posterior parietal cortex (ctx; A, B), and the dorsal striatum (C) for Med0.1, Med0.05, and Med/Iso, respectively. (A) Correlations to the seed in the anterior parietal cortex indicate similar correlation patterns for all three conditions. Further, anti-correlations between anterior parietal and cingulate cortex were observed for Med/Iso. (B) Data acquired under Med0.1 did not reveal contralateral correlations to the posterior parietal cortex seed, in contrast to the results in Med0.05- and Med/Iso-anesthetized mice. (C) For all three anesthetic regimens, above threshold correlations were found between the seed in the dorsal striatum and the ipsi- and contralateral striatum. Distances relative to bregma in mm are indicated in the lower left corner of the images. Values for correlation coefficients to the seed are color-coded for each voxel, blue for negative correlation (-0.5 ≤ R ≤ -0.1) and yellow for positive correlation (0.1 ≤ R ≤ 0.5).

3.3.3 Dose dependence
To evaluate the influence of the anesthetic dose on FC patterns we administered two doses of each anesthetic. In general, FC patterns observed for the different anesthetics administered at low dose displayed less dissimilarity. Also, lower anesthetic depth yielded higher FC values with better confined regions within the individual networks. In particular, under low dose medetomidine anesthesia (Med0.05) contralateral cortical correlations were observed with respect to the frontal, medial, and posterior parietal cortex seeds in GSR data, while data acquired under Med0.1 only presented above threshold inter-hemispheric correlations with respect to the anterior parietal cortex seed (Fig. 3A, B). Bilateral cortical FC has been also observed for the low dose of isoflurane, propofol and urethane (Iso1, Pro30, Ure1.2). When evaluating UR data, Iso1.5 similar to Ure1.5 revealed correlations exceeding the threshold to extended intra- and inter-hemispheric cortical regions for a seed in the anterior parietal cortex, while data analysis of the corresponding low dose groups (Iso1, Ure1.2) revealed correlations to better confined cortical regions (Fig. 2, Suppl. Fig. S3)
Figure 4 | Inter-hemispheric correlations in the parietal cortex and the dorsal striatum for the different anesthetic regimens. A statistically significant effect of anesthesia was observed for both interactions evaluated, i.e. for the bilateral parietal cortex (ANOVA p-value = 1.2 x 10^{-7}) and the bilateral dorsal striatum (ANOVA p-value = 1.7 x 10^{-4}). Med/Iso anesthesia yielded the highest correlation coefficients for the parietal cortex, R = 0.89 ± 0.04, which was statistically significantly different with regard to Med0.05 (p-value = 2 x 10^{-2}), Med0.1 (p-value = 5.9 x 10^{-7}), and Ure1.5 (p-value = 1.5 x 10^{-3}), respectively. Anesthesia with Med0.05 showed the strongest correlation between the left and right dorsal striatum, R = 0.66 ± 0.22. The difference was statistically significant when compared to Iso1 (p-value = 2.7 x 10^{-2}), Pro30 (p-value = 2.4 x 10^{-2}), and Ure1.5 (p-value = 2.8 x 10^{-2}), respectively. All data are given as mean across animals ± standard deviation (SD). Significant differences between different anesthetic regimens are indicated (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

3.3.4 Combination of medetomidine and isoflurane
Combination of Med0.05 with isoflurane at a dose of 0.5% resulted in bilateral correlations with respect to the seeds in the parietal cortex (Fig. 3A, B, C) as well as with respect to the seeds in the dorsal striatal that exceeded the threshold (Fig. 3C). Similarly to administering Med0.05 alone, the combination revealed correlations to confined cortical structures with respect to the anterior parietal cortex seed in the UR data (Suppl. Fig. S3). Data acquired under Med/Iso presented the highest left to right parietal cortex correlation (ANOVA: anesthetic effect: p-value = 1.2 x 10^{-7}, Fig. 4), which was statistically significantly different from the results under Med0.1 and Med0.05. Finally, applying the anesthetic combination resulted in high left to right dorsal striatal correlation, comparable to values computed when using Med0.05 (ANOVA: anesthetic effect: p-value = 1.7 x 10^{-4}, Fig. 4). Generally, applying Med/Iso resulted in significantly higher correlations of the signal extracted from cortical and subcortical brain areas compared to isoflurane, medetomidine, propofol, and urethane anesthesia at the doses used.

3.3.5 Frequency components of rs-fMRI signals
The temporal profile of the cortical seed area was decomposed into frequency components by Fourier transformation. For Iso1 a 1/f frequency distribution has been found with the highest amplitude at the lower limit of the spectrum at 0.01 Hz. Also, for data acquired under Pro30 and Ure1.5, a 1/f frequency distribution has been observed, though somewhat less distinct for Pro30. In contrast, for medetomidine at either dose the frequency distribution peaked around 0.2 Hz in
the cortical seed area, while for Med/Iso it was shifted to slightly lower frequencies, i.e. 0.15 Hz (Fig. 5A).

**Figure 5 |** Mean frequency distribution and approximate entropy (ApEn) values of the signal extracted from the seed in the anterior parietal cortex for the different anesthetic regimens. (A) A 1/f frequency distribution was observed for the Iso1 group, and to a lesser extent for the Pro30 and the Ure1.5 groups. Medetomidine anesthesia at both doses showed frequencies centered around 0.2 Hz, while analysis of data acquired in mice anesthetized with Med/Iso revealed a frequency distribution that peaked around 0.15 Hz. (B) Approximate entropy was computed from the rs-fMRI signal extracted from the anterior parietal cortex seed to assess recurrent patterns in the signal at a window length m = 3. Kruskal-Wallis test for non-parametric data revealed a between group difference (p-value = 1.8 \times 10^{-4}). Both Pro30 and Med/Iso show highest ApEn, i.e. lowest predictability of the signal compared to the other anesthetic regimens. All data are given as mean across animals ± standard deviation (SD). Significant differences between different anesthetic regimens are indicated (*p \leq 0.05, **p \leq 0.01, ***p \leq 0.001).

Approximate entropy (ApEn) was computed from the signal measured in the frontal parietal seed as a measure of the predictability within a time series. Lower ApEn values indicate a higher probability of finding repeated patterns of a given window length m within the signal, while higher values are indicative for lower predictability. Comparing ApEn for m = 3 using a non-parametric Kruskal-Wallis test revealed a significant difference among the anesthetic regimens (p-value = 1.8 \times 10^{-4}, Fig. 5B). In particular, data acquired under Med/Iso and Pro30 yielded higher ApEn values, indicating increased stochastic behavior in the temporal signal. In contrast, medetomidine alone at both doses yielded ApEn values, which were statistically significantly lower than those for Pro30 or Med/Iso. These observations were also consistent for all window lengths of 3 \leq m \leq 5, indicative of data acquired under Pro30 and Med/Iso to display a less predictable temporal signal fluctuation.

### 3.3.6 Systemic effects of anesthetics

Measured values for heart rate, pulse distention, and O$_2$ saturation under the different anesthetic regimens are summarized in Table 2. Physiological variables were found similar and rather stable over time for both doses of isoflurane and urethane. Physiologically unstable conditions in propofol-anesthetized animals caused frequent loss of pulse waves affecting the reliability of the recorded values. Medetomidine anesthesia caused bradycardia that was more pronounced at the higher dose. Values for pulse distention were lowest for urethane at both doses used and highest for propofol, and varied markedly among animals under medetomidine anesthesia. O$_2$ saturation was...
in physiological ranges for all four anesthetic regimens at all the doses used. While measuring the systemic parameters we observed that i.v. infusion of medetomidine led to muscle twitching, which had been described previously for this anesthesia [17]. The combination of low doses of medetomidine and isoflurane (Med/Iso) produced more stable conditions compared to anesthesia with medetomidine alone at the lower dose.

3.4 Discussion

Anesthetics affect neural activity, metabolism, cerebrovascular tone, perfusion pressure and/or cerebral autoregulation to a variable degree and, therefore are likely to have an impact on FC patterns derived from rs-fMRI experiments. In fact, we obtained anesthetic-specific FC maps, which were highly reproducible, for mice studied under commonly used doses of isoflurane, medetomidine, propofol, and urethane, all administered in conjunction with a neuromuscular blocking agent. Analysis of GSR data for Iso1 and Pro30 revealed similar FC patterns with inter-hemispheric connectivity of corresponding cortical regions, but not of subcortical regions. In contrast, animals anesthetized using Med0.1 displayed significant bilateral FC for subcortical regions, while intra- and inter-hemispheric cortico-cortical connectivity was too weak to be detected. Anesthesia with Ure1.5 resulted in very weak overall FC. Analysis of the results in the context of physiological and pharmacological effects elicited by these anesthetics might reconcile the apparently contradictory reports for mice in the rs-fMRI literature [6-10].

The FC patterns observed for Iso1 and Pro30 in our study displaying bilateral connectivity among corresponding cortical regions are in agreement with data published previously using isoflurane [8]. Similar cortical FC maps were reported from a study using ketamine anesthesia and intrinsic optical imaging as readout modality [18]. In contrast, Jonckers et al. [7] and Nasrallah et al. [10] observed unilateral cortical connectivity in mice using high doses of medetomidine. This observation might have been exacerbated as these authors applied a band pass filter of the range 0.01 Hz to 0.1 Hz. We found that the frequency distribution of the fMRI signal yields the maximum amplitude at 0.2 Hz under medetomidine anesthesia, an similar observation was also reported by Kalthoff et al. for rats [19]; hence significant contributions of the rs-fMRI signal might have been rejected by the choice of band pass filter used by Jonckers et al. and Nasrallah et al. Bilateral cortical connectivity was observed by Nasrallah et al. [10] for a lower dose of medetomidine administered subcutaneously, which corresponds to our results for Med0.05. Sforazzini et al. [9] reported bilateral subcortical FC in addition to cortical FC in mice anesthetized with 0.7% halothane, whereas we did not observe subcortical inter-hemispheric correlation when using isoflurane, a volatile anesthetic of the same class. However, halothane is a potent hepatotoxic anesthetic, which limits its use in laboratories. We did obtain a very similar FC pattern as reported for halothane including subcortical bilateral correlations in mice anesthetized with Med0.1, Med 0.05 or Med/Iso. For all anesthetic regimens used in this study correlations within the cingulate/retrospinal regions have been observed, the origin of which remains controversial. While studies in mice [8-9] and in rats [20-21] referred to it as rodent elements of the default mode network, the interpretation of the underlying neuronal origin of this region is likely skewed by the close proximity of large blood vessels as proposed before [19].
In order to rationalize the effects of anesthetics based on their resting state FC characteristics both the depth of anesthesia and the drug’s pharmacological effects should be considered. In our study, anesthesia depth was inferred from tests regarding residual reflexes of the anesthetized mice and from the fMRI signal response elicited by innocuous electrical hindpaw stimulation [14]. When evaluating stimulus-evoked fMRI responses under the four anesthetics, we found differences in temporal dynamics: the responses in animals anesthetized with Iso1 and Pro30 evolved much more quickly than those of animals under Med0.1 and Ure1.5. Based on this we conclude that anesthesia levels for Med0.1 and Ure1.5 were deeper than for Iso1 and Pro30.

When comparing rs-fMRI results obtained at the lowest dose used for each anesthetic FC patterns appeared more similar. This is to be expected as anesthetic-specific effects should become less prominent when reducing the dose, thereby approaching the awake state. While it would be highly attractive to study the functional brain architecture in awake mice, reliable FC results have been reported as difficult to achieve [6]. Nevertheless, such measurements had yielded FC maps, which appeared similar to maps that we obtained under 1% isoflurane anesthesia [6]. An analogous result comparing awake and isoflurane (2%)-anesthetized state has been reported for rats [22].

In general, lower anesthetic depth, which also goes in hand with fewer pharmacological effects on the animal physiology, revealed higher FC values with better confinement of brain regions involved. For instance, significantly improved cortico-cortical connectivity has been found for data acquired using a lower dose of medetomidine (Med0.05) and of urethane (Ure1.2). Similarly, the Iso1 and Pro30 groups revealed higher thalamo-cortical correlation compared to Ure1.5, in line with the increased depth of anesthesia for Ure1.5. Interestingly, no significant FC between cortex and thalamus has been observed for medetomidine at either dose. The absence of thalamo-cortical connectivity under medetomidine anesthesia has been reported before also for rats [17] and constitutes a clear distinction from the pattern exerted by the other three anesthetics studied.

Deeper anesthesia was also observed to induce unspecific correlations, which becomes obvious when processing UR data. For example, the Ure1.5 group does not display contralateral correlation to the seeds in the cortex in GSR data, while UR data indicate a widespread pattern of correlation across the cortical and subcortical structures. The increase of synchronicity in resting state signals might reflect the deep anesthetic state reached at the dose of 1.5 g/kg, and therefore a strong modulation of cortical processing, which comes with the synchronization of neuronal activity. This is also observed for isoflurane: upon increasing the dose from 1% to 1.5%, more widespread bilateral cortical correlations have been found (Suppl. Fig. S3). Dose-dependence studies using rs-fMRI combined with electrophysiological recording performed in rats under isoflurane [23] and under propofol [24] substantiate the concept that in deeply anaesthetized animals the spatial confinement of correlated regions disappears at the expense of unspecific widespread synchronicity across large cortical areas. Such widespread unspecific correlation could not be observed under high dose of medetomidine, which might be related to its strong vasoconstrictive activity [25].

The specific pharmacological mechanism of each anesthetic elicits specific neural responses. Both, isoflurane and propofol target predominantly the GABAergic neurotransmitter system [26-27]. Based on the regional GABA receptor expression in the brain, subcortical structures with a high density of GABA(A) receptors such as the striatum are likely to respond more sensitive to these
anesthetics than cortical structures. This might explain the absence of any significant subcortical inter-hemispheric connectivity already at low doses. Urethane is described to provide stable long-lasting anesthesia with only modest effects on multiple ligand-gated ion channels [28-31]. It influences GABAergic neurotransmission, but to a lesser extent than isoflurane and propofol. Yet, striatal connectivity could not be detected either. The effects of medetomidine on neural function are rather distinct from those of the other anesthetic agents used here, which is also reflected in the unique features of the FC pattern. Medetomidine has negligible effects on the GABAergic system, but produces sedation by interaction with central α2-adrenoreceptors [32-33]. The receptor distribution and therefore the sensitivity to medetomidine varies across the brain and so may the neural and consequently the neurovascular response. Accordingly, Nasrallah et al. [10] attributed the dose- and region-dependent FC patterns to the density of α2-adrenoreceptors in different brain regions. Whereas the thalamus is characterized by a high density, cortex and caudate putamen express intermediate or low receptor density, respectively. This might explain the absence of thalamo-cortical connectivity, as also reported in other studies [17], as well as the persistence of bilateral striatal connectivity even at higher doses of medetomidine.

The hemodynamic response mediated via neurovascular coupling depends on the cerebrovascular baseline state, which typically is affected by the anesthetic regimen. For mice anesthetized with Pro30, baseline CBV values indicated cerebral vasodilation. In contrast, medetomidine-anesthetized mice displayed low baseline CBV values in line with the known vasoconstrictive properties of the drug. Administration of Iso1 and Ure1.5 resulted in an intermediate effect on baseline CBV (Suppl. Fig. S4). Vasodilation may reduce the adaptive capacity of vessels, i.e. the amplitude of BOLD signal fluctuations, and consequently the apparent FC. In contrast, the vasoconstriction effect of medetomidine reduces vascular reactivity, which is also reflected by the delayed onset of the fMRI response to sensory paw stimulation as reported earlier [14]. The compromised vascular reactivity could translate into smaller amplitudes of BOLD signal fluctuations and consequently reduce FC values.

