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Up states-based developmental trajectories of the autistic cerebral cortex

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TABLE OF CONTENTS

1. ABSTRACT

Autism is a severe neurodevelopmental disorder which affects information processing in the brain as the result of an abnormally developed cortex, brought about in ways that are poorly understood. The disorder is characterized by a very early onset, however, neurobiological studies at such young ages are often precluded in humans, thus, rendering respective research in appropriate animal models of the disease invaluable. The bulk of this research has focused mainly on *how* experimental models differ from normal rather than on *when* they begin to differ. However, understanding the neurobiology of autism at its *onset* is important for both describing and treating the disorder. Moreover, modelling human behaviours in animals is often very difficult. Therefore, in order for neurobiological research of autism to proceed it is essential to "decompose" the disorder into simpler, behavior-independent biological parameters. Here, I propose how network dynamics of local microcircuits may serve such a role in order to derive developmental trajectories of the cerebral cortex that will allow us to detect and investigate the disorder at its very beginning.

2. INTRODUCTION

Autism is a neurodevelopmental disorder that affects 1 out of 150 children and is characterized by impaired social interaction and communication, absence or delay in language, restrictive and repetitive actions (stereotypy), heightened responses to sensory stimuli

and increased comorbidity with epilepsy (1-4). Beyond this unified description autism is part of a spectrum of related disorders/conditions known as autism spectrum disorders (ASDs) that ranges from mild personality traits to severe impairments and which affects 1 out of 68 children (5-7). Autism has a true emotional and financial burden for families, society and the state. For example, in the United States it can cost about $3.2. million (USD) to take care of an autistic person over his/her lifetime (8) while recent estimates of the annual direct medical and non-medical, as well as productivity costs of autism sum up to an average of $268 billion (range $162–$367 billion; 0.8.84–2.0.09 % of GDP) for 2015 and $461 billion (range $276–$1011 billion; 0.9.82–3.6.00 % of GDP) for 2025 exceeding respective costs for diabetes or attention deficit and hyper-activity disorder (ADHD) (9). Moreover, besides its financial aspect, autism has a significant psychological cost for both the family and the patient himself, as well. The lack of social interaction, severely abnormal body use and often self-directed aggression render autistic patients unable to hold a job and make interpersonal relationships leading to a life dependent on caregivers. Nevertheless, despite the socio-economic burden of autism as well as reports showing that the prevalence of ASD is rising sharply (7), very little is still known about the neurobiology underlying autism.

Autism is characterized by a very early onset. The first symptoms of the disease are present already during the first couple of years of life (10-12) and early diagnosis and treatment have the best possible outcome for the child (13, 14). Despite their significance neurobiological studies in autistic patients are extremely difficult and rare at very young ages, condemning the majority of respective research to be *retrospective* describing autistic brains at adolescence or adulthood, in other words, at ages 10, 20 or even 30 years after the onset of the disease (15)! Moreover, often the best and sometimes only source of information about molecular and cellular alterations in the actual patient brain come from neuropathological studies in post-mortem tissue. However, studies performed at advanced stages of a disease may reflect an endpoint of the disorder making it very difficult to distinguish between “cause, consequence, compensation or confound” (16), a fact that underlines the need to *shift* autism research focus from the time of symptoms to the time of onset. Therefore, an improved understanding of the neurobiology of autism would require longitudinal, *perspective* studies beginning at early stages of life (15). Such studies, however, are very difficult in humans rendering respective studies in appropriate animal models of the disease an invaluable resort to further neurobiology research and our understanding of autism.

In the current review I introduce the novel idea that spontaneous neuronal network activity recorded *in vitro* in rodent cortical slices may help us understand not only *how* local microcircuits of the cortex may be affected in autism, but also *when*. Pinpointing when development deviates from normal is important for several reasons: (1) abnormal excitatory and inhibitory synaptic homeostasis of the cerebral cortex have emerged as key cellular components in the pathogenesis of several psychiatric and neurodegenerative disorders, including ASDs, schizophrenia and Alzheimer’s disease (17–20) and it has been suggested that the *unique* deficits in cognition and behavior associated with these disorders depend on *when* dysregulation of synaptic structure and function occurs across the lifespan (16, 21). (2) It may provide insight into when therapeutic intervention would be most *effective* in preventing the emergence of defected phenotypes (21, 22). (3) It will contribute to our understanding of the role of genes, molecules and cells in abnormal cortical development contributing to the perspective of mechanism-based therapeutics and the fulfillment of the molecular medicine for personalized treatment of the disorder. Finally, (4) understanding the underlying biology at the time of functional/physiological phenotype onset may better describe autism compared to neuropathological studies or descriptions performed at advanced stages of the disease.