However, our results were not fully in line with above mentioned predictions. Data acquired under Pro30 presented high FC potentially due to the increased arousal state or physiological fluctuations, respectively, which we conclude from the occurrence of signal correlations to surrounding muscle tissues that have been observed for this anesthetic regimen only. In contrast, for the Med0.1 group these hypotheses could be confirmed for cortical and thalamic regions of interest, but not for the striatum. Reduced CBV values were observed in all three brain regions (cortex, striatum, thalamus; Suppl. Fig. S4). Differential connectivity patterns, however, have been observed with significant bilateral FC for striatal but not for cortical and thalamic areas, which points to a neural origin of the regionally different rs-fMRI signals. Also, Iso1 and Ure1.5 show similar effects on baseline CBV values, but quite distinct effects on FC patterns. Taken together, we conclude that the cerebrovascular baseline state is a minor contributor to the FC pattern: The effects induced by different baseline CBV states appeared to be overruled by the effects of anesthesia depth or drug-induced pharmacological effects on the neural system.

The vasoconstriction state in medetomidine-anesthetized mice might be phenomenologically reflected by the frequency components in the fMRI signal. Similar to Kalthoff et al. [19] in rats, we
observed a peak at 0.2 Hz in the frequency distribution of the cortical rs-fMRI signal in medetomidine-anesthetized mice, which was not the case for any of the other anesthetics evaluated. Typically, rs-fMRI signals are characterized by a frequency distribution with high amplitudes at low frequencies (0.01 Hz, 1/f distribution), which is attributed to a slow hemodynamic response to high-frequency neuronal signal. However, vasoconstriction associated with medetomidine anesthesia leads to a hemodynamic response of short duration [14], which may enhance the probability of detecting higher frequency components.

There is an increasing amount of data suggesting that medetomidine might be a suitable candidate for rs-fMRI studies in rodents [10, 19, 34-35]. An ideal anesthetic would exert only minimal systemic effects while providing sufficient depth of anesthesia and should be suited for longitudinal studies. Medetomidine at moderate doses can provide sufficient sedation for rs-fMRI experiments. Nevertheless, side effects on the cardiovascular system including bradycardia, hypotension and vasoconstriction have to be considered, even at the low dose used in this study. In addition, i.v. infusion was found to cause muscle twitches, presumably related to epileptic activities. Fukuda et al. [17] proposed supplemental administration of low dose isoflurane in order to suppress potential seizures without changing the desired effects of medetomidine. The presence of epileptic activity might fit to the low entropy values for data acquired under medetomidine alone, which could reflect the presence of seizure-related repeated patterns in the signal. The higher entropy values for Med/Iso might indicate the absence of epileptic activity. In addition, it has been described that isoflurane with its vasodilatory characteristics compensates the vasoconstrictive effect of medetomidine to some extent [17].

Analysis of rs-fMRI data acquired in mice anesthetized with Med/Iso yielded strong bilateral cortical and subcortical FC. In essence, the combined anesthesia recovers the main FC features of low dose medetomidine, thus providing data that are not contaminated by the potential contribution of epileptic seizures.

Our study reproduces several features reported from human rs-fMRI studies such as the loss of bilateral striatal connectivity prior to loss of thalamo-cortical connectivity observed under propofol anesthesia [36]. Furthermore, Peltier et al. [12] showed that increasing the sevoflurane dose to induce deeper anesthesia led to a gradual loss of connectivity in the motor cortex. Stamatakis et al. [37] observed loss of spatial confinement of DMN and motor networks linked to anesthesia, similar to our observations of widespread cortical synchronization with higher doses of isoflurane or urethane. Studies in humans have suggested several mechanisms potentially leading to the loss of consciousness such as the loss of information content within the signal either in the form of temporal information, reflected in the entropy measures, or of the spatial integration [13, 38]. Our data may be related to this: rs-fMRI data acquired under medetomidine anesthesia display low signal entropy indicative of reduced temporal information, while FC maps obtained from data of isoflurane- and urethane-anesthetized mice indicate a loss of spatial specificity with increasing dose of the anesthetic.

In conclusion, our results show how aspects such as anesthesia depth and pharmacological effects of four different anesthetic drugs might explain differences in FC patterns. This allowed rationalizing the differential responses to the various anesthetic regimens by assuming two
categories with isoflurane, propofol and urethane in one and medetomidine in the other class. Within the first group consistent FC patterns characterized by cortical and thalamo-cortical but no striatal correlations were observed when accounting for the anesthesia depth. Medetomidine anesthesia revealed a distinctly different pattern displaying no thalamo-cortical but pronounced subcortical FC. Combining medetomidine with isoflurane, a representative of the first category yielded a synergistic effect: both cortical and subcortical FC could be reliably detected with no detrimental side-effects observed. Both the high reproducibility and the extend of the brain structures showing connectivity under Med/Iso offer opportunities to investigate biological mechanisms underlying the rs-fMRI signal and for conducting biological studies in mouse models of brain disorders in order to better understand the functional mechanisms induced by different pathologies.
3.5 Supporting information

Supplementary figure S1 | Raw and processed image quality. (A) Representative multi-slice GE-EPI data showing twelve coronal sections through the mouse brain. Mean (B) and standard deviation (C) of the coregistered images to an in-house EPI template.

Supplementary figure S2 | Overview of the different components identified by independent component analysis. All cortical components shown have been derived from data sets acquired under Iso1, all subcortical components from data sets acquired under Med0.1. For seed-based analysis the 2 x 3 voxels regions displayed in green were used as seeds, which were usually positioned within the area of highest intensity of a particular component. For the motor cortex (ctx) no seed region has been defined, as a seed-based analysis has not been performed for that area. Distances relative to bregma in mm are indicated in the lower left corner of the images. Values for correlation coefficients to the seed are color-coded for each voxel, blue for negative correlation (-0.5 ≤ R ≤ -0.1) and yellow for positive correlation (0.1 ≤ R ≤ 0.5).
Supplementary figure S3 | Seed-based analysis of un-regressed data (UR) for alternative doses of anesthetics. (A) Images display regions with correlation coefficients exceeding the threshold value for the seed in the anterior parietal cortex (ctx) under Iso1.5, for which correlation across the entire cortical ribbon was observed, and under Ure1.2, for which the correlation pattern presented a tendency to more defined cortical structures as highlighted by independent component analysis. (B) Similarly, the correlations to the signal extracted from the anterior parietal cortex seed for the Med0.1 and Med/Iso groups presented a tendency towards delineated cortical structures. Distances relative to bregma in mm are indicated in the lower left corner of the images. Values for correlation coefficients to the seed are color-coded for each voxel, blue for negative correlation (-0.5 ≤ R ≤ -0.1) and yellow for positive correlation (0.1 ≤ R ≤ 0.5).

Supplementary figure S4 | Contrast agent (CA)-induced changes in baseline relaxivity ΔR₂₀ CA as marker for relative baseline CBV values (CBVbsl) in the primary somatosensory cortex (S1), Thalamus (Thal) and Striatum (caudate putamen, CP) for Iso1, Med0.1, Pro30 and Ure1.5, respectively. All data are given as mean across animals ± standard error of the mean (SEM). Significant differences between regions
and between different anesthetic regimens are indicated (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).


4 Early alterations in functional connectivity and white matter structure in a transgenic mouse model of cerebral amyloidosis

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Abstract

Impairment of brain functional connectivity (FC) is thought to be an early event occurring in diseases with cerebral amyloidosis such as Alzheimer’s disease. Regions sustaining altered functional networks co-localize with regions marked with amyloid plaques burden suggesting a strong link between FC and amyloidosis. Whether the decline in FC precedes amyloid plaque deposition, the hallmark of Alzheimer’s disease, or is a consequence thereof, is currently unknown. The sequence of events during early stages of the disease is difficult to capture in humans due to the difficulties in providing an early diagnosis and also in view of the heterogeneity among patients. Transgenic mouse lines over-expressing amyloid precursor proteins develop cerebral amyloidosis and constitute an attractive model system for studying the relationship between plaque and functional changes. In this study, ArcAβ transgenic and wild-type mice were imaged using resting-state fMRI methods across their life-span in a cross-sectional design to analyze changes in FC in relation to the pathology. Transgenic mice show reduced FC developments during the first months of post-natal life compared to wild-type animals, resulting in functional impairments, in particular affecting the sensory-motor cortex already in pre-plaque stage. The changes were matched with decreased fractional anisotropy, a marker for white matter integrity, derived from diffusion tensor MR imaging. Our results suggest cerebral amyloidosis in mice is preceded by impairment of neuronal networks and white matter structures. FC analysis in mice is an attractive tool for studying the implications of impaired neuronal networks in cerebral amyloid pathology.
4.1 Introduction

The occurrence of amyloid plaques in brain parenchyma and around the cerebral vessels constitutes a hallmark of Alzheimer’s disease (AD) [1]. Cerebral amyloidosis involves the aggregation of Aβ peptides, a cleavage product of the amyloid precursor protein (APP). In AD, plaque deposits, fibrils, and oligomers of Aβ are considered essential mediators in the pathophysiological cascade leading to cognitive decline [2]. Aβ peptides in various forms have been linked to alterations in cerebral calcium homeostasis [3], axonal guidance [4], axonal transport, and/or synaptic signaling [5], processes that ultimately might translate into impaired neural function.

Marked functional impairments have been observed in patients with AD using functional magnetic resonance imaging (fMRI) [6-7]. In particular, resting state fMRI (rs-fMRI) has been extensively used to investigate functional connectivity (FC) as derived from the apparent synchronicity of the fMRI signal of distinct brain regions. Human brain appears organized into 10–15 networks [8], among which, the default mode network (DMN) has gained attention in AD due to its co-localization with amyloid plaques [9-10]. Additionally, DMN connectivity was reported affected in subjects at risk of developing AD including patients with mild cognitive impairments [11-12], in cognitively healthy elders with amyloid deposits [13-15], and in mid-life carriers of APO ε4 risk allele [16-17].

Elucidating the relationship between plaque deposition and functional network change in a temporal and spatially resolved manner is essential for understanding the pathophysiological cascade and may provide an early diagnostic tool. However, the heterogeneity among AD patients combined with poor method reproducibility at the individual level, the latency of disease onset and difficulties to accurately perform early diagnostics in patients have rendered such studies in humans difficult.

Animal models of amyloid pathology offer an experimental platform to studying AD [18]. Although the molecular processes underlying amyloid pathology in animals have been examined extensively, studies addressing its implication on brain FC are lacking as fMRI in mice is challenging. We have assessed the FC in mice across their lifespan, from 1 month to 21 months, in a cross-sectional design using rs-fMRI. The ArcAβ transgenic mouse model of cerebral amyloidosis has been used, which over-expresses the human APP protein with the Swedish and Arctic mutations. The mice show reduced synaptic plasticity [5], and amyloid pathology affecting both the brain parenchyma and vasculature by 6 months of age [19-20]. To obtain a structural correlate for FC changes we have complemented fMRI measurements assessing the white matter architecture using diffusion tensor imaging. Combining structural and functional readouts provides a spatial and temporal map of the macro-cellular and dynamical alteration occurring in a mouse model of amyloidosis.

4.2 Method

4.2.1 Animal preparation

All animal experiments in this study have been performed in adherence to the Swiss law of Animal Protection and were approve by the Zurich cantonal veterinary office. A total of 38 (17 male and 21 female) transgenic ArcAβ mice and 36 (12 male and 24 female) wild-type littermates aged between 1 and 21 months were used in this study in 4 cohorts, with group sizes shown in Table 1. Animals were housed in standard mouse caging, with 12h:12h light and dark cycles with food and water...
provided ad libitum. Mice were anaesthetized with isoflurane (Abbott, Cham, Switzerland) in 1:4 oxygen to air mix at 3% for induction and 1.4% during measurements administered via a face mask. Animals were placed on a mouse support with ear bars placed to reduce animal motion during the experiments. The animal body temperature was monitored with a rectal thermometer and kept at 36.5°C +/- 0.5°C with an adjustable warm water bath flowing in the support. Mouse preparation and measurement typically lasted for 45 minutes. Animals were measured a second time in a week interval to obtain values of reproducibility.

4.2.2 MRI
A BioSpec 94/30 animal MRI system (Bruker BioSpin GmbH. Karlsruhe, Germany) equipped with a 9.4 T magnet with a horizontal bore of 30 cm diameter, a BGA12S gradient system capable of a maximum gradient strength of 400 mT/m with a 80 µs rise time, a 2 x 2 cryogenic phased-array receive coil and a linear volume resonator coil for excitation have been used. Anatomical images have been recorded using a multi-slice rapid acquisition with relaxation enhancement (RARE) sequence with repetition time TR = 2500 ms, echo time TE = 11 ms, effective echo time TEeff = 33 ms, pulse angle FA = 90°, number of average NA = 1, matrix dimension MD = 180 x 180, pixel dimensions V = 111 x 97 µm², slice thickness ST = 500 µm, inter-slice distance ISD = 200 µm, and number of slices NSl = 12. For fMRI studies gradient-echo echo-planar imaging (EPI) has been used with TR = 1500 ms, TE = 9.3 ms, FA = 50°, number of repetition NR = 500, NA = 1, MD = 90 x 70, V = 250 x 220 µm², ST = 500 µm, ISD = 200 µm, NSl = 12. Diffusion tensor imaging was performed with a multi segment DTI-EPI sequence, with TR = 3000 ms, TE = 25 ms, FA = 90°, number of segments = 4, NA = 1, MD = 128 x 128, V = 160 x 130 µm², ST = 500 µm, ISD = 200 µm, NSl = 12, b-value of 690 s/mm² and 36 diffusion encoding directions.

![Figure 1](image_url) | Color coded template used for network analysis showing 18 regions-of-interest based on the functional organization of the mouse brain. The distance of the respective section from Bregma in mm is indicated.

4.2.3 Images processing
Images were pre-processed in SPM05 (The FIL group, London, UK) for MATLAB (The MathWork Inc, Natick, USA). Functional MR images were re-aligned, corrected for slice timing and normalized to
an in-house EPI template image. Motion and ventricular signal were regressed from the time series in Matlab and the time series band pass filtered between 0.01 Hz and 0.15 Hz using Rest toolbox (State Key Laboratory of Cognitive Neuroscience and Learning, Beijing, China) for Matlab. Mean time series were extracted from the regressed images using an in house anatomical template based on the functional organization of the mouse brain with 18 different regions of interest (Figure 1). Functional connectivity was measured as the Pearson correlation coefficient between mean time series from each brain region pairs, corrected with Fisher-z transformation for bounded values.

Fractional anisotropy (FA) was computed from diffusion tensor images using Paravision 5 (Bruker BioSpin GmbH. Karlsruhe, Germany). Images were converted to Analyze 7.5 format and normalized to a reference space using b0 images with SPM05. Fractional anisotropy values were extracted for selected regions-of-interest (ROIs).

Unprocessed anatomical, diffusion tensor, and functional images are freely available at central.XNAT.org in analyze7.5 format (Project ID= fMRI_AD_mouse).

4.2.4 Histology
Animals were deeply anesthetized with Ketamine/Xylazine, and transcardially perfused with cold PBS followed by 4% paraformaldehyde. Brains were extracted and put in paraformaldehyde over night. Brains were transferred in succession in 10%, 20%, and 30% sucrose solution, before being frozen at -80°C. Randomly sampled serial sections (cut at 40 μm) were collected and stored at -20°C in cryoprotectant solution (50 mmol/l sodium phosphate buffer, pH 7.4, containing 15% glucose and 30% ethylene glycol; Sigma-Aldrich). The free-floating sections were treated for 10 minutes with pepsin (Dako) 0.15 mg/ml in 0.2 N HCl at 37°C. After three washes in PBS, the pepsin treated brain sections were then incubated overnight at 4°C in the primary antibody solution (mouse monoclonal Amyloid-β1-16, clone 6E10, diluted 1:1’000, Covance) diluted in PBS containing 2% normal goat serum and 0.2% Triton X-100. After three washes in PBS, tissue sections were incubated for 30 minutes at RT in biotinylated secondary antibody (diluted 1:500; Jackson ImmunoResearch Laboratories). A commercial kit (Vectastain Kit; Vector Laboratories) was then used with 3,3-diaminobenzidine (DAB; Sigma–Aldrich Inc.), and sections were stained for 5 to 10 minutes. After three washes in PBS, sections were mounted onto gelatinized glass slides and air-dried overnight. The sections were then dehydrated through ethanol, cleared in xylene, mounted with resinous mounting medium (EukittTM Sigma-Alrich), and coverslipped.

4.2.5 Statistics
Statistical analysis was performed using the statistical software R version 2.13.0 (The R Foundation for Statistical Computing, Vienna, Austria). A linear mixed model analysis was performed with gender, age, genotype, and their twofold interactions as fixed effects using the function lmer of the lme4 package (lme4: Linear mixed-effects models using S4 classes, Douglas Bates and Martin Maechler and Ben Bolker, 2013). Since the variables modeled, i.e. correlation coefficient and fractional anisotropy, are bounded, we applied the Fisher's z transformation to the response variable. The effects of each interaction and factor were tested with Likelihood Ratio Tests. Residual analysis of the mixed models was performed on randomly selected samples (n=30) with QQ-plots to inspect normal distribution, Tukey-Anscombe plots for the homogeneity of the variance and skewness, and Scale Location plots for homoscedasticity (i.e. the homogeneity of
The assumption of normally distributed residuals was considered plausible in all tested fractional anisotropy samples and in all of the randomly tested functional connectivity pairs describing cortical interactions. Multiple comparison was corrected with false discovery rate (FDR)[21], with a q-value of 0.05 for network comparison, and 0.15 for voxel-wise analysis. Voxel-wise maps were further filtered for statistically significant clusters of 15 voxels or more. Contrasts were used in the mixed model analysis to test the statistical significance differences between slopes in wild-type and transgenic mice between 1-5 months and between 5-19 months (slope contrast), and the absolute genotype effect between 1-5 months and 5-19 months (genotype contrast). The null hypothesis, i.e. the absence of effect for a given factor or interaction, was rejected according to the FDR threshold, and is denoted in the text as factor effect for simplicity (e.g. genotype effect, genotype x age interaction effect, etc). Values in plots and images are shown as mean value per group and error bars represent +/- 1 standard deviation, p-values are shown as log10(p-value) to account for the large range of values.

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>N</th>
<th>tSNR</th>
<th>S1</th>
<th>Maximum motion</th>
<th>Variance motion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(female / male)</td>
<td>(+/- sd)</td>
<td>(+/- sd)</td>
<td>(μm +/- sd)</td>
<td>(μm² +/- sd)</td>
</tr>
<tr>
<td>1 month §</td>
<td>WT</td>
<td>7</td>
<td>164.2 (20.4)</td>
<td>41 (17)</td>
<td>.16 (.17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>14 (7 / 7)</td>
<td>162.1 (34.8)</td>
<td>39 (24)</td>
<td>.14 (.13)</td>
<td></td>
</tr>
<tr>
<td>2 months §</td>
<td>WT</td>
<td>7</td>
<td>180.8 (14.2)</td>
<td>40 (14)</td>
<td>.17 (.11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>14 (7 / 7)</td>
<td>184.1 (12.4)</td>
<td>44 (25)</td>
<td>.22 (.29)</td>
<td></td>
</tr>
<tr>
<td>5 months</td>
<td>WT</td>
<td>8</td>
<td>113.4 (16.5)</td>
<td>39 (33)</td>
<td>.32 (.22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>8</td>
<td>113.2 (15)</td>
<td>47 (44)</td>
<td>.31 (.28)</td>
<td></td>
</tr>
<tr>
<td>8 months #</td>
<td>WT</td>
<td>9</td>
<td>104.7 (16.5)</td>
<td>44 (21)</td>
<td>.38 (.53)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>9 (6 / 3)</td>
<td>117.9 (15.5)</td>
<td>30 (14)</td>
<td>.31 (.60)</td>
<td></td>
</tr>
<tr>
<td>11 months #</td>
<td>WT</td>
<td>9 (6 / 3)</td>
<td>139 (17.8)</td>
<td>35 (24)</td>
<td>.18 (.21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>9 (6 / 3)</td>
<td>154.5 (22.1)</td>
<td>31 (20)</td>
<td>.59 (.71)</td>
<td></td>
</tr>
<tr>
<td>19 months ¥</td>
<td>WT</td>
<td>9 (7 / 2)</td>
<td>102.6 (24.5)</td>
<td>45 (32)</td>
<td>.51 (.75)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>10 (5 / 5)</td>
<td>101.8 (21.8)</td>
<td>49 (29)</td>
<td>.59 (.71)</td>
<td></td>
</tr>
<tr>
<td>21 months ¥</td>
<td>WT</td>
<td>7 (5 / 2)</td>
<td>146.4 (26.3)</td>
<td>22 (12)</td>
<td>.07 (.06)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>10 (5 / 5)</td>
<td>173.6 (38)</td>
<td>37 (27)</td>
<td>.23 (.41)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1** | Number of the animals used in the study per groups (WT=wild-type, TG=transgenic); mean tSNR in the S1 sensory cortex; and mean animal motion during fMRI acquisition. Groups denoted with §, #, and ¥ come from the same cohort respectively.
4.3 Results

4.3.1 Group description

Group size per genotype and age was between 7 and 14 animal (Table 1), with a female to male ratio of 45:29. During the study, a small portion of animals developed co-morbidities or abnormal structures observed with T2 anatomical images: 2 animals with abnormal ventricles (1 month old transgenic, 19 months old transgenic), 1 animal with focal hypointensity in the thalamus (1 month old transgenic). In addition, 2 wild-type animals had to be sacrificed between measurements at 19 months and 21 months due to age related events. All animals measured were included in the analysis.

Figure 2 | Network analysis of cortical regions showing cross-correlations in wild-type (lower left half of FC matrix) and ArcAβ mice (upper right half of FC matrix), at 1 (a), 2 (b), 5 (c), 8 (d), 11 (e), 19 (f), and 21 (g) months of age. Color-coded values represent the mean Pearson correlation coefficients per group corrected with Fisher-z transformation. The two elements for each cortical area correspond to regions-of-interest in left and right hemisphere, respectively. Strongest correlations in the wild-type groups are observed between left and right parietal (aPc, mPc, pPc), motor (mc), and retrosplenial/cingulate cortex. (h) Pair-wise mixed-model analysis showing the genotype and age effects below FDR correction threshold. Color-coded
values represent the log p-value for each effect ranging from 0 to -15, corrected with false discovery rate.

4.3.2 Development of FC during early months of life

In wild-type mice, the correlation coefficient between rs-fMRI time series for ROIs located within the somatosensory and motor cortex developed from values in the range $z = 0.32$ to 0.15 at 1 month to values of $z = 0.89$ to 0.4 measured at 5 months of age, (Figure 2, Figure 3 a, b, c). Similarly, although less pronounced, changes have been found in cross correlation between bilateral retrosplenial/cingulate and with the parietal cortices (Figure 2, Figure 3 d). The hippocampus (Figure 2, Figure 3 e) displayed weak correlations with coefficients $z \leq 0.3$ bilaterally, and $z \leq 0.1$ with other regions. Correlation did not exceed $z = 0.3$ between the dorsal and ventral striatum and the cortical regions (Figure 2).

![Figure 3](image.png)

**Figure 3** | Changes in correlation over time between selected representative pairs. Black triangles represent the mean value from transgenic ArcAβ mice; grey circles represent the mean value from wild-type controls. Young animals showed low correlation coefficient in cortical structures in both wild-type and transgenic animals at 1 month of age, increased between 1- and 5 months of age in wild-type but remained low in transgenic animals. Statistics for each effect is indicated below each plot with 'G' standing for genotype, 'A' for age, 'G:A' for genotype x age interaction. Genotype x interaction effect was observed in the anterior and posterior parietal cortex bilateral correlation (a p-value= 2.5x10^{-8}, b p-value=5.5x10^{-3}), left to right motor cortex pair (c p-value=2.4x10^{-4}), and retrosplenial/cingulate cortex (d p-value=1.3x10^{-2}, above FDR threshold). There was no genotype effect in the hippocampus (e) or limbic regions (f). Error bars represent +/- 1 standard deviation. Statistics for each effect is shown under the plot as such, 'G' for genotype, and 'A' for age effect. The # symbol represents above FDR p-values.

4.3.3 ArcAβ mice display age related impaired FC development

While correlation coefficients observed in 1 month old ArcAβ mice were similar to the age-matched wild-type animals across all regions analyzed, they a reduced development of FC between month 1 and 5 for the cortical region-of-interests (ROI) (Figure 2 a, b, c, Figure 3 a, b, c). The rate of
correlation change per month (Δcorrelation/month) was evaluated with linear regression between 1-5 months, and 5-19 months, respectively and tested with a set of contrasts within the mixed-model analysis. ArcAβ showed smaller slopes for the cortical areas compared to wild-type during the 1-5 month period, for example Δcorrelation/month = 2.7x10⁻³ month⁻¹ in the medial parietal cortex in ArcAβ against Δcorrelation/month = 6.5x10⁻³ month⁻¹ in wild-type (Figure 4 a, c, contrast p-value = 4.5x10⁻¹⁰). There was, however, no significant difference in the medial parietal cortex between wild-type and transgenic animals between 5 to 19 months of age with minor changes over time, Δcorrelation/month = 1.5x10⁻⁴ month⁻¹ for ArcAβ and Δcorrelation/month = -1.9x10⁻⁴ month⁻¹ for wild-type respectively (Figure 4 b, c, contrast p-value = 0.98). Statistical analysis of the correlation matrices (Figure 2 h) revealed strong genotype effect for correlations among left and right medial and posterior parietal cortices, and motor cortices (Figure 3 b, c; posterior parietal cortices p-value = 3.3x10⁻⁹, motor cortices 5.1x10⁻³). There was no genotype x age interaction below FDR threshold. Correlations from the parietal cortices to the motor cortex showed an age effect (Figure 2 h). None of the correlation pairs, however, showed a gender effect or gender interaction effects below FDR threshold.

**Figure 4** | Correlation change in function of age in young adulthood (a 1-5 months) and mid-life (b 5-19 months) indicate lower slope in ArcAβ mice compared to wild-type during the early age in the cortical regions, with range Δcorrelation/time = 4.3x10⁻³ to 1.3x10⁻³ month⁻¹ in transgenic animals and Δcorrelation/time = 7.6x10⁻³ to 3.4x10⁻³ month⁻¹ in wild-type mice controls. In comparison, there was no substantial changes in correlation over time in mid-life in both ArcAβ with Δcorrelation/time = 2x10⁻⁴ to -9x10⁻⁴ month⁻¹ in the cortical areas, and wild-type mice with Δcorrelation/time = 0 to -2x10⁻⁴ month⁻¹. Post-hoc contrast analysis of differences between ArcAβ and wild-type between 1-5 months (c,d, lower left corner) and between 5-19 (c,d, upper right corner). The slope contrast tests for a true...
difference in slope (Δcorrelation /time) between the groups, while the genotype contrast tests for a true absolute difference in FC between the groups.

Seed-based analysis of the left anterior parietal cortex (Figure 5) illustrates the change of cross-correlation from 1 month to 5, 8, and 21 months of age (images for 11, 19 and 21 months are not shown given the similarity of 8 and 21 months data). In both ArcAβ and wild-type mice, a seed ROI within the somatosensory S1 cortex showed weak diffuse correlation with the adjacent cortex, and little bilateral correlations at 1 month. In wild-type animals, correlation to the contralateral somatosensory cortex increased by 5 months of age to z = 0.6 to 0.25 consistent with the network analysis discussed above. In ArcAβ mice, FC to the contralateral somatosensory cortex remained below the threshold of z = 0.1 also at 5, 8, and 21 months of age.

4.3.4 Changes in FC are associated with changes in white matter structures

A genotype effect in FA changes was found for the minor forceps, the anterior part of the external capsule, and the anterior internal capsule (Figure 6 b, c). Age effect on FA values was widespread in the white and grey matter (not shown), while a gender effect was identified for the anterior internal capsule. No factor interaction (e.g. genotype:age) effect was observed below FDR threshold.

Region-of-interest analysis revealed an increase in FA from 1 to 5 months of age in the corpus callosum and anterior external capsule in both wild-type and transgenic mice, although the rate of increase was greater in wild-type. In the period from 5 months to 19 months, FA values remained constant. There was a significant genotype effect in the corpus callosum (Figure 6 e, p-value = 1.8 x10^{-3}) and in the anterior external capsule (Figure 6 f, p-value = 1.6 x10^{-3}). The minor forceps showed different dynamics, with FA values decreasing over time in wild-type while remaining constant in transgenic mice (Figure 6 d, genotype effect, p-value = 5.6 x10^{-6}). Aging effect of FA changes were present in both the corpus callosum (Figure 6 e, p-value = 9.6 x10^{-5}) and anterior external capsule (Figure 6 f, p-value = 1.7 x10^{-7}). No genotype effect below FDR threshold was observed in the posterior external capsule, or in the cortex.

Figure 5 | Seed-based analysis of the S1 sensory cortex in wild-type (a) and ArcAβ mice (b). At 1 month, both genotype groups show diffuse and weak cross-correlation between the seed region and the surrounding voxels in the cortex. Wild-type animals at 5, 8 and 21 months show correlations marking the delineation of the parietal cortex with respect to seed. In comparison, ArcAβ mice show weak contralateral correlations to the seed. Color-coded values represent the cross-correlation coefficient between the seed and the corresponding voxel ranging from z= ±0.1 to ±0.5. Seed regions are indicated as red regions-of-interest, with the anatomical...
referenced overlayed from the mouse brain atlas. Values represent the distance from Bregma in mm.

Figure 6 | Voxel-wise analysis of fractional anisotropy in ArcAbta and wild-type mice reveals a genotype effect in the corpus callosum, anterior external capsule, minor forceps and internal capsule (b). A gender effect is found in the left internal capsule (c). Color-coded values represent the log p-value corrected with false discover rate at cluster size =15. Region of interest analysis shows the maximum FA value along a line profile crossing the minor forceps (ROI mf, d), corpus callosum (ROI cc, e), anterior external capsule (ROI ec\textsubscript{ant}, f), posterior external capsule (ROI ec\textsubscript{post}, f). Black triangles represent the mean value from transgenic ArcAβ mice; grey circles represent the mean value from wild-type controls. Similar fractional anisotropy values were found in young wild-type and transgenic animals, both in the anterior external capsule and the corpus callosum, and increased by 5 months in wild-type while it remained low in transgenic animals. Error bars represent +/- 1 standard deviation. Statistics for each effect is shown under the plot as such, ‘G’ for genotype, and ‘A’ for age effect.