Since autism has been often associated with developmental delays of motor and speech skills, as well as excess responses to sensory stimuli, which all rely on intact cortical processing, the cerebral cortex has been the main area of focus of autism research (3). It has been hypothesized that autism affects information processing in the brain as the result of the altered synaptic organization of an abnormally developed cortex brought about, however, in ways that are poorly understood. This limiting gap of neurobiological knowledge is due to (1) the fact that research of mental disorders is often complicated by behavior, thus stressing for the need to study simpler (behavior independent) biological phenotypes or “endophenotypes” of the disorder and (2) the lack of such appropriate neurophysiological markers to study. An endophenotype is the manifestation of a disease at a *reduced* level of biological organization as opposed to the macro-level of behavior (23–27). The slice is a reduced preparation of the brain, which importantly preserves its ability to spontaneously generate activity reminiscent of the intrinsic activity of the intact brain. Therefore, studying local microcircuits in spontaneously active cortical slices may lead to *network-based* endophenotypes of autism which can both guide research and therapeutic interventions in the lab and clinic.

Cortical neurons form recurrent networks which synchronize individual cells and are intrinsically active in the form of oscillating activity, visible at

increasingly macroscopic neurophysiological levels: from single cells to local field potentials (LFPs); to the clinically relevant electroencephalography (EEG). Synchronized oscillating neuronal networks are viewed as the "middle ground" between single-neuron activity and behavior (28). Interestingly, both *in vivo*, during quiescence, but also *in vitro*, in the brain slice preparation, cortical networks are spontaneously active in the form of a slow oscillation in the neocortex, composed of periods of sustained (persistent) activity alternating with prolonged periods of no activity, namely Up and Down states, respectively (29–37). This spontaneous network activity of the cortex is of particular neurobiological, clinical and research interest since: (a) it consists the 'default' activity of the cortex, the activity that the cortex generates endogenously, in the absence of external inputs, typical of quiescent states of the brain such as deep, non-REM sleep, anesthesia and quiet wakefulness (38). (b) It reflects the cortex's hardwiring as shaped by genes and experience, the intrinsic properties of its cells and the dynamics of their synapses; forming the background upon which incoming sensory stimuli interact in determining cortical responses and behavior. (c) Up states are network, synaptic, events that reflect the balance of excitation and inhibition in the cortex (37, 39-41) which is essential for normal cortical function. Deviation from this important balance in the neocortex has been suggested to underlie the pathophysiology of brain disorders such as autism and epilepsy (16, 21, 42, 43). Finally, (d) this activity is preserved from the intact brain down to the reduced level of the brain slice providing researchers with a number of experimental tools and approaches from less to more invasive, from *in vivo* to *in vitro*.

3. UP AND DOWN STATES OF THE CEREBRAL CORTEX

The cerebral cortex is an intricate brain area that has been systematically viewed in the context of the thalamocortical system due to its extensive connection with another equally complex part of the brain, the thalamus. Sensory information reaches the neocortex almost exclusively via the thalamus, which drives the neocortex during activated states such as arousal in the behaving animal (44). However, the neocortex can be active even in the absence of thalamic input, a condition typical of quiescent brain states such as non-rapid eye movement (non-REM) sleep (34, 45-47), some types of anesthesia (34, 37, 45, 46) and *in vitro* in the brain slice (31, 33, 36). Under these conditions the default activity of neocortical neurons consists of slow (<1 Hz), large-amplitude membrane potential fluctuations known as the *slow oscillation*. The slow oscillation occurs synchronously among both nearby (48, 49) and distant (50) neocortical neurons. Therefore, when quantitatively analyzed these membrane potentials actually define two states of the neocortex: the Up state which corresponds to an active cortical state with prolonged (hundreds of milliseconds) depolarized membrane potential and action potential firing, interspaced by long-lasting (at the range of seconds) periods of the Down state when the cells are relatively hyperpolarized and with no synaptic activity. The depolarization of neocortical cells during the Up state to levels similar to those of wakefulness (35), renders the Up state reminiscent of the activated state of the neocortex during arousal and cognition.

Activation of the cortex is known to be gated by diffusely projecting neuromodulatory systems that stem from the brainstem and the basal forebrain (51, 52), which fire intensively during brain arousal (53, 54). However, Up/Down states emerge in the absence of neuromodulators, thus the short epochs of persistent activity during the Up states must have a different mechanism of generation. It has been shown that Up/Down states are intracortically generated (46); they are subjected to neuromodulation (55–57); they involve large ensembles of neurons both excitatory and inhibitory from all cortical layers (33, 34, 58); they recruit cells in repeatable spatiotemporal sequences (36, 59); they originate in layer V (40) and they propagate along the cortex (60) via upper layers (49, 61) and to cortical targets such as the thalamus (45, 58), striatum (62) and upper brain stem core structures (63).