4.3.5 Amyloid plaque deposition development

While only anecdotal parenchymal plaques have been detected in brains of 2 and 5 months old ArcAβ mice, amyloid pathology was densely present in the cortex and hippocampus in 11 and 21 months animals (Figure 7). This is in line with earlier reports [19].

Figure 7 | Immunohistological detection of Aβ peptides reveals the anecdotal occurrence of cortical plaques at 2 and 5 months of age in ArcAβ transgenic mice (b and c). Aged animals showed numerous wide-spread cortical plaques at 11 and 21 months (d and e). Scale bar represents 1 mm.
4.3.6 Potential confounds

We have used isoflurane as an anesthetic, as we observed it maximized cortical correlations, while medetomidine favored sub-cortical correlation. We have titrated isoflurane levels to achieve animal sedation, with lower doses resulting in excessive motion. The dose to achieve animal sedation was similar in all age and genotype groups. Temporal signal-to-noise ratio (tSNR) in the somatosensory cortex was found to vary between functional imaging sessions (Table 1, Figure 8 c) with animals imaged at 2 and 21 months of age displaying the highest tSNR values. There was no differences in tSNR between wild-type and transgenic animals, or male and females at any time point. Motion derived from the realignment algorithm was used to assess maximum motion between the 3 translation vectors, as well as the variance of the motion vectors. No correlation between tSNR and motion could be observed. Finally, neither tSNR nor motion showed statistical significant effect in the linear mixed model analysis. Variance of the within-subject effect was lower than the residual variance by two orders of magnitude, which is corroborated with a relatively low correlation in test-retest analysis in the correlations, ranging between $r = 0.4$ and $0.58$ across cortical pair-wise correlations (Figure 8 a). For example medial parietal cortex bilateral correlation yielded a test-retest correlation of $r = 0.48$ (Figure 8 b). Fractional anisotropy of the external capsule showed similar test-retest values of $r = 0.39$.

Figure 8 | Reproducibility of functional connectivity was evaluated by comparing the coefficients of correlation of the results in the two rs-fMRI sessions recorded at 1 week interval for every animal at a given age (a). Reproducibility ranged between $r = 0.4$ and $0.58$ between the cortical regions pair-wise correlation, and $-0.01$ to $0.2$ in the sub-cortical interactions. Intersession mean and difference is shown for the medial parietal cortex pair-wise correlation, illustrating the reproducibility of the measure in cortical regions (b). The temporal signal-to-noise ratio (tSNR) is shown per age group and genotype, and indicates a peak at 2 and 21 months, with tSNR values ranging between 105 and 169.

4.4 Discussion

Development of FC in the somatosensory and motor cortex, as derived from the temporal correlation of rs-fMRI signals, occurs during the first months of life in wild-type mice and appears to be significantly impaired in age-matched ArcAβ mice. These functional deficits are observed prior to and during the early plaque build-up stage, while there was no major decline in FC after 5 months of age during amyloid plaque build-up. White matter structures were found to be affected in a pattern matching FC impairments both spatially and temporally, with the exception of the minor forceps. However this discrepancy may be explained by the nature of the fiber orientation,
spreading to reach frontal cortical areas thus displaying low FA values also in the wild-type. The findings are in accordance with the previous description of amyloid pathology and cognitive deficits early in this mouse model [19], and reduced synaptic long term potentiation observed at 3.5 months [5].

The major finding of our study relates to the early and robust functional changes occurring in ArcAβ transgenic mice, appearing before amyloid deposition as derived from histological analysis and reported earlier for this animal model [19]. The results compare well with growing evidence of early functional impairments in humans at risk of developing Alzheimer’s disease, such as mid-life APOε4 carriers [16-17] or asymptomatic elderly with amyloid deposition [13-15], and corroborate the reported absence of a correlation between amyloid plaques and functional connectivity in humans [22]. In a previous study of FC in APP/PS1 mouse model of AD, Bero et al. [23] linked regions of high FC at 3 months in transgenic mice with regions at risk of developing amyloid plaques later in life, although the temporal dynamics of the change with respect to amyloid plaque appearance has not been demonstrated. Their results differ from ours as FC deficits in ArcAβ mice appeared already robustly reduced in the early phase of life, while it appeared increased in young APP/PS1 compared to wild-type. Yet, given the large phenotypic variations between mouse models of AD [24], different FC patterns and dynamics are to be expected. In fact, a comparative study of FC patterns between different transgenic lines may highlight salient features necessary and sufficient to alter neuronal networks.

A potential confound in the study of FC in rodents is interference by anesthesia. Although it has been reported that rats could be trained to undergo fMRI sessions in awake state [25], FC studies have been reported as being difficult to apply in mice due to difficulties in acclimating the animals [26]. However, isoflurane was shown to induce highly similar patterns of FC compared to the awaken state [26-27].

All anesthetics interact with targets of neurotransmitters, which might be affected in a differential way in different (transgenic) mouse lines. For example the GABAergic system, being a target of several anesthetics including isoflurane [28], appears to be affected by amyloid toxicity already in early life of transgenic animals of cerebral amyloidosis [29]. Hence, isoflurane anesthesia may exacerbate the effects of amyloidosis in fMRI studies in these animals. We nevertheless decided to use this anesthetic as it FC has been reported to be similar to those observed in awake mice (Jonckers et al. 2011, 2013), it preserves cortical connectivity to a large extent, suggesting an overall validity of the results.

Alzheimer’s disease often involves amyloid deposition around the vasculature [30], which inherently affects the neurovascular coupling and potentially masks FC measured on the basis of hemodynamic readouts such as BOLD fMRI [31] and intrinsic optical imaging. A number of structural and functional consequences of vasculopathy has been reported in murine models of amyloidosis such as reduced cortical blood flow already early in life [32], as well as damaged capillary systems [33]. The ArcAβ model displays vascular pathology at a young age [20] leading in old animals to cerebral microbleeds [34], impaired vascular reactivity [35], and vascular remodeling [36]. Consequently, the nature of the FC alteration remains uncertain, whether it is of neural or vascular origin, or both. A potential contribution of altered neuro-vascular coupling should
also be considered when interpreting functional impairments in AD patients, which is commonly considered to be of neuronal origin.

Interestingly, the altered FC in transgenic animals was associated with corresponding changes in white matter structures, and observation corroborating the results derived from resting-state fMRI, and suggesting a neuronal rather than vascular basis for the functional changes. Diffusion tensor imaging in the APP/PS1 model revealed a significantly reduced FA in the fimbriae of the hippocampus in transgenic mice, and a trend for an FA reduction in the corpus callosum [37], consistent with our findings. The animal data differ from patterns of FA changes in patients with AD, where regions marked by reduced fractional anisotropy include the temporal lobes, hippocampus, posterior cingulum and occipital lobe [38]. Although a temporal and spatial correlation between functional changes and white matter changes could be established in our study, additional studies combining DTI and rs-fMRI are required to investigate the causal nature of the link between white matter changes and FC.

In addition to the genotype effect, our results suggest late postnatal maturation of neuronal networks and white matter in mice. Evidence suggests that infants and children have immature FC networks, with weak diffuse proximal correlations and few distal correlations [39-40], similar to our findings regarding somatosensory and motor cortical FC in young wild-type mice. White matter changes observed are in line with previous reports describing an increase in FA in white and grey matter in C57/B6 mice between 30 and 70 days [41]. Surprisingly, the pattern of maturation of both FC and FA appears late in the mouse development, at 1 month of age corresponding to early adolescence. FC maturation during the first 5 months of age is supported by the analysis of FA indicating matching age-related changes in white matter structure, an independent structural measure putatively more robust than functional connectivity.

The dynamics of alteration, both spatial and temporal, differ from patterns observed in AD patients, which is marked with early altered FC in the default mode network and aggravated FC in the transition from mild cognitive impairments to AD [7, 11-13, 16]. The discrepancy in regional FC impairments between mice and human might be related to a link between neuronal activity and Aβ release affecting FC strength and amyloid deposition in later life [23, 42]. While, in humans, the DMN is the metabolically most active network in the brain at rest [43], the somatosensory cortex appears as metabolically active as the cingulate in mice [44]. In fact, FC involving somatosensory ROIs shows the highest correlation coefficients in our study, supporting the hypothesis linking high brain activity to Aβ pathology.

Another discrepancy with regards to human pathological progression related to the marginal decline in FC in ArcAβ mice between 5 and 20 months of age despite massively increasing amyloid plaque load, consistent with the dissociation between amyloid load and connectivity [22], but inconsistent with the observed aggravation of FC associated with transition from preclinical to clinical AD [11]. The changes observed suggest an impaired network maturation in ArcAβ, yet the biological mechanisms underlying the phenomenon remain elusive due to the many effects attributed to elevated Aβ peptide concentration. Mechanisms might involve altered LTP [5], axon misguiding [4], and/or alterations in cerebral calcium homeostasis [3].
In conclusion, we have shown using rs-fMRI that in the ArcAβ transgenic mouse model of cerebral amyloidosis that the development of FC during the first months of life is impaired as compared to age-matched wild-type mice, with effects being most pronounced for FC involving ROIs in the somatosensory and motor cortex. Changes in FC are among the earliest detectable feature in cerebral amyloidosis, both in human and animal models; however the diagnostic value of this readout is hampered by poor reproducibility at the individual level. This remains a caveat of the method, which requires large groups of animals/patients or large effects to properly power future studies. Effects on FC are paralleled by changes in white matter structures as derived from analysis of the FA of water diffusion, and occur in the early amyloid plaque build-up stage. Several factors may explain the observed alteration, including reduced synaptic activity, impaired vascular system or altered white matter architecture. The variety of transgenic models of cerebral amyloidosis offers an experimental platform to elucidate features necessary and sufficient to alter neuronal networks, to study the effects of disease modifying agents, and to bridge mechanistic information at a molecular and cellular level to functional/physiological readouts obtained at the system level, that can be ultimately translated to neuroimaging studies in humans.


5 Multiparametric MRI evaluation of passive anti-Aβ immunization in a transgenic model of Alzheimer’s disease

Joanes Grandjean, Giovanna Ielacqua, Miriam Friedel, Luka Kulic, Tobias Welt, Roger M Nitsch, Mark Henkelman, Markus Rudin

This work is currently in preparation.

Abstract

The study of the early events preceding Alzheimer’s disease onset is gaining relevance in the hope to modify the disease course before the occurrence of severe brain insults. While cognitive abilities are only mildly affected at preclinical stages, readouts such as brain volume, functional networks, and white matter integrity have been reported to be altered early in disease development. This offers an opportunity to identify patients at risk of developing AD. While research in humans is difficult at this early stage of the disease due to the limitations of current diagnostic tools, mouse models of the disease lend themselves for studying these parameters as a function of age using structural and functional neuroimaging. We have previously shown that both functional networks and white matter integrity were affected prior to amyloid pathology in ArcAβ transgenic mice, a mouse model of cerebral amyloidosis. To evaluate of the sensitivity of these readouts for assessing the response to therapeutic intervention, we have applied a passive immunization protocol to transgenic animals in the pre-amyloid stage and to wild-type controls. While we did not observe changes to the Aβ peptides concentrations following the intervention, cortical volumes presented a marked positive correlation with Aβ peptide levels. There was, in contrast, no correlation to functional connectivity measures. Further, volumetric assessment of the ArcAβ model reveals enlarged nuclei in the basal forebrain and pontine region in transgenic animals, suggesting neurochemical imbalance in young animals as a potential causal mechanism for loss of functional connectivity. Finally, power analysis suggested that MRI parameters might be more sensitive to detect effects of therapeutic interventions than behavioral assessments of mice.
Alzheimer’s disease (AD) is a complex disorder marked with cerebral deposition of β-amyloid (Aβ) peptide formation of neurofibrillary tangles, as well as the occurrence of neuronal cell death, changes that become manifest as well as a marked cognitive decline. The population living with this debilitating disease is currently estimated to be around 36 millions world-wide, and is set to triple in the coming decades [1]. Treatment options are so far limited and restricted to transiently alleviating symptoms by targeting the cholinergic system with acetylcholine esterase inhibitors or the glutamatergic system with NMDA receptor agonist [2]. Drug discovery programs have identified several potential disease modifying strategies for treatments [2-3], which include immunization against the Aβ peptide [4-6], inhibition of γ-secretase [3], inhibition of Tau phosphorylation [7], and anti-inflammatory approaches [8]. However, despite a large number of clinical trials, none of these approaches has led to significant beneficial effects in patients, which might suggest that damages in moderate and advanced stages of AD pathology are too severe to be reversed. This observation has caused a paradigm shift with focus on treating early, preferentially even preclinical stages of the pathology found in patients suffering from mild cognitive impairment [9].

Yet, such early disease stages are notoriously difficult to study due to the related challenge in performing an accurate diagnosis given the potentially subtle changes compared to healthy controls, especially as events leading to AD develop over a period spanning 10 to 20 years [9]. Both brain atrophy and alterations of functional networks have been reported for groups at risk of developing the disease [10-14]. It has therefore been suggested to include MRI measures such as volumetry and FC in the protocols for clinical trials. Atrophy rates [15], hippocampus volume [16], and FC [17] have been shown to reflect the cognitive state of the patient and have been suggested as markers of disease progression. In fact, MRI measures may be more sensitive in detecting effects than cognitive assessment such as the Mini-Mental State Exam [18].

Similar early structural and functional changes have been observed in murine models of cerebral amyloidosis such as volume changes in specific brain regions [19-20] as well as abnormal functional connectivity (FC) prior to amyloid plaque deposition [21]. Along these lines, we have found that FC as well as white matter integrity is impaired in ArcAβ mouse model, which overexpresses mutated human amyloid precursor protein (hAPP). These results illustrate the potential of mouse MRI/fMRI as a platform for evaluating treatment response applying readouts used in clinical trials in a controlled experimental environment, i.e. animals expressing a homogenous disease phenotype.

Immunization against the Aβ peptide is one of the most propagated disease-modifying therapeutic strategies. The peptide in its oligomeric form is thought to play a pivotal role in the pathological progression [22] and is enriched in amyloid plaques, a pathomorphological hallmark of AD. Hence, inhibition of the potentially detrimental effects of the peptide in its various forms should translate into improved outcomes. Yet, clinical trials have failed to show improvement with both active and passive immunization. It has to be considered though that immunization was also linked to vasogenic reactions in humans [23]. Immunization experiments in transgenic mice have demonstrated the effective clearance of plaques [4-5], reduction of both extra- and intracellular Aβ [24], improvement of memory skills [25], and the restoration of synaptic function with improved...
long term potentiation [26-28]. However, despite these marked improvements, immunization therapy was linked in mice to increased vascular pathology in mice similar to the observations in clinical trials [6].

In this study, we applied a passive immunization protocol in young ArcAβ in prior to amyloid plaque deposition and used MRI/fMRI to assess effects of therapeutic interventions on the volumes of brain substructures, white matter integrity, and functional networks. The objective was to evaluate the sensitivity of the individual readouts for reliably detecting treatment related changes, a prerequisite for considering it as secondary measure in clinical trials. Moreover, the imaging data provide insights into pathology related structural and functional characteristics in murine models of AD at a phenotypic level.