There are two simple ways to view Up/Down states in regard to their generation: either Down states are imposed on sustained Up states or, vice versa, Up states emerge from sustained Down states, and there are mechanisms that can support these alternative scenarios. For example, neuromodulators act by alleviating cells from hyperpolarization imposed to them by outward potassium currents (as previously reviewed (64, 65)). Following Up states, the activation of repolarizing intrinsic currents could generate Down states that are imposed on Up states. On the other hand, Up states could originate from spontaneous subthreshold synaptic events amplified by a potent intrinsic inward current activated when such synaptic inputs synchronously impinge on a cell (66). An example of such a current is the Na^+-mediated persistent current (I_{NaP}). Therefore, if the cell has a strong I_{NaP} then only a few spontaneous subthreshold synaptic events are required to generate an Up state in the cell and subsequently in the network. The presence, for example, of a stronger I_{NaP} conductance in layer V cells compared to cells in other layers, could explain why slow oscillations originate in this deep layer (40).

3.1. Up and down cortical states *in vitro*

Initially the knowledge and technology available confound neuroscientists to simply watch

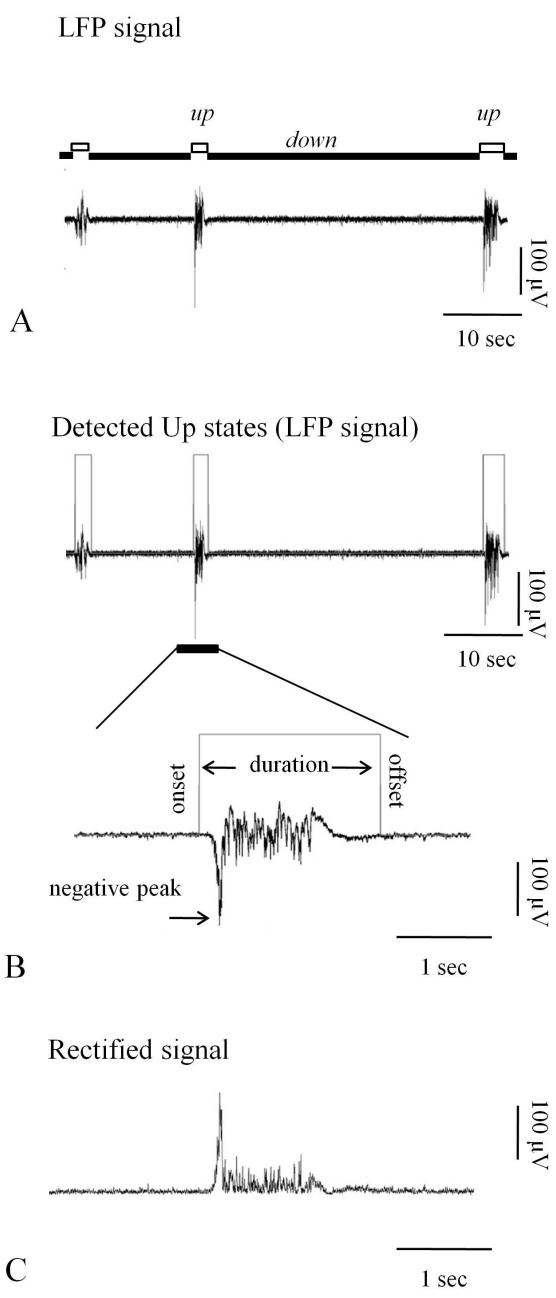

Figure 1. Detection and quantification of the local field potential (LFP) Up state. (A) Continuous LFP recording (1–200Hz) of spontaneous Up/Down state activity from a cortical slice. (B) Top panel: Automatically detected LFP Up states of the signal in (A) are outlined by gray rectangles. Bottom panel: Signal at high magnification provides view of individual Up state. Gray line is the automatically detected onset and offset of the event, based on which duration is calculated. (C) Rectified signal (absolute valued signal) of the Up state, from which the rectified area is calculated. Reproduced with permission and modified from (115).

and describe brain oscillations, and correlate them to behavior. However, the advent of the *in vitro* technique allowed researchers to create these rhythms under controlled conditions and, thus, study and understand the underlying cellular and network mechanisms for their generation. The slice preparation preserved *in vitro* provides a steady and easily controllable environment which optimizes combined electrophysiological and pharmacological studies.