5.2 Methods

5.2.1 Animals, immunization and MRI preparation

A total of 42 animals were used in this study, including 21 transgenic ArcAβ mice (7 male, 14 female), and 21 wild-type controls (14 male, 7 female). Mice were housed in standard animal house, with 12:12 light and dark cycles, with food and water ad libidum. Animals were imaged at 5 months for baseline, followed by a weekly immunization for 4 weeks, and a post treatment imaging session at 6 months. Immunization was performed with intraperitonal injection of 6E10 anti-body (Enzo Life Sciences AG, Lausen, Switzerland) at 2 mg/kg, in PBS solution, or vehicle only (PBS) solution. No weight change was observed between the treated animals and the untreated animals during the course of the treatment.

Animals were anesthetized with 3% isoflurane in oxygen and air for induction, and then endotracheally intubated for mechanical ventilation. Animals were positioned on a MRI compatible support, and fixed with ear bars. Ventilation was set at 80 breaths per minutes. A cannulae was positioned in the tail vein, and a bolus solution of medetomidine at 0.05 mg/kg and pancuronium 0.1 mg/kg was injected, followed by an infusion of medetomidine at 0.1 mg/kg/h and pancuronium at 0.1 mg/kg/h. Isoflurane was reduced to 0.5% during MRI preparation scans and fMRI, then the medetomidine and pancuronium infusion was discontinued and isoflurane was set at 1.5% for the remaining structural scans. This was done to reduce the exposure to muscle relaxant, and facilitate post-imaging recovery. After measurements, animals were left to recover with mechanical ventilation until the effect of pancuronium dissipated.

5.2.2 MRI sequences and parameters

Imaging was performed on a Bruker Biospec 94/30 system, equipped with a quadrature volume transmit coil and a phased array 2x2 cryogenic receiver coil, and operated with Paravion 6 beta version 0.46 software. Tripilot images were acquired to adjust the animal positioning in the scanner. Shims were adjusted with Mapshim protocol and recalculated with each scan protocol to adapt for the slice geometry. Anatomical images were acquired prior to fMRI using a RARE spin echo sequence [29] with the following parameters: field of view (FOV) 20 x 17.5 mm², matrix size 250 x 190, resolution 0.08 x 0.09 mm², number of slice (NS) 12, slice thickness (ST) 0.5 mm, repetition time (TR) 3500 ms, echo time (TE/TEeffective) 8.2/32.9 ms, flip angle (α) 90°/180°, RARE factor 8. Resting-state fMRI was acquired with a gradient-echo EPI sequence with FOV 20 x 17.5
Diffusion tensor imaging was acquired with a spin-echo EPI sequence, with FOV 20 x 17.5 mm², matrix size 128 x 128, resolution 0.156 x 0.136 mm², NS 16, ST 0.4 mm, TR 3000 ms, TE 22 ms, α 90°, number of segments 4, water direction encoding 36, b value 1000 s/mm². Structural scan for volumetry was performed using a spin echo 3D RARE sequence, FOV 15 x 20 x 8 mm³, matrix size 150 x 200 x 80, resolution 0.1 mm³ isotropic, TR 2000 ms, TE/TEeffective 6/48.6 ms, α 90°/180°, RARE factor 16.

5.2.3 Data processing
Resting-state fMRI images were temporally realigned and normalized to an in-house EPI template using AFNI (National Institutes of Health, Bethesda, USA) and FSL 5.0 (FMRIB, Oxford, UK) software suits for Linux. Global signal regression was performed and the time series were band-passed filtered between 0.01 and 0.3 Hz using FSL. Network analysis was performed by extracting the signal from an atlas template and performing pair-wise cross-correlation analysis using R 3.0.1 software (The R Foundation for Statistical Computing, Vienna, Austria).

Diffusion tensors were computed using FSL, using eddy current correction. The b0 image was used to compute the linear and non-linear transform to an in-house DTI template, which were applied to the fractional anisotropy (FA) images. Region-of-interest (ROI) analysis was performed on previously indentified fibers affected in young ArcAβ mice.

Voxel-based morphometry (VBM) was performed using MICe-Build-model (The Toronto Centre for Phenogenomics, Toronto, Canada). Linear transformation with 6 parameters was applied to each 3D volume with respect to a reference image. In a second round, each image was co-registered to the others using a 12 degree of freedom transformation. Finally, the averaged linearly transformed images were non-linearly transformed with respect to each other in three sequential rounds. The quality of the registration was done by examining the standard deviation of the images. The jacobians determinants were computed from the non-linear transformation, and scaled to represent change in absolute volume change per voxel. Voxel-wise was performed in images smoothed at 0.5 mm full width at half maximum (fwhm) and ROI analysis were performed using smoothed images at 0.1 mm fwhm in order to maintain the structural information at every voxel.

5.2.4 Biochemical analysis
Animals were deeply anesthetized with ketamine-xylazine and transcardially perfused with cold PBS. The brains were collected, and split into two hemispheres. The cortical from one hemisphere was collected and frozen. The soluble fraction was obtained from homogenization in a Teflon tube in a SDS - Tris Buffer with protease inhibitors (Roche, Basel, Switzerland). Aβ peptides were measured using a MesoScale Discovery (MSD) 3plex multi-SPOT Aβ human kit (Gaithersburg, MD, USA) for Aβ38, Aβ40 and Aβ42. Human sAPPα levels were determined using an MSD 2plex kit (Gaithersburg, MD, USA), and human Swedish sAPPβ levels were determined using an MSD 1plex kit (Gaithersburg, MD, USA), in accordance with the manufacturer's instructions.

5.2.5 Statistics
Data analysis and statistics were performed in R. Power analysis was computed from data from the baseline session assuming n=15, one-sided hypotheses, and a 66% restoration to wild-type values.
in both two sample t-test comparison and paired t-test. Linear models were used to model gender, genotype, and treatment effects, and their respective two-fold interaction. FC and FA values and correlation values were corrected in the statistical analysis with Fisher's transformation for bounded values. The assumptions of normality were tested for all interaction using QQ-plots to inspect normal distribution, Tukey-Anscombe plots for the homogeneity of the variance and skewness, and Scale Location plots for homoscedasticity (i.e. the homogeneity of residual variance). The alternative hypothesis, i.e. the presence of a difference between the groups, could not be rejected at p-value ≤ 0.05, and is referred in the text and in the figures as factor effect (i.e. genotype effect) for simplicity.

5.3 Results

5.3.1 Aβ peptide concentration changes to 6E10 intervention

An immunoassay was used to measure the concentrations of Aβ peptide, as well as the sAPPα fragment and the swe/arc APP protein (Figure 1). There was no statistical significant difference in either of the peptide or protein tested between the 6E10 treated and PBS control group. A correlation was observed between the concentrations of Aβ_{40} and Aβ_{42} yielding an r-value r = 0.7.

![Figure 1](image)

*Figure 1* | Peptide concentration estimated with immunoassay for different peptides species and APP protein. Neither Aβ_{40} (A), Aβ_{42} (B), sAPPα (C), or APP (D) concentrations presented significant changes following 6E10 intervention (dark red) compared to PBS control ArcAβ animals (light red).

5.3.2 Functional connectivity and fractional anisotropy recapitulates previous findings

Bilateral FC was assessed in both 6E10 and PBS treated wild-type and ArcAβ mice 4 weeks after treatment onset (6 month). No treatment effect could be detected in any of the brain regions considered in any of the mouse strains. Yet, in agreement with our earlier study we found a genotype effect in the anterior parietal cortex (Figure 2 A, genotype effect: p-value = 1.3x10^{-3}), the medial parietal cortex (Figure 2 B, genotype effect: p-value = 9.5x10^{-3}), the cingulate/retrosplenial cortex (Figure 2 E, genotype effect: p-value = 1.1x10^{-3}). In addition, we found a genotype effect in the hippocampus (Figure 2 F, genotype effect: p-value = 0.032), which was not observed in our previous study.

White matter integrity was affected similarly to our previous findings, the rostral fibers presented alterations in fractional anisotropy. The minor forceps and anterior commissure displayed increased FA values in the transgenic animals (Figure 3 B, C, p-value = 1.7x10^{-5} and 6.1x10^{-3} respectively), while the genu of the corpus callosum and the anterior portion of the external capsule showed
decreased FA values in the ArcAβ mice (Figure 3 D, E, p-value = 7.4x10^{-3} and 9.5x10^{-3} respectively). Similarly to FC, no treatment effect could be detected.

Figure 2 | Functional connectivity changes in post-treatment session across brain region bilateral pairs. A statistical significant genotype effect was revealed between ArcAβ (red) and wild-type (blue) animals in the anterior (A) and medial (B) parietal cortex, as well as the cingulate/retrosplenial cortex (E) and hippocampus (F) bilateral connectivity. In contrast, the posterior parietal and motor cortex (C, D) as well as the striatum (G) and limbic areas (H) did not exhibit significant changes linked to the genotype of the animals. Linear model analysis did not reveal gender or treatment effect for any of the region pairs.

Figure 3 | Fractional anisotropy changes in post-treatment session. Regions of interests are shown on the fractional anisotropy heat map (A), indicating the minor forceps (B mf), anterior commissure (C ac), genu of the corpus callosum (D gc), and the anterior external capsule (E ec). A genotype effect was found in all 4 regions examined between ArcAβ (red) and wild-type (blue) animals. Linear model analysis did not reveal significant gender or treatment effects.

5.3.3 Voxel-based morphometry
VBM analysis in the ArcAβ mice aged 5 months revealed striking differences when compared to aged-matched wild-type animals. Volume reductions were observed in the retrosplenial cortex and
to a lesser extend in the parietal cortex of transgenic animals (Figure 4 A, C, D). However, the basal forebrain, dentate gyrus (Figure 5 E, genotype effect: p-value = 4.9x10⁻⁵), and a major portion of the pontine nuclei surrounding the aqueduct Sylvius (Figure 5 F, genotype effect: p-value = 2.4x10⁻¹⁰), including the dorsal raphe nuclei, periaqueductal grey, and the locus coeruleus, were found enlarged in the ArcAβ mice. VBM also revealed a gender effect with larger cingulate and parietal cortex, hypothalamus, and the anterior portion of the left internal capsule in female mice compared to males (Figure 4 B). There was however no noticeable difference in total brain volume or ventricle size between wild-type and transgenic animals (Figure 5 A, B). Also, there was no treatment effect in any of the brain regions in the ROI analysis (Figure 5).

**Figure 4** | Genotype and gender effect on local brain volume changes during baseline session identified with voxel-based morphometry analysis. Voxel-wise linear model analysis reveal both genotype (A, C, D) local effects on the scaled jacobian determinants estimated during voxel-based morphometry procedures. Wild-type mice presented enlarged cingulate and parietal cortex (red) compared to transgenic animals. In contrast, transgenic animals had enlarged basal forebrain, detate gyrus and nuclei in the pontine regions (blue). A superior (C) and lateral (D) 3D representation of the voxels presenting a statistical significant effect are shown. Red regions indicate voxels enlarged in wild-type animals compared to transgenic, blue regions indicate voxels enlarged in transgenic animals. Ventrices are shown in yellow as a reference object in the volume. A gender effect was also observed (B), notably with the cingulate and parietal cortex, and hypothalamus were found enlarged in females compared to males (blue). Colorbars represent F-statistics for the given effect.
**Figure 5** | Region of interest analysis of volume changes in the post-treatment session. The whole brain volume (A) and the ventricle (B) volume did not present significant difference link to either treatment or genotype. The volume of the anterior parietal (C) and cingulate (D) cortex was extracted using ROI derived from the resting-state fMRI analysis. There was neither genotype, gender or treatment effect in either volumes. The dentate gyrus and pontine nuclei present larger volume in ArcAβ compared to wild-type animals (E, F).

**Figure 6** | Correlation between Aβ42 concentration and MRI parameters. Soluble Aβ peptides were estimated with immunoassay. Soluble Aβ42 presented strong correlation with cortical volumes revealed most affected in the resting-state fMRI analysis, the anterior parietal cortex (A $r = 0.54$) and the cingulate cortex (C $r = 0.43$). However, there were no correlations between the peptide concentrations and the functional connectivity measures in the same regions, neither in the anterior parietal cortex (B $r = -0.25$) or the cingulate cortex (D $r = -0.04$).

5.3.4 Aβ concentration correlates with cortical volumes, but not functional connectivity
Correlation analysis was performed between the levels soluble Aβ peptides and cortical delineated regions derived from the FC components identified in our previous studies. The concentrations of
Aβ peptides were found to correlate with volumes of cortical subregions with $r = 0.54$ for the correlation $[\text{Aβ}_{42}]$ versus volume of anterior parietal cortex (p-value = 0.019), and $r = 0.43$ for $[\text{Aβ}_{42}]$ versus volume of cingulate/retrosplenial cortex (p-value = 0.072). However no significant correlation has been found between soluble peptide concentrations and functional connectivity values ($[\text{Aβ}_{42}]$ versus volume of anterior parietal cortex: $r = -0.25$, p-value = 0.3, $[\text{Aβ}_{42}]$ versus volume of cingulate/retrosplenial cortex: $r = -0.04$, p-value = 0.86).

### 5.3.5 Power analysis of MRI parameters

The probability to detect a 66% value restoration toward wild-type values for each MRI parameter was computed based on estimated means and standard deviations obtained during the baseline session. Results from the Y-maze and Morris water maze behavior test in 6 months old ArcAβ from Knobloch et al 2007 [30] are presented as a comparison. Volumetric measurements in the dentate gyrus and in the pontine nuclei appear the most sensitive to detect both between group (power = 0.93 and 0.99 respectively) and intra individual (power 0.99 and 1 respectively) differences. Further, power analysis suggests functional connectivity, at least between anterior parietal cortices, could be a sensitive marker to assess changes induced by an intervention (Table 1, two sample t-test: power = 0.81, paired t-test: power = 0.96). Fractional anisotropy appears a less sensitive method to detect changes in such a scenario (Table 1, two sample t-test: power = 0.63, paired t-test: power = 0.86). In contrast, the behavioral testing with either Y-maze or the Morris water maze may not be sufficiently sensitive to detect similar changes (Table 1, Y-maze, two sample t-test: power = 0.5, paired t-test: power = 0.74, Morris water maze, two sample t-test: power = 0.38, paired t-test: power = 0.59).

### 5.4 Discussion

We have used structural and functional MRI readouts as outcome measures to assess the effects of a 1 month weekly passive immunization protocol in young ArcAβ transgenic mice. There were, however, no changes related the antibody treatment in the measured soluble Aβ concentrations, suggesting the duration and/or the dose administered was not sufficient to elicit an intended decrease of the peptide concentrations. While higher doses of anti-bodies, 10 mg/kg, have been used in previous studies [5, 27], they have been shown to elicit detrimental vascular insults [6]. The potential difference in peptide concentration between groups may have been further reduces as we assessed the total soluble peptide concentration, which includes both extra and intercellular Aβ, while only the extracellular compartment may have been targeted by the anti-bodies.

Consistent with this finding, we did not observe any significant treatment effects in any of the MRI parameters recorded. Nevertheless, both FC and FA measures reproduced the findings from our previous study confirming that functional and structural alterations in the anterior portion of the brain of ArcAβ mice can be reliably detected. FA in the minor forceps was found increased in transgenic animals compared to wild-type, which is counter-intuitive to the expected decrease in FA associated with loss of white matter integrity. While it is rather difficult to elucidate the causes underlying these changes, we interpret the increase as a reduction in fibers spreading to reach multiple frontal cortical targets in transgenic mice, leading to a reduced orientation spread of the fibers, resulting in a higher tendency to anisotropic diffusion of water in the regions innervating the frontal cortical areas. However, as in our previous study, the biological origins to the FA changes...
observed in ArcAβ mice in the minor forceps as well as in the other regions are unclear, and the difference between wild-type and transgenic mice is only indicative of an overall white matter alteration.