Work *in vitro*, in brain slices has significantly contributed to our understanding of the neurobiology of pathological synchronized brain activity, such as epilepsy (67-73). Physiological cortical network rhythms, for example theta or gamma oscillations, have also been studied in brain slices. However, these studies were largely confounded to the hippocampus and they presupposed a pharmacological manipulation to induce the activity (74-76) (but see (77, 78)). On the other hand, *in vitro* work of neocortical, non-paroxysmal, network activity was limited, and in all cases neocortical oscillations were pharmacologically prompted and highly localized (79, 80). In general, the consensus was that such a reduced brain preparation, as the slice, could not support unconditioned, spontaneous, long-range cortical oscillations similar to that seen *in vivo*, due to the lack of a complete neuronal network (81). However, Sanchez-Vives and McCormick in 2000 (40) showed that neocortical slices made from ferret brain produced spontaneous slow oscillations or Up/Down states when bathed with a buffer that closely mimicked the natural cerebrospinal fluid instead of solutions that had been traditionally used till then. Later work of ours reproduced spontaneous Up/Down states in neocortical slices of the mouse brain and mapped their presence throughout the whole cortex (73). Therefore, what is currently available is an *in vitro* model of Up/Down states that provides us the means to investigate the mechanisms of its generation at both a network and cellular level from various cortical regions (Figure 1).

3.2. The significance of up and down cortical states

Up/Down states are intrinsic features of the cortex, since the cortex spontaneously generates them in the absence of sensory input during quiescence. Large amplitude Up states and prolonged Down states are what underlie slow oscillations, the electroencephalographic hallmark of non-REM sleep (35). In addition, cortical slow oscillations play a pivotal role in brain activity during sleep since they are extensively synchronized over the cortex (50) and they organize and group other sleep rhythms, such as spindles and delta (82). Interestingly, the amplitude of Up states, as reflected in the amplitude of slow oscillations, correlates positively with sleep pressure (83) and depth of sleep (84). Thus, understanding how Up states are generated could provide us with valuable insight into cortical sleep physiology. In addition to spontaneous Up/Down states during quiescence, the cortex can generate Up states when being evoked either from within the cortex (41, 84) or from the thalamus (31, 36), its main input. Evoked cortical Up states are

identical to those that occur spontaneously (31, 36), which probably indicates the priority of intrinsic cortical dynamics over extrinsic inputs during quiescent states. Therefore, taken to their extreme, uncontrolled for Up states may underlie aspects of the pathophysiology of paroxysmal or hallucinative cortical activity during epilepsy (68, 85) and schizophrenia (86) resulting in a suppressed or distorted consciousness (87).

Up/Down states can be maintained in cortical slice preparations, in the absence of sensory inputs or active neuromodulation, indicating that they are chiefly the outcome of intrinsic properties of local networks and hence reflects the 'default' activity of the cortex (38). Moreover, while Down states are periods of relatively low input conductance, Up states consist high-conductance states of cortical neurons both *in vitro* and *in vivo* (88-90). Therefore, Up states may provide the context to study and understand normal firing relationships between different types of neurons. The fact that cortical Up states can be sustained in the absence of sub-cortical or long range inputs, has fuelled studies of Up state activity, *in vitro*, in brain slices as a model of the basic operation of the cortex whose mechanisms may form the substrate for cognitive functions during attention (91).

4. THE ENDOPHENOTYPE

Research of mental disorders is often hindered by complex behaviors, which underlines the necessity to study simpler, behavior independent, biological markers or "endophenotypes" of the disorder. By definition, an endophenotype is the biological manifestation of a disease at a *reduced* level of biological organization as opposed to the macro-level of behavior (23-27), justifying the term's etymology of a *hidden* ("endo", in Greek) from the un-aided eye phenotype. Thus, in order for biological research of mental disorders to proceed, it is essential to 'decompose' the disorder into simpler, measurable biological parameters that can serve as its *endophenotypes*. The idea of the endophenotype was introduced within the context and the perspective of genetic analysis of diseases as an alternative method to measure phenotypic changes in order to ease the detection of the underlying genes responsible for the hereditary traits of these changes. In this sense endophenotypes can be viewed as intermediates between genotypes and phenotypes: some being closer to genes and others closer to behavior. Endophenotypes more proximal to the effects of genetic variation aid attempts to link genes to disorders and such endophenotypes fulfill a number of criteria in order to be useful in genetic analyses (92). In this review, however, I will use the term endophenotype at its broader definition: the biological manifestation of a disease at a reduced level of organization. The slice is a reduced preparation of the brain, which importantly preserves its ability to spontaneously generate activity reminiscent of the intrinsic activity of the intact brain. Therefore, studying *local microcircuits* in spontaneously active cortical slices may lead to *network-based endophenotypes of autism* which can both guide and be evaluated by respective efforts in the clinic. For example, Up/ Down states are cellular correlates of the slow-oscillation, the electroencephalographic (EEG) hallmark of quiescent states of the brain such as deep, non-REM sleep, anesthesia and quiet wakefulness (rest) (34, 93), and recordings of spontaneous network cortical activity during rest are currently employed in the clinic for the discovery of biomarkers of autism, schizophrenia and Alzheimer's disease (94-97). The quest for a functional/electrophysiological biomarker of neurodevelopmental disorders of the brain, can be inspired and informed by the history and potential of epilepsy research.

4.1. Spike and wave discharges: an endophenotype of epilepsy

An important contribution of the EEG in epilepsy research was that it allowed neuroscientists to correlate epileptic behavior (e.g. myoclonic seizures) to the brain's electrical activity. A classical distinction that neurologists make as far as the electroencephalographic expression of seizure activity is concerned is between *interictal* and *ictal* activity (98). The interictal phase is demonstrated in the EEG as a series of sporadic epileptiform discharges and is not accompanied by obvious behavioural manifestations of the seizure. From time to time an interictal event will give its place to an ictal period, characterized by a more continuous series of multiple EEG discharges and associated with a stereotyped behavioral disturbance. Each interictal event is typically composed of a negative discharge or "spike" followed by a more long-lasting positive "wave" (*spike and wave discharges*, *SWDs*).

In turn, this behavior-EEG link enabled investigators to study seizures electrographically, *independently* of behavior, in anesthetized animals in order to understand the underlying neurobiology. In 1870 Hughlings Jackson had predicted that focal epileptiform activity is characterized by intense and synchronous activity in large groups of neurons within the cortex (99). However, it was only less than a century later that Ajmone-Marsan and colleagues first demonstrated the correlation of EEG surface recordings with recurrent intracellular and extracellular recordings from a given epileptic focus and found that the cellular correlate of the electrographic seizure is a large and prolonged depolarization (the *paroxysmal depolarization shift* (PDS)) followed by a long-lasting hyperpolarization (100-102). Therefore, the alternation of the depolarizing shift and the following hyperpolarizing potential, respectively, gives rise to

the “spike and wave” electroencephalographic pattern. Later work *in vivo* furthered our understanding of cells and networks responsible for the development and control of seizures (85, 103-106).

Although such *in vivo* models of epilepsy gave us important insight to the brain circuitry underlying ictogenesis, studying the respective cellular and molecular mechanisms requires long-lasting intracellular recordings and pharmacological manipulations, which are not easily applied *in vivo*. This prompted investigators to model and study epileptic activity *in vitro* and indeed since the end of the 1970’s, work in slices has brought about groundbreaking progress in our understanding of epilepsy (67-73). However, early *in vitro* attempts were hampered by the fact that epileptiform events had to be induced artificially, either by electrical stimulation or by significantly altering the ionic composition of the bath solution, since brain slices were not spontaneously active. Thus, studying seizures *in vitro* required conditions that deviated from normal. Something more than 15 years ago, it was discovered that this problem could be circumvented by bathing the slices in a buffer (artificial cerebrospinal fluid, aCSF) that closely mimics the cerebrospinal fluid *in vivo*. Under these conditions the cortex generates spontaneous slow (<1Hz) oscillations (31, 32, 40) which can turn into paroxysmal SWDs after suppression of GABA-mediated inhibition (71-73), similarly to *in vivo* results (85, 107). Therefore years of electrophysiological research have lead to an *in vitro* model of electrographic seizures, generated spontaneously in the reduced preparation of the brain slice. This model provides the context to understand at the cellular and molecular level the neurobiology of seizures, the action of current anti-epileptic drugs and the development of new ones. Following I will provide two examples from previous work of ours of how an *in vitro* model of epileptic activity can be used to address clinically relevant questions.

4.1.1. Comparing ictogenesis in the cortex and studying mechanisms of epileptic activity

Comparing cortical ictogenesis, i.e. which cortical areas and/or layers are more susceptible than others to generate seizures, is of critical clinical importance since not all cortical regions are equally epileptogenetic. For example, secondary generalized epileptic seizures begin locally from an epileptic focus and soon after generalize to the rest of the cortex and the brain. It is therefore important to pinpoint and study those cortical areas that are more susceptible to seizures in order to design targeted treatments of or to pharmacologically prevent seizures. Using the *in vitro* preparation in order to study cortical ictogenesity has several advantages: cortical areas can be studied in isolation; the researcher has easy access to different cortical layers; and since the *in vitro* condition provides a steady and well-controlled environment the slice preparation optimizes prolonged-pharmacological and intracellular studies providing insight to cellular and molecular mechanisms. The development of those conditions under which cortical slices would be spontaneously active in the form of the slow oscillation and subsequently the transformation of this normal activity into abnormal (paroxysmal) epileptiform activity (i.e. SWD) after suppression of cortical inhibition, has allowed us to study the intrinsic ictogenesity of distinct cortical areas (72, 73).