<table>
<thead>
<tr>
<th>Measure type</th>
<th>Wild-type (mean ± sd)</th>
<th>ArcAβ (mean ± sd)</th>
<th>Pooled standard deviation</th>
<th>Δ (meanwt – meantg)</th>
<th>Power : two sample</th>
<th>Power: paired</th>
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<td>anterior parietal cortex</td>
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<td>1.21 ± 0.22</td>
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<td>0.23</td>
<td>0.81</td>
<td>0.96</td>
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<td>Functional connectivity:</td>
<td>1.31 ± 0.26</td>
<td>1.17 ± 0.26</td>
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<td>0.13</td>
<td>0.25</td>
<td>0.38</td>
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<td>Fractional anisotropy:</td>
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<td>0.54 ± 0.03</td>
<td>0.04</td>
<td>0.04</td>
<td>0.63</td>
<td>0.86</td>
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<td>genu of corpus callosum</td>
<td>1.02 ± 0.04</td>
<td>1.11 ± 0.06</td>
<td>0.05</td>
<td>0.08</td>
<td>0.93</td>
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<td>Volume:</td>
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<td>2.5 ± 0.09</td>
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<td>0.27</td>
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<td>pontine nuclei [mm³]</td>
<td>60.0 ± 6.1</td>
<td>53.1 ± 9.6</td>
<td>7.3</td>
<td>6.9</td>
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**Table 1 |** Power analysis was conducted on parameters estimated from the baseline session in 5 months old animals, assuming a 66% restoration (2/3 x Δ) of the biomarker level toward wild-type reference value using 15 animals per group and one sided alternative hypothesis. For Aβ42 level, the test assumes a 66% decrease in peptide concentration. The power for both two sample and paired test are presented, powers above 80% are highlighted in bold. Behavioral test results from 6 months old animals were extracted from Knobloch et al. 2007 and are shown for comparison [30]. Volumetric measurements appear the most sensitive test to reveal differences to potential intervention, followed by functional connectivity between the anterior parietal cortices.

Volumetric measurements were added to our MRI protocol, as additional parameters measurements may improve the discriminative power of the data. Furthermore, volumetric measurements have been proposed as sensitive biomarkers in AD [18] and are routinely added to clinical trials as a secondary outcome. VBM analysis revealed a strong genotype effect in several brain regions. Notably, the pontine nuclei were enlarged by about 13% in ArcAβ compared to wild-type animals. Also the volume of the basal forebrain was found increased. While these observations are at odds with the expected neurotoxicity of Aβ species towards the cholinergic system, e.g. it has been reported that amyloid deposition was associated with decreased basal forebrain volume in mildly impaired elderly [31], they might indicate a cholinergic origin to the functional changes. The pontine nuclei host a large variety of specialized neurons involved in several neurochemical systems, including the cholinergic, serotonergic and the adrenergic system. Combined with the observation of an increased basal forebrain volume, a brain region hosting predominantly cholinergic neurons, this might suggest acetylcholine imbalance to constitute an early event...
occurring in the brains of ArcAβ mice. The cholinergic system, which is severely affected in humans affected with AD [32], is considered central to hypotheses regarding the disease development [33]. Cholinergic synapses are also the target of the major class of drugs approved for alleviating the symptoms of mild AD, acetylcholine esterase inhibitors [2]. Further, the cholinergic system is reported to be involved in the inducing the non-amyloidogenic APP processing route [34-35], while inhibition of cholinergic neurons leads to more severe amyloid pathology in mice [36], as well as memory impairments [37]. Taken together, neurochemical imbalance, in particular changes acetylcholine levels and turnover might constitute a potential causal element for the functional connectivity impairments observed in the ArcAβ mice at a young age. Alternatively, increased pontine nuclei may be the result of a compensatory mechanism to the functional connectivity alteration taking place in the mice. Currently these interpretations remain speculative: future investigations addressing the effects of modulation of cholinergic signaling on the functional connectivity patterns may highlight mechanisms underlying functional changes in these mice. In summary, neurochemical imbalance in ArcAβ mice may therefore constitutes an additional putative causal element leading to functional impairments, in addition to the previously suggested loss in long range pyramidal connection loss, supported by the changes in white matter integrity, disruption of local neuronal assemblies, and impairment of neurovascular coupling [38-39]. Interestingly, the volume of the periaqueductal gray, a region allegedly associated with analgesia [40], was also found to be increased in young ArcAβ mice, which may explain the earlier report of increased sensitivity to pain in these mice [30].

Another mechanism highlighted by VBM analysis is the potential link between soluble Aβ peptide and local brain volume. Such a link has been proposed previously for another mouse model of AD, where increases in local cortical thickness were associated with a greater incidence of amyloid plaques later in the animal life [19]. Also these results appear counter-intuitive to the expected neurotoxic effects of Aβ peptides. However, it has been shown that the APP protein and associated peptides potentially possess neurotrophic properties [41-42]. This may suggest that increased APP levels, as observed hAPP overexpressing transgenic mice, might lead indirectly to synaptic stability or reduce pruning. This may in turn lead to deficiencies in the neuronal network as illustrated in Cx3cr1 knock-out mice, a model exhibiting reduced synaptic pruning due to decrease microglia in early postnatal stage [43]. Improper synaptic removal might also explain the epileptic moments reported in hAPP transgenic mice [22], as well as the deficits in neuromaturation observed in ArcAβ mice in our previous study. However, in this present study, we were unable to link soluble Aβ peptides to FC changes, either due to genuine absence of correlation, to an absence of cortical localization of the soluble Aβ peptide concentration with respect to functional territories revealed with FC analysis, or, due to the inconsistent nature of FC measures at the individual level. Previously, local FC alteration in a group of young APP/PS1 was shown to predict regions exhibiting highest amyloid plaque loads later in the animals’ life, supporting the existence of a link between FC and amyloid pathology in mice [21].

Finally, the changes in hippocampal volume found in this study differ from the results obtained in a different mouse line (PDAPP), which showed smaller hippocampi in transgenic mice during the preamyloid stage [44], while they are consistent with another mouse model, TASTPM (APP(695(K595N, M596L)) x PS1(M146V)) displaying larger hippocampi and basal forebrain nuclei
[20] compared to wild-type. However, APPxPS2 transgene expression effect on brain volumes was marginal [45] including with aging, suggesting a dissociation between volume and amyloid plaque load in transgenic animals, contrary to the views expressed in humans [46]. The variability in the volume changes between various transgenic APP models echoes the variability reported for both FC and FA alteration patterns observed in our previous study. While this variability may be seen as a disadvantage of hAPP murine models of AD, each expressing a wide dynamic range of macro structural changes, the large variability among mouse model might offer a handle for gaining mechanistic insight by analyzing commonalities and differences with regard to functional and structural connectivity changes, as well as volumetric changes with respect to the various cellular and molecular parameters such as Aβ peptide concentration, neurotransmitter levels and turnover, and/or compartmental accumulation.

A power analysis was performed using MRI, biochemical and behavioral data recorded at baseline. While the assumed effect size turned out to be unrealistic with regard to this particular study, the comparison of different parameters suggests that both volumetric measurements and functional connectivity are potent biomarkers to monitor therapy induced effects. In fact, these readouts appear more sensitive for detecting the normalization of values following an intervention than behavioral measures. This observation echoes a similar proposition in humans: it has been reported that the inclusion volumetric measurement in the study protocol may drastically reduce the number of patients needed to be enrolled in a given clinical trial to power the study [18]. However, the shortfall of using local brain volume or functional connectivity is the indirect association with the outcomes of interest, cognitive restoration or reduced rate of cognitive loss. For instance, greater cortical and ventricular volume reduction was observed in responder to active immunization, suggesting a non-linear link between brain volume changes and cognition [46]. Furthermore, structural rearrangements affecting white matter integrity or brain volume are likely to occur slowly. Volumetric measure in humans are typically carried out at with yearly frequency with brain atrophy rates estimated at 1.5% per year in patients with AD, and 1% in patients with MCI as compared to 0.3% in healthy age-matched controls [47]. This would translate into potentially small volume changes in the mouse brain, which is of the order of 0.5ml especially in the context of a 1 month intervention period. Further, mice present an increasing brain volume trends during their lifespan [45], which might mask potential treatment related volume changes. In turn, this may limit the application of volumetric measurements in mice.

The use of secondary MRI-based outcomes has increased in the past years, amid interest to gain in sensitivity to detect intervention-related changes in clinical trials. In mice, the efficacy of interventions such as anti-body mediated reduction of Aβ peptide levels have been assessed with both behavioral tests and biochemical/histomorphological analysis. Corresponding readouts are used in clinical studies in the form of cognitive tests and in-vivo molecular imaging approaches assessing the amyloid plaque burden in patients using positron emitting tomography in combination with plaque specific tracers [48]. Validating the efficacy of volumetric changes or functional changes to a given intervention in a mouse model may thus enhance the validity of similar observations as secondary outcomes in clinical trials. Furthermore, understanding the elements underlying the structural and functional changes observed in murine models over-expressing hAPP may contribute to our better understanding of cerebral amyloidosis and AD.
In conclusion, we evaluated the sensitivity of functional and structural MRI readouts in young ArcAβ mice and age-matched wild-type mice. Volumetric analysis revealed significant volume changes of to specific brain structures in ArcAβ mice. Notably, a strong genotype effect was observed for the nuclei in the pontine area in transgenic animals, which displayed increased volumes as compared to age-matched littermates. This might suggest a neurochemical imbalance as a potential cause for the changes in functional connectivity observed in these animals, an interpretation that remains speculative at this stage. Combined with FC and FA analysis, volumetric measurements in the ArcAβ model may improve our interpretation of the changes occurring at a macro structural level in young animals. A power analysis suggest MRI parameters may be sensitive measures to follow in vivo the changes induced by potential interventions in future studies, while benefiting from a strong translational link to studies in humans.


6 Discussion

Alzheimer’s disease is a major brain disorder, and the major cause of senile dementia. Its impact of the world population is large, with currently 36 million people diagnosed with the disease. The cost of care is a burden to the health systems of developed countries, and is set to increase in the coming decades. Despite its major impact on societies, there is currently no cure to the disease, and the elements leading to the disease are not yet fully understood.

Several lines of treatments have been proposed and tested in clinical trials but have not found to impact significantly on the disease progression. It is currently considered that the damages inflicted in mild to moderate AD are too severe for the treatments to show a marked impact. Interests have turned toward preclinical occurrences of the disease, such as mild cognitive impairments, which develop over a period of 10 to 20 years prior to disease onset. Patients in such stages have become the focus of potential treatments, as they are more susceptible to respond positively to disease modifying agents [1]. Yet the definition of preclinical stages is based on mild biomarker alterations, which limits the reliability of the diagnosis in patients in this stage. The understanding of the early events leading to the disease may consequently improve diagnosis of such early stages, as well as suggest novel mechanisms for disease modifying treatments.

Functional changes, as observed with fMRI, are among the first modification observables in groups at risk of developing the disease [2]. This makes the measures an interesting biomarker for both diagnosis and monitoring of treatment outcomes. However, the application of functional measures is difficult in stimulus evoked fMRI, due to the need of a stimulus paradigm, which is not necessary available onsite, as well as the need for the patients to understand the task to perform in the scanner. Recently, measures performed at rest, referred as resting-state fMRI, have been used to shed light on the functional organization of large scale neuronal networks. Alterations in functional networks appear to be similarly an early biomarker identifying groups at risk of developing the disease [3-6]. Yet the biological implications behind functional connectivity changes in the disease remain elusive.

Applying the method to the mouse using dedicated MRI systems allows studying the phenomenon in an experimental setting in models of Alzheimer’s disease, with the aim to understand the early events leading to the functional changes and to use it as a biomarker in preclinical studies to test potential disease modifying drugs.

6.1 Summary of the findings

The goal the present work was to establish, optimize, and test the application of resting-state fMRI in mice using a dedicated mouse MRI platform. Functional MRI is a method widely used in the human MRI research community; however, its application in rodent is hampered by several hurdles. The principal concern relates to the smaller brain size in mouse, approximately by a factor of 1000, imposing higher demands on spatial resolution. As the signal is directly proportional do the size of a volume element this inherently leads to lower signal-to-noise (SNR) in the image, which is further exacerbated by the high demands on temporal resolution of functional MRI. The use of dedicated high field MRI scanners operating from 4.7 to 16.4T, as well of as cryogenic coils
[7], have allowed to compensate for the low SNR values encountered in high resolution mouse fMRI.

The method is however hampered with additional shortcomings. Functional MRI not only measures the BOLD response underlying neuronal activity, but the signals are contaminated by contributions from spurious noise sources arising from the physiology of the animal, as well as from instabilities of the MRI instrumentation. These elements need to be carefully examined to ensure the response analyzed reflects a maximum of the neuronal component of the signal, and contains as little contaminating nuisance as possible. We have performed a comparison of several approaches to estimate noise from both a data-dependent and data-independent approach. However, this analysis is complicated as metrics for assessing the effectiveness of noise reduction are lacking in the model-free approach of resting-state fMRI. Nevertheless, we found that the vascular contribution, which can be estimated from the areas co-localizing with major arteries and venous vessels, reflects an important portion of the signal. Vascular noise estimation is effective in removing a spurious portion of the signal, under the assumption that signal in major vessels is orthogonal to local signal underlying neuronal origin.

The use of anesthetic to restrain the animals is the second major bias to the measures of the functional network with fMRI in mice. The difficulties in measuring mice in the awake state preclude the comparison of the anesthetized state to a reference state. Consequently, the anesthesia regimen in mice needs to be optimized for functional MRI measures to meet some criteria. A first criteria regarding the functional networks is that they should present a similar the organization to the awake state in previously described studies in rats [8], monkey [9], and humans [10]. Further, the systemic effects of the anesthesia should be minimized as they might impact the hemodynamic fMRI readouts, and the anesthesia should be non-terminal to allow for longitudinal studies. Finally, anesthesia depth should be sufficient to minimize animal distress, the anesthetic should be easy to apply, and the resulting functional networks should be reproducible. With these criteria in mind, we have performed a comparison of 4 anesthetic regimens: isoflurane, propofol, medetomidine, and urethane. We have identified a regimen based on a combination isoflurane and medetomidine, which presented both cortical and sub-cortical connectivity, and which was reproducible, while not displaying notable negative side effects.

We have applied resting-state fMRI, in combination with structural imaging, to study longitudinal changes in functional and structural connectivity occurring in a mouse model of cerebral amyloidosis. Significant functional and structural alterations were shown to appear during the first months of life in ArcAβ transgenic mice compared to age-matched wild-type littermates, prior to the appearance of amyloid plaque deposits. However, there was no evidence of decline in either parameter during adulthood in the transgenic animals, suggesting either a floor effect of the functional and structural defects in transgenic mice, or an absence of significant neurodegeneration affecting the parameters used in the read-outs. With this study, we have demonstrated the effectiveness and robustness of functional and structural measures in mice. However, both the functional and structural imaging modalities used in the study lack specificity to specific biological process, so that the changes observed cannot be directly attributed to an underlying molecular mechanism.
In order further test the sensitivity of functional connectivity measures in the context of disease modifying approaches, we have applied the read-out in the context of a passive-immunization protocol. Although we did not succeed in altering Aβ peptides concentration, we were able to observe a positive correlation between peptide concentration and local cortical volumes. This study demonstrated the applicability of multi parametric MRI approach to phenotype mouse models of disease, with a strong translational application to MRI measures in humans for biomarker elucidation, or treatment evaluation.

### 6.2 Limitations in noise models in rodent fMRI

Previous studies in rodent fMRI, and especially in mice, have neglected the impact of noise in the fMRI signal [11-14]. In the case of stimulus evoked fMRI, the contribution of noise may appear minor with respect to the magnitude of the stimulus evoked response, however, in the case of resting-state measures, sources of accountable noise represent a significant portion of the signal. The result of our study of noise in mice fMRI highlight a major contribution of motion in the signal and weak respiration effects, similarly to the results found in rats [15]. We have highlighted regions most at risk to nuisance, and used the signal traces in these regions to effectively remove noise in the rest of the brain. We found that accounting for the signal in the major arterial and venous vessels led to a notable decrease of spurious correlation in addition to the other noise sources previously described [15-16].

A promising method for fMRI signal denoising is based on the collection of multiple echoes during the fMRI acquisition [17]. Noise induced artifacts present a different signal dependency with regard to the echo time, which allows discriminating it from the BOLD signal, and thus ensuring their removal. Such measures may increase the reliability of fMRI measures. However, multi-echo acquisition requires compromise in the image parameters either in term of spatial or temporal resolution of the image, each being necessary elements to successful fMRI experiments. Further, a significant portion of the noise in mice seems to be of systemic vascular origin, which would be reflected with a similar dynamic as the as the BOLD signal, although not linked to the hemodynamic response to neuronal activity. Consequently, multi-echo imaging may be of value in denoising fMRI time series in mice, but it would not capture systemic vascular noise contribution.

Measuring fMRI signal independent parameters can be used to account for the noise, as applied with the RETROICOR method [18]. While respiration parameters had only a minor impact, measures relating more directly to vascular contribution could have a more significant effect, such as measuring blood gas concentration or blood pressure. However, non-invasive blood gas concentration is not measured at sufficient sampling rate and precision to be of use as a noise regressor in fMRI modeling, blood pressure measures carried out in the scanner were not sufficiently reliable, while electrocardiogram measures were strongly affected by the high magnetic field and gradient switching.

Measuring an additional hemodynamic parameter to BOLD, such as using sequences sensitive to BOLD and cerebral blood flow (CBF) [19], might help in studying the uncoupling between BOLD and CBF, which is indicative of non-neuronal artifacts such as those induced by major blood vessel [19]. However the method may be limited by the insufficient accuracy of CBF measures, which is a
difficult method to apply in mice. Alternatively, near infrared fluorescence spectroscopy sensors can estimate non-invasively total hemoglobin, as well as deoxy- and oxyhemoglobin, which may help underline systemic vascular nuisance factor from neuronal response in the BOLD signal. The method would need to be adapted to the small size of the mouse brain, and be MRI compatible.

6.3 Improved anesthesia regimen in mice

Anesthesia related neuronal and vascular effects represent a major confound in mouse fMRI measures. Measures in awake mice have been attempted, but difficulties in acclimating the mice to the restrainer and the MRI environment are significantly greater in mice than in rats [20]. We have established a regimen that maximizes connectivity, both cortically and sub-cortically, while apparently minimizing side effects.

Our study comparing rs-fMRI results under different anesthesia regimen provided additional insight. The major finding in this regard is the discrimination of anesthetics into two different classes in term of their mode of action. On the one hand, isoflurane, propofol and urethane seemed to induce similar effect, such as loss of striatal connectivity but retaining of weak yet apparent thalamo-cortical connectivity. On the other hand, medetomidine preserved striatal connectivity, but there was no evidence of thalamo-cortical synchrony, and further, the temporal signal was affected by displaying a highly repetitive pattern, which results in lower entropy in the signal, a measure of the level of information carried by the signal. Further, dose dependence seems to be reflected differently between the two categories of anesthetics. Our results, as well as electrophysiological recordings [21-22], suggest that increasing dose in isoflurane, propofol, or urethane lead to a loss of localization of the signal, akin to whole cortex synchronization. However, this effect was masked when performing global signal regression, a measure of signal correction, possibly due to the loss of orthogonality between the local signal and global signal representation [21]. Higher dose leads to burst-suppression and loss of signal synchronization, which is supported in studies combining electrophysiological recordings and fMRI in rats [21-22], as well as studies in mice using higher dosages than those used in our study, notably urethane [20]. In contrast, medetomidine, and, as it appears, also α-chloralose, present a different dose dependence relationship, leading to decreased bilateral cortical connectivity at higher doses in rats and mice [23-24], but no unspecific signal synchronization. This observation is difficult to confirm due to confounding vascular effects, local neurovascular coupling effect, as well as systemic cardiovascular effect of each agent. However, electrophysiological recordings in previous studies investigating doses appear to provide a neuronal substrate [21-22, 24] to the observations made with regards to dose dependence observed with fMRI.

Both proposed methods of actions of the anesthetics are in accordance with the current views of loss of consciousness proposed by Tononi [25], suggesting either a loss of spatial integration of the signal, characterized with the appearance of unspecific correlations across whole cortical structures, or a loss of information within the signal itself, reflected by the lower entropy values as with medetomidine anesthesia. However, our observations are limited to BOLD recordings, which are a reflection of the neuronal activity on the local vascular systems, and consequently limited with regards to interpretations at the neuronal level. While electrophysiological recordings bring substance to our observation with rs-fMRI [21-22], however, the mechanisms leading to a loss of
consciousness in mice are likely more complex. For instance, anesthesia effect appears to dampen higher order cognitive process in humans, reflected by the reduction of connectivity in the default mode network and in the salience networks. It is very likely that homologous networks in mice respond in a similar fashion to anesthesia, however, our study, limited to a comparison of anesthetic regimens could not capture such a dynamic.

We found that the methods used to process data, such as correcting for global signal or taking the unprocessed signal, are likely to affect the results and might alter significantly their interpretation. Correcting for nuisance variable such as with global signal regression [26], may induce artifacts in the correlation and anti-correlation patterns across the brain [27], especially given the observation that anesthesia could induce a global cross-cortical synchronization pattern, however, absence of correction makes the signal susceptible to systemic effects, such as cardiovascular effects, and as such the validity of either result is difficult to compare. In light of this, the regression of vascular signal contribution appears a good heuristic to balance noise removal with signal integrity.

The study of anesthesia effects by means of resting-state fMRI offers several opportunities: more precise localization of the signal than electroencephalography and the coverage of a larger portion of the brain, thus resolving large scale networks. However, BOLD fMRI cannot disentangle neuronal from vascular effects of the anesthetic, and is susceptible to different pre-processing methods, which lead to spurious gains and losses of connectivity. Finally, each anesthetic acts in a complex manner on different neurotransmitters. Hence the model proposed based on our results is a simplified concept describing the functional networks at a phenotypic level yet does not capture the actual mode of action of the different anesthetic composed of systemic and central molecular and physiological effects. Nevertheless, the classification scheme offers a framework for more detailed investigation, in mice as well as in humans.

### 6.4 Functional connectivity changes in a mouse model of AD

The major findings of this work related to the functional networks found to be profoundly affected in ArcAβ transgenic mice from an early stage. However, the difference between transgenic and wild-type animals appeared constant during adulthood, similar to white matter alteration, suggesting a lack of degeneration of the parameters measured. Functional connectivity showed the most robust changes in ArcAβ compared to fractional anisotropy, and apparently, represent a stronger effect than detectable with some behavioral test, using a comparable number of animals at the same age [28].

#### 6.4.1 Spatio-temporal dynamics of structural and functional changes in ArcAβ mice

A striking observation in our results is the patterns of spatio-temporal alterations difference between ArcAβ mice and other mouse models, and from humans. More specifically, the alterations, both of the white matter and of the FC seem to be linked to maturation deficits rather than linked to the progression of amyloid plaque deposition appearing during adulthood. In comparison, another transgenic model, APP/PS1 mice, present a different dynamic, showing increased functional connectivity at young age in transgenic animals compared to wild-type, followed by deterioration in mid-life, however, the changes were only measured at two time points,
insufficiently to infer on the dynamic of the alteration [29]. Such a pattern of change might resemble the dynamic observed in mid-life groups at risk of developing the disease, such as APO ε4 carriers, who also display increased connectivity in the temporal lobes in mid-life [6], prior to decline in mild cognitive impairment and AD. The differences between the mice models studied are numerous, in term of transgene expression, Aβ peptide concentration, mutated codon, Aβ40 to Aβ42 ratio, and accompanying pathological elements. Any of these attributes may potentially be involved in the mechanisms leading to functional deteriorations observed in one or the other mouse model. An in-depth assessment of different models at young age, with respect to their functional connectivity patterns and biological differences might highlight the role of the different molecular mechanisms related to both in hyper functional connectivity, and in functional decline.

Another observation in our results relates to the different pattern of functional and structural changes in ArcAβ mice compared to humans. Indeed, white matter alteration, as detected with fractional anisotropy analysis, appears to develop from posterior to anterior regions in humans with MCI and AD, in a process referred to as retrogenesis [30-31]. On the other hand, regions most affected in ArcAβ compared to wild-type mice were fibers in the anterior portion of the brain, including the minor forceps, anterior commissure, and genu of the corpus callosum. White matter changes in both humans and ArcAβ mice are matched with corresponding functional impairments, first in the medial temporal lobes in humans, and in the anterior parietal cortex in ArcAβ mice. The origins of the discrepancy between the patterns of alterations are appealing. We propose it may be linked to maturation, or due to evolutionary differences between mice and humans. Regions with strongest white matter alterations were those showing signs of late maturation in wild-type animals, while posterior fibers did not show such maturation effect. In line with the observation of functional connectivity changes, it is possible that transgene expression, accumulation, and cytotoxicity in the early months of life may induce neurodevelopmental defects in these regions resulting in the pattern of alteration observed in ArcAβ mice.

Another explanation for the different pattern of functional and structural alteration between mice and humans may relate to evolutionary aspects. Human present higher cognitive levels, which is thought to be reflected in part by the default mode network, which is linked to inner thought processing, and involves the most metabolically active regions at rest [32]. In mice, we assume that cognition is of lesser importance and replaced with an emphasis on sensory exploration instead. This appears also reflected in the cerebral metabolism: the sensory and cingulate cortex were shown to have equal uptake of glucose [33], and consistent in the patterns of functional changes observed by others [29] and by us. Further, regions marked with hyper connectivity in young APP/PS1 were affected with higher amyloid load later in life [29]. This finding is corroborated by the observation that denervation of the whiskers, led to reduced number of amyloid plaques in the contra lateral barrel field cortex [34], corroborating a link between neuronal activity and amyloid plaque burden. These interpretation places functional impairments and amyloid plaques in mice in the context of neuronal activity, similarly to observations made in humans [35].

6.4.2 Limitations of the mouse model
The ArcAβ mouse model [28, 36-38] is however limited in its representation of the AD as it expresses a transgene derived from the early onset familial Alzheimer’s disease. In this sense, the
observations made in the model relate better to familial AD than to the late onset sporadic AD, which represents approximately 95% of the cases.

Our results suggest another limitation to the model, it appears that the defects in both functional and structural connectivity relate to improper neurodevelopment in ArcAβ rather than to neurodegeneration. Both wild-type and transgenic animals displayed similar ranges of values in for functional connectivity and fractional anisotropy at 1 month of age, however, as FC and FA increased up to 5 months in wild-type mice, the values remained at the low initial levels in ArcAβ, suggesting improper network maturation. The lack of functional and structural change over time and with increasing amyloid pathology suggests that these phenomena are independent of amyloid plaque load. In humans, progression from cognitive healthy to mild cognitive impairments to Alzheimer’s disease is associated with increased amyloid deposition and aggravations of the biomarkers, including functional connectivity [4, 39], white matter integrity [31, 40], and atrophy rates [41].

These elements should be taken into consideration when interpreting the results in ArcAβ in the context of AD. It appears that the mouse model may represent a phenotype more akin to preclinical AD, bearing amyloid plaques and mild behavioral impairments, but devoid of major signs of neurodegeneration.

While in the context of our study, this appears as a limitation, it offers opportunities to perform comparative studies between the large numbers of transgenic models available. Different models exhibit different aspects of the disease, such as tauopathy or neuroinflammation, which can be studied independently of other factors. In this sense, a comparative study of different transgenic animals may help highlight the elements necessary for different aspects of the macro structural changes observed in groups at risk of developing the disease, and in patients with AD.

6.4.3 Limitations of MRI methods to study AD

The methods used in our study of the ArcAβ model suffer from a series of limitations, which extend as well to studies of patients with Alzheimer’s disease. Alterations of the white matter as uncovered with fractional anisotropy changes can be attributable to several biological mechanisms, such as demyelization, loss of fiber orientation, or axonal degeneration. While the method can localize regions most susceptible to be affected, elucidating the cellular and molecular mechanisms can be both tedious and uncertain. Similarly, brain / cortical volume is usually correlating with cognition in AD [41], but atrophy rates was found greater in patients immunized against Aβ peptide, despite improvements in the cognition [42]. The lack of specificity of these structural biomarkers to biological processes associated with AD complicates the interpretation of these read-outs.

Functional connectivity suffers from the additional complexity of the read-out, which depends on local neuronal activity, neurovascular coupling, as well as long distance connection, and potentially neuromodulation. Data are insufficient to easily distinguish the various contributions. This constitutes a serious limitation in the interpretation of FC data regarding the origin of the alterations in functional readouts, especially given the occurrence of vascular pathology in ArcAβ mice [38] and in AD patients [43].
The limitations of functional and structural imaging apply both to mouse and human MRI studies. Combining different modalities may nonetheless allow distinguishing contributions of different physiological origin. For instance, finding both white matter changes and functional connectivity change occurring with a similar spatio-temporal dynamic suggest neuronal impairments of both axons and synapses, rather than a result of vascular pathology. In this sense, different imaging methods may compensate the limitation of others, leading to more confidence in the interpretation of underlying biological mechanisms. The versatility of MRI allows investigating different aspects during the same imaging session.

Adding molecular imaging modalities to the protocol may help complement the description of mouse models phenotypes and overcoming the limitations of functional and structural imaging, using modalities such optical imaging [44], positron emission tomography [45], or magnetic resonance spectroscopy. Spectroscopy measures the concentration of a range of metabolites, which may help characterizing cellular/physiological events. For example glutamate and GABA are markers of synaptic changes, lactate levels are related to metabolic alterations, myo-inositol constitutes a marker for inflammation, and N-acetylaspartate is an indicator of cell death/integrity. Several of these metabolites have been used as markers for monitoring AD progression, with N-acetylaspartate being one of the most affected, changes being already detectable in patients with mild cognitive impairment [46]. These modalities, together with functional and structural imaging may contribute to improving the understanding of the biological events underlying the pathology in mice.

Additionally, multi parametric MRI imaging may allow compensating for the low sensitivity of different methods, or the low statistical power of a study. False positive may arise due to the large number of elements inspected with the different imaging modalities, especially in voxel-wise analysis, which typically involve several thousands of statistical tests. Finding similar patterns in different read-out increase the confidence in the results despite a relative low statistical significance. In studies in humans, multi parametric imaging, whether limited to MRI or including PET, and combined with clinical score and biochemical assay have also been linked to improved diagnostic at the individual level using automatic classification algorithms controlled by machine learning [47-49]. This illustrates the benefits of combining multiple imaging modalities.