Since SWDs consist the electrographic hallmark of the epileptic brain we compared the capability of distinct cortical areas such as the granular (e.g. primary somatosensory) and the agranular (e.g. primary motor) cortex (72) or of the neocortex (namely the somatosensory cortex) and the evolutionarily more primitive paleocortex (namely the piriform cortex) (73), to generate spontaneous SWDs as a measure of their epileptogenesity. GABA is the major inhibitory neurotransmitter of the brain which acts on two main types of receptors: the fast, ionotropic, GABA-A receptors and the slower, metabotropic, GABA-B receptors (108, 109). The generation of epileptiform activity in the brain by suppression of GABA-A mediated inhibition is a widely applied model for the study of epileptic seizures (69, 85). Both *in vivo* and *in vitro*, suppression of GABA-A inhibition transforms cortical slow oscillation into recurring SWDs (72, 73, 85). Therefore, when we compared *in vitro* the epileptiform activity of the neocortex with that of the paleocortex, we found that the latter generates SWDs at significantly higher frequencies (73). In addition, total suppression of cortical inhibition by blocking both GABA-A and GABA-B types of receptors, leads to the development of 10Hz afterdischarges in the agranular (motor) but not the granular (somatosensory) cortex (72). These differences in the excitability of distinct cortical areas likely demonstrate differences in the way that underlying neuronal circuits are organized due to their different cytoarchitecture.

Recording spontaneously active cortical slices is an experimental model that allows the study and comparison of the *intrinsic* activity of distinct cortical areas. We were actually the first in the field to describe this kind of activity in the adult mouse brain (72) opening new ways to study the cellular and molecular mechanisms of the cortex’s epileptiform activity and to better understand and treat it pharmacologically in the mature brain in a focused manner. The epileptic 10Hz afterdischarge activity that the agranular (motor) cortex develops during disinhibition is a good example of such a focused approach. Afterdischarges are of particular clinical interest since they correspond electrophysiologically to the ictal periods of seizures during which myoclonus takes place (107). In particular, during tonic-clonic

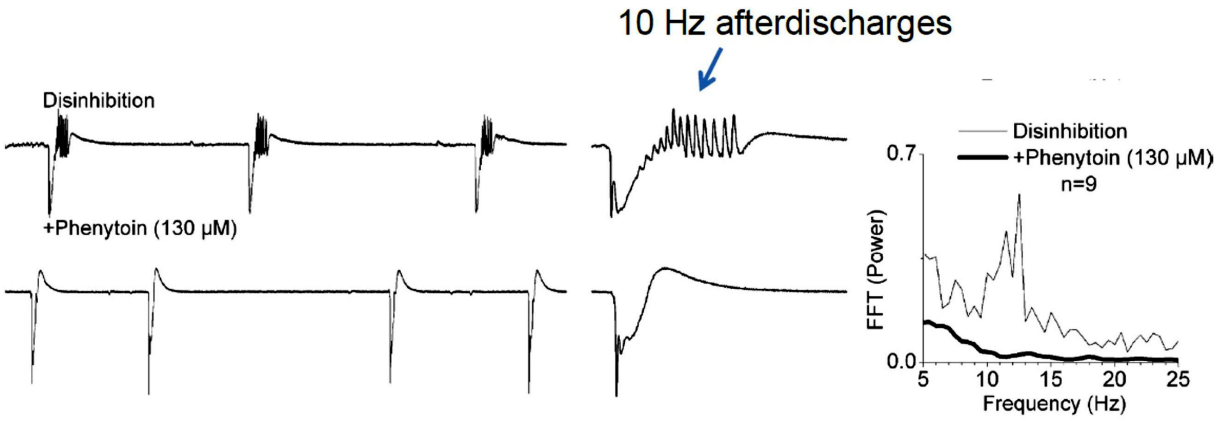

Figure 2. The effect of phenytoin on afterdischarges *in vitro*. Reproduced with permission and modified from (72).

seizures the EEG is composed of periods of small amplitude, higher-frequency activity (the ictal period) interrupted by large amplitude but low-frequency discharges (the interictal period). Interestingly, ictal periods of the EEG are behaviorally demonstrated as myoclonus, while in the absence seizures type of epilepsy (common among children) the EEG is dominated by interictal discharges, of the SWD type. Thus in our *in vitro* preparation the SWD discharges corresponds to the interictal period of a seizures, while 10 Hz activity belongs to the ictal period of an epileptic episode (Figure 2).

Interestingly, it seems that the 10 Hz activity is the intrinsic oscillation (resonance) of the motor cortex's excitatory network since the same frequency can be generated not only under the condition of disinhibition but also in other widely used experimental models of epilepsy such as the activation of NMDA receptors in low extracellular Mg^{2+} which we also applied in our *in vitro* preparation (71, 110). Therefore, combining the appropriate electrophysiological and pharmacological experiments we found that the 10 Hz afterdischarges: (a) are produced purely by a network of excitatory neurons without the involvement of inhibitory neurons, (b) they are generated by the synaptic network rather than pace-makers, (c) they are generated in the superficial layers of the cortex, and (d) they depend on both synaptic currents but also on intrinsic currents cortical neurons such as both inward currents like the persistent Na^+ current (I_{NaP}) as well as outward currents like the M-type of potassium current (I_M) (71, 72).

4.1.2. Screening for new antiepileptic drugs

Despite the years of epilepsy research the drugs currently available in order to treat seizures are still more or less those introduced decades ago with often unknown mechanisms of action and significant side effects. Moreover, treatment of epilepsy in children is largely based on reduced doses of antiepileptic drugs used for adults according to the false view of the immature brain as a "smaller adult brain". The promise of molecular medicine for the treatment of epilepsy in the future is the discovery of drugs of (1) known molecular mechanisms of action, with (2) region-specific and (3) age-specific effects within the brain. In this perspective research in experimental animals is instrumental and recording spontaneously active cortical slices of the mouse brain could be used as an experimental model that will allow us to test and study new antiepileptic drugs according to the scheme described in Figure 2. In this example, in an acute model of epilepsy (namely disinhibition) *in vitro*, in mouse brain slices, the agranular (motor) cortex develops SWDs followed by afterdischarges at the 10 Hz range which are abolished by phenytoin, a well-known antiepileptic drug. Similar work can be employed as a first, high-throughput screening of the efficacy and potency of novel antiepileptic drugs or of antiepileptic drugs with discrete mechanisms of action. Further extracellular and intracellular recordings combined with appropriate pharmacological experiments would provide insight to the microcircuit, cellular and molecular mechanisms of action of the antiepileptic drug.

5. USING SPONTANEOUS CORTICAL UP/ DOWN STATES TO DEFINE CRITICAL PERIODS FOR THE DEVELOPMENT OF AUTISM

Understanding and treating autism is intrinsically related with defining the *onset* of the disease. To this end, functional/ neurophysiological phenotypes may be more appropriate than structural deficits since dysfunction often occurs without apparent changes in structure. Accumulating evidence suggests that mental disorders such as Alzheimer's disease, schizophrenia, or autism but also depression and bipolar disorders can arise from abnormal intrinsic activity in specific brain circuits in the absence of detectable structural lesions (26, 111-114). In autism research clinical investigators stress the need for functional longitudinal studies of the brain (functional

magnetic resonance imaging, fMRI) in human infants, toddlers and children at high age resolution in order to define the ontogeny of the disease (15). However, such technologically advanced and complex studies are often difficult or practically precluded at very young ages, rendering research in appropriate animal models an imperative alternative.

Animal models have proven to be essential for understanding the neurobiology of brain disorders and for preclinical testing of potential therapeutics. Indeed, animal research of autism neurobiology has led to an abundance of published information on the genetic, molecular, cellular and synaptic changes that take place in the disordered brain, however, much less is known on whether, to what extent and how these changes actually contribute to higher levels of organization such as the *neuronal network*, as a common *final functional pathway* that defines the pathophysiology of the disease and ultimately respective behavior. Moreover, to present, animal studies of autism neurobiology target ages and cortical areas of largely the investigators' choice in a rather "*top-down*" manner and are usually limited each time to a single age, a specific cortical area and to males. Finally, the bulk of currently available research has essentially focused on *how* the brains of animal models of autism differ from normal, rather than on *when* they begin to differ. Alternatively, network dynamics of local microcircuits i.e. Up/Down states recorded *in vitro* in spontaneously active, acute brain slices prepared from appropriate animal models of autism could be used as a neurophysiological measure of brain maturation in order to draw and compare *developmental trajectories* of the autistic and normal brain. Such an approach would allow us to pinpoint and study the neurobiological onset of autism in distinct subareas of the developing cortex. In recent work of ours we showed that spontaneous Up states recorded in cortical slices by means of local field potentials can be used to draw the lifetime trajectory of network dynamics of the mouse neocortical microcircuit (115) and preliminary results of ours from a mouse model of autism indicate that Up states may also reflect differences in the development of normal and disordered cortical networks (116). Therefore, recording spontaneous Up/Down states *in vitro* could allow us to investigate those developmental periods and cortical areas that will prove to be significant for the ontogeny of autism as highlighted in a *"bottom-up"* manner by tracing the development of multiple areas of the autistic cortex.

5.1. The Fmr1KO rodent model of autism

Rodents have been instrumental in the neurobiology research of autism. Since mice are more amenable to genetic manipulations than rats, rodent models of autism currently fall into two main categories: genetic models in *mice* and environmental models in *rats*. The Fmr1KO mouse, which is an excellent animal model of the fragile X syndrome (FXS), the most common inherited form of intellectual disability and an identified monogenetic cause of autism in humans; has been widely employed as an animal model for autism research (117-119). The fragile X syndrome is due to the silencing of a single gene, the fragile X mental retardation 1 (Fmr1) gene, located on the X chromosome. Mutations in the FMR1 gene lead to loss of expression or function in the protein it encodes, FMRP, a suppressor of translation present in synapses (120, 121). FXS has a traceable line of neurobiology from genes and molecules to cells, from synapses and circuits to behavior; a fact that renders the Fmr1KO a highly promising animal model for the understanding of neurodevelopmental disorders from genes to behavior and their treatment (22). Indeed several studies have described changes of cortical development in FXS and autism at the level of cellular and synaptic structure and dynamics (18, 21, 94, 122-129). An alternative rodent model of autism was developed mainly in rats and involves their exposure to the antiepileptic drug valproic acid (VPA) during gestation. The VPA-model is considered to be an environmental animal model for autism (130) for which molecular, cellular, synaptic and network alterations in neural tissue have been reported (131-137).