### 6.4.4 Biological limitations of resting-state fMRI in mice

Patterns of connectivity in mice were found to be very robust in our studies. Cortical structures were distinct in 2-3 parietal components, 1 motor cortex element and the cingulate, while the subcortical components were divided between the dorsal and ventral striatum and the amygdala, consistent with previous observations made in mice and in rats [50-52]. However, compared to the others [14, 51], the presence of higher order networks could not unambiguously derived from our data, neither could significant functional connectivity in the hippocampus. We observed a component centered on the cingulate/retrosplenial cortex, which has been proposed to be a rodent equivalent of the human default mode network [14, 51, 53-54], however, in contrast to observations from others, we did not observe correlations between the cingulate/retrosplenial cortex and the medial prefrontal cortex or the hippocampus, the other elements of the putative rodent default mode network.
We interpret the absence of a marked murine default mode network as both an effect of anesthesia, and from an evolutionary perspective. In the first case, default mode connectivity is the first structure to be dampened by anesthesia in human studies [55-56], consistent with its putative role central to inner thought processing. Given the necessity to perform mouse fMRI in anesthetized state [20], the potential structures linked to default mode networks are likely to appear be dampened, as opposed to the sensory regions, which were reported unaffected by anesthesia [56-58]. In consequence, the neuronal activity, and the resulting FC in the regions attributable to the default mode network are likely to be reduced or masked by other confounding effects, such as systemic vascular response.

Secondly, as we propose in our model of functional impairment mechanism in mouse models of Alzheimer’s disease, the murine sensory cortex is as metabolically active as the cingulate cortex [33], which differ from humans, where the default mode network overlap with the most active regions [32]. In this context, sensory information processing from environment exploration appears as one of the major process in the mouse cognition, while arguably, higher order processes such as inner thought processing are not as developed, suggesting that the element in mice corresponding to the default mode network is in fact a proto network.

Finally, the regions potentially involved in the murine default mode network also colocalize with major vessels [59] such as the superior sagittal sinus. The susceptibility of BOLD fMRI to large vessels therefore renders the study of the cingulate/retrosplenial regions difficult and prone to systemic artifacts, and less likely to represent signal of neuronal origin.

These limitations inherent to mouse fMRI under anesthesia limit the applicability of functional connectivity studies in mice. In the case of AD, the pathology involves large parts of the cortex, which has enabled us to detect differences in the bilateral parietal cortex connectivity and to a lesser extent in the cingulate. However, mouse models of different brain disorders such as schizophrenia may present fewer benefits from functional connectivity studies, given the limited functional connectivity in higher order processing networks in mice. The method may still find applicability in some models of disorder such as models of Parkinson’s disease, multiple sclerosis, or spinal cord injuries, which involve sensory cortex and sub-cortical regions, as well as depression, which involved the amygdala. For instance, resting-state fMRI network analysis in rat has been used to study the functional rearrangements of cortical networks following experimental stroke [60-61]. Consequently, despite the limitations of the method, rs-fMRI in mice offers studying the reorganization of many large-scale networks from a unique perspective. Its wide-spread use in human to study brain disorder makes it a method of choice in translational studies.

6.4.5 Functional connectivity across species
Resting-state fMRI has been performed in a variety of species, from humans [10, 62], to monkey [9, 63-64], to rat [65-66], and finally to mouse [13-14, 51]. This offers a unique perspective to compare results across species, from an evolutionary perspective. While there are major discrepancies between studies, such as the use of anesthetics, the resolution that needs to be achieved to resolve the object, and the magnetic field strength, the power of the rs-fMRI approach to elucidate functional connectivity is its simple application, which can be easily translated from humans to animals and back.
Monkeys have been animals of choice to conduct fMRI studies, as their brain is relatively of similar size and anatomical structure as in humans. Earlier studies in monkeys have demonstrated a link between electrophysiology and the BOLD effect [67-68]. This has been pursued in functional connectivity studies, where local correlations between electrodes and rs-fMRI fluctuations have been shown [64, 69]. In term of evolution, monkeys have been shown to have similar functional networks as in humans [63]. The lower order sensory networks exist very similarly to those in humans, while higher order networks, such as the default mode network seem to exhibit slightly different patterns. Different studies in monkeys report different results; however, it appears the default mode network and other higher order network might involve fewer brain structures than in the humans, as illustrated with weaker correlations between the cingulate and medial temporal lobes to the frontal lobe in the monkey default mode network [9, 63].

In rats, the networks present further simplification. Reports show the brain to be divided into 3 major structures in the awake state, the cortical ribbon, the basal ganglia, and the limbic system, all with bilateral organization as in humans [8]. Studies have reported a default mode network-like structure in the rat, which includes the dentate gyrus of the hippocampus, the cingulate and retrosplenial cortex, as well as the parts of the frontal cortex [53-54]. In mice, a default mode network was also reported, which included the same regions [14, 51]. In our experiences, the homolog to the default mode network is confined to the cingulate cortex, with minimal connectivity to either the medial prefrontal cortex, or the hippocampus. In that sense the structures underlying the default mode network in humans present less connectivity in their rodent analog.

However, rodents present similarities in the temporal profile of the cingulate cortex. Indeed, we observed the presence of anti-correlations between the cingulate and the somatosensory cortex, similar to the reported anti-correlations between task related networks in human studies, such as the somatosensory network, and the default mode network [70]. While the default mode network may appear different in rodents compared to humans, it might be considered a proto-network of higher order cognitive processing. This offers opportunities to study of the evolution of this potentially important region in human evolution and cognition, to understand the neuronal mechanisms underlying the network, as well as of mechanisms involved in the anti-correlation phenomena.

### 6.5 Outlooks

Functional connectivity methods were applied in mice using dedicated mouse MRI systems and cryogenic coils [7]. Procedures to minimize noise factor in the signal have been devised, and anesthesia bias has been reduced. The method was successfully applied to study a mouse model of Alzheimer’s disease. The studies presented in this work raise several questions on the pathophysiological process in models of Alzheimer’s disease, as well as regarding the molecular and cellular mechanisms reflected with functional connectivity measurements both under normal and pathological conditions.

#### 6.5.1 Uncovering the molecular and cellular mechanisms behind functional connectivity

The BOLD signal is explained locally by the local field potential in electrophysiological recordings [67-68]. However, the mechanisms underlying the correlative effects between brain regions would
need to be elucidated. There are presently two major elements which may account for the functional connectivity between two regions, especially in the cortex, the long range pyramidal neuron connections, and the local neuronal networks processing the long range information. The first element is necessary to convey the information between two regions, however, the BOLD response relates more to the synaptic activity, which is related to the information integration process [68]. Consequently, local neuronal networks are expected to partake significantly in the phenomenon behind functional connectivity. We propose to use well studied paradigms to study the elements behind bilateral functional connectivity, such as whisker denervation [71-72], for which the molecular alterations have been quantified [73-74]. Applying resting-state fMRI to measure the functional consequences of denervation may provide a first approach to understanding the molecular and cellular basis underlying functional connectivity, especially if the correlations are intrinsic, or dependant on sensory input such as from the whiskers.

6.5.2 Understanding the maturation of neuronal networks
The contrast mechanism behind BOLD is related to local synaptic activity. The functional coupling between two regions as measured by functional connectivity, however, appears to be a more complex phenomenon relating to an acquired coherence of the synaptic activities at two distant sites. Our results hint towards a late maturation of both white matter and functional networks in mice that occur within the first 2 months of life. This is substantiated with the late changes in white matter, observed by others [75-76] and by ourselves, which support a neuronal basis to the maturation of the networks observed. Understanding the molecular and cellular changes associated with the maturation of functional network represent a key challenge to understanding the connectivity phenomenon. While the approaches to elucidate the molecular mechanism are non-trivial, our results suggest neurochemical imbalance as an additional putative elements leading to the impaired neuronal maturation in young ArcAβ, as illustrated with the presence of hypertrophic nuclei in the pons in transgenic animals. In particular, we propose to investigate the role of the cholinergic system, as potentially involved in neuronal network maturation. Understanding the elements of network maturation may represent an important step to studying brain disorders, many of which have a neurodevelopmental basis.

6.5.3 Establishing the necessary elements for functional impairments in murine models of AD
Being a reproducible measure, functional connectivity readouts in mice are attractive for characterization network adaptation due to pathological conditions. We have shown in our study in ArcAβ mice that such measures are sensitive to detect early functional changes, similar to observations made in human groups at risk of developing AD [3-6]. Understanding these pathological functional changes at a physiological, cellular, and molecular level represent a necessary element to validate FC as a biomarker. We could not demonstrate a link between Aβ and functional connectivity, suggesting a potential dissociation between the two elements. Yet there are many aspects to consider, which may have led to functional changes in the first place, including axonal injury, vascular insults, or impaired network maturation. Using different models, one may potentially highlight important mechanisms linked to functional impairments, for instance using single transgenic models with different mutations than those present in the ArcAβ transgene may lead to fewer vascular insults and thereby allow studying the functional implication of Aβ in the
relative absence of vascular damage. Using APP inducible models may reduce the influence of transgene expression during the maturation of networks, and would allow studying the effect of the peptide on mature networks only. Further models expressing different aspects of the pathology, such as neurofibrillary tangles or expressing human APO ε4 risk allele may help complement our understanding the role of each pathophysiological element which partakes in functional defects.

Resting-state fMRI in mice was shown to be sensitive to changes induced by potential disease modifying treatments. Testing different potential treatments, such as axonal stabilization molecule epothilone D [77-78], secretase inhibitors [79], or anti-inflammatory drugs in different models may also help to characterize to factors contributing to alterations in functional connectivity. This knowledge would be important for designing novel therapeutic strategies for this disabling disease.

6.5.4 Establishing the neuronal mechanisms behind hyper-connectivity

Functional impairments appear to be one of the earliest changes observable in groups at risk of developing AD [2], and hyper-connectivity and hyper-responsiveness in the fMRI signal are among the earliest signs of it. Hyper-connectivity in the medial temporal lobe has been recorded in young humans carrying a genetic risk to AD [6], and in the cingulate cortex of young APP/PS1 [29], but not in ArcAβ mice. This increase in functional response to a task, compared to healthy controls, appears to be a necessary condition for memory encoding in mild cognitive impairment patients [80]. Yet, the elements causing this hyper response are unknown.

The phenomenon of hyper-connectivity is difficult to explain in a context other than from a neuronal network perspective, making functional connectivity approach central to elucidating the mechanism behind. A first aspect to be considered for the elucidation of the mechanism lies in the difference between ArcAβ, which do not present apparent hyper connectivity, and APP/PS1, which do at a young age [29]. A second element comes from the correlation between hyper-connectivity at young age and local amyloid plaque load in aged animals [29], suggesting a link between aberrant network and amyloid pathology. Finally, it was shown that some APP transgenic mice present a thicker cortex at a young age, compared to aged-matched controls [81]. Further, increased local cortical thickness was linked to a higher propension to amyloid plaque load later in life. As such, it is possible that cortical thickness relates to neuronal networks as an expression of improper synaptic pruning or improper local neuronal network assemblies resulting in the thickness changes, as well as functional alterations, thus drawing an important link between structure and function.

Combining MRI methods in assessing the maturation of hyper-connectivity may shed some light in the mechanism, notably, understanding when hyper-connectivity occurs during the maturation of networks, and how it relates to cortical volume change, neurotransmitter levels, such as glutamate and GABA, and how it relates to blood perfusion and white matter changes. Combining the different MRI methods with electrophysiological recordings may allow reducing the possible explanations behind the phenomenon and help guide molecular and cellular approaches to understand the mechanisms underlying hyper-connectivity, which might in turn better our understanding of the
early events occurring in sporadic AD, and help identifying robust biomarkers in the preclinical stages of the disease, and guide drug development.

7 Conclusion

The feasibility of functional connectivity in mice has been demonstrated in this project. Biases of the method, such as nuisances in the signal and anesthesia effect, have been highlighted. We have attempted to minimize the interference of these factors by designing improved noise models and optimizing the anesthesia regimen. The resting-state fMRI approach in mice presents a sensitive and robust method for detecting the functional organization of the mouse brain, as well as impairments in connectivity already in young ArcAβ mice, a mouse model of Alzheimer’s disease.

While the method was shown to be sensitive to detect changes in brain networks, these changes are not specific to biological processes underlying amyloidosis in the brain, rendering the interpretation of the results difficult. We tested partial removal of Aβ peptide with passive immunization in order to test the Aβ dependence of the functional changes observed, but did not find any evidence of improved functional connectivity in treated ArcAβ mice.

The functional and structural changes observed in our study present similarities to results obtained from studies in human, suggesting a translational link to the observations made in mice studies. In that sense, we propose that mouse resting-state fMRI approach may play an important role in translational research for understanding the biological mechanism behind the biomarker in humans, the early events in sporadic AD, and for evaluating the effect of disease modifying drugs in mouse models before testing the compounds in humans.


8 Sharing of information

All the data presented in this work are publicly available on an online repository, https://central.xnat.org/, Resting state fMRI and diffusion tensor imaging in a mouse model of cerebral amyloidosis (ID: fMRI_AD_mouse), and Resting-state anesthetic protocol comparison in mice (ID: fMRI_ane_mouse), corresponding to the data used in chapter 4 and 3 respectively. The study of Alzheimer’s disease has largely benefited from large scale collaborative efforts to share imaging, biochemical, and clinical scoring information in patients, under the flagship project of the Alzheimer Disease Neuroimaging Initiative (http://www.adni-info.org/), leading to over 600 publications listed in pubmed as of this writing, on various topics such as image-based automatic diagnostic, or biomarker identification. Other initiatives, such as the 1000 functional connectome project (http://fcon_1000.projects.nitrc.org/), offer large number of dataset to the wider community. These databases empower researchers to collaborate and extend the power of studies by benefiting from the large scale of data made available. Experimental results in rodent imaging have so far rarely freely been shared. We attempt to provide the large datasets gathered for our studies, containing in total over 300 imaging sessions. These may allow in the future for performing meta-analysis of anesthetic effects in mice, or the comparison with different mouse model of Alzheimer’s disease. Further, given the large number of preprocessing possibilities, as well as data analysis methods, we ensure that our data can be re-analyzed to match the pipelines of others, and thus improve the comparisons between studies. Finally, our data are available to facilitate the replication of our results, a crucial factor in the scientific method.
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10 Curriculum Vitae

I was born on the 6th of June 1985 in Lausanne, VD, son of Geneviève Bille and Olivier Grandjean. I spent the majority of my childhood in Juriens, VD before heading to Singapore, where I attended the United World College of South East Asia, and I obtained my International General Certificate of Secondary Education in 2002. Back in Switzerland, I attended the "Gymnase de la Cité" in Lausanne, and majored in biology and chemistry in 2004. I attended the courses in biology at the "Université de Lausanne", and obtained a Bachelor in science in 2007. The following summer was spent in the laboratory of Prof Patrick Aebischer at the Swiss Federal Institute of Technology Lausanne (EPFL), within the Summer Research Program for Scholars. There, I was given the opportunity to perform my first experiments in neuroscience in the Neurodegenerative disease laboratory. The next year was marked with a break to perform my military training at the non-commissioned officer school in Spiez, BE, to become sous-officier de défense NBC. Following this event, I was admitted at the ETH in 2008 at the Biology department. In 2009, I benefited from the fast track to PhD program and began my PhD in the laboratory of Molecular Imaging and Functional Pharmacology under the supervision of Prof. Markus Rudin. I developed an interest in the relation between brain structure and function, with an emphasis on brain disorders. My work involved developing methodologies to assess the functional connectivity in the mouse brain.