The arrival of the Fmr1KO rat model of autism consists a recent advance in the field (138). Prior to the advent of genetic manipulations in mice, the rat was the animal of choice in neurobiology since it shows a more complex behavioral repertoire than the mouse, and its larger brain permits more sophisticated electrophysiological recordings. Recent developments, however, in genomic editing technologies have facilitated the ability to manipulate the rat genome, thus renewing interest in the rat as a model for genetically linked disorders. Although rats are sometimes falsely perceived as larger versions of mice, the evolutionary distance between rats and mice may actually be as great as that between humans and Old World monkeys (139). Therefore, the Fmr1KO rat model is of special interest for autism research since it will allow researchers to compare, combine and reconcile findings from mice and rats; from genetic and environmental animal models of autism.

5.2. The developmental delay and heterochronicity of autism

Autism and mental retardation are clinically manifested by a *developmental delay* of motor and speech skills, as well as excess responses to sensory stimuli, all of them functions that require intact cortical processing (140). Since the cognitive deficits associated with these disorders first arise during childhood, they likely result from the altered development of neuronal networks of the cerebral cortex. Normal development of functional cortical networks requires the coordination of many precisely

timed events at the molecular, cellular and synaptic level (141-143). Deviations in the timing of these events will alter the precision of neuronal connectivity which in turn may lead to changes in network dynamics and ultimately the emergence of cognitive and behavioral dysfunctions. Moreover while normal cortical function requires the dynamic balance between excitation and inhibition, there is evidence that in the Fmr1KO cortex this balance is tipped in favor of excitation rendering its networks hyperexcitable (144-147). It has been suggested that impairment of this balance may be responsible for a shifted *critical period* of cortical development in autism (42) which, in turn, could be manifested as a shift/delay in developmental trajectories between normal and autistic cortices (22).

Although clinically justified and conceptionally anticipated such a developmental delay in the developing autistic cortex has yet to be experimentally demonstrated. To this end network-based developmental trajectories may have several advantages. In particular, understanding how the absence of FMRP affects individual components of the network is important but not sufficient to predict their effect on the function of the network. It is likely that entirely new properties *emerge* at higher levels of neuronal organization (networks) compare to those of the constituent parts (molecules, cells and synapses) (148, 149). For example, drawing developmental trajectories based on individual cells or molecules may differ significantly from respective trajectories of functional networks. Therefore not only can cortical microcircuits not be understood in terms of a mere extrapolation of the properties of their particles, but rather the understanding of their function will lead to new directions of integrative knowledge. In this perspective, studying the activity of local cortical microcircuits may serve to fill the cell and molecules to behavior gap in our understanding of the pathophysiology underlying autism. In recent work of ours we showed that spontaneous Up states recorded in cortical slices by means of local field potentials can be used to draw the lifetime trajectory of network dynamics of the mouse neocortical microcircuit (115), which closely follows changes in synaptic density in the cortex over the lifespan (150). We have also found that Up states-based developmental trajectories can differentiate the development of distinct cortices (115). The cerebral cortex is not uniform but instead it consists of functionally and structurally distinct areas which develop at different rates. As opposed to the intact brain where long-range connectivity synchronizes different cortical areas (50), in the brain slice preparation distinct types of cortices can be separated into different slices and the network dynamics of their local microcircuits can be studied in isolation from each other. This way we can derive developmental trajectories that are *intrinsic* to different types of cortices and thus test whether changes in the autistic brain occur earlier and/or more severely in some cortical areas compared to others suggesting that they are more vulnerable to autism. This is of particular interest since clinical data suggest that the development of autism does not involve equally and simultaneously the entire cortex, but instead its effect on different cortical areas of the developing brain is characterized by *heterochronicity* (15). Therefore spontaneous Up states at the reduced level of the brain slice may serve as a neurophysiological measure to draw developmental trajectories, and thus mathematically describe and compare the development of distinct cortical areas in the normal and the Fmr1KO rat. We have recently published as part of a conference proceedings the comparison of Up states-based developmental trajectories of the normal and Fmr1KO primary somatosensory (S1BF) mouse cortex (Figure 3) (116).

5.3. Proposing a scheme for for the neurobiological research of autism onset

Following I will propose a scheme of pinpointing and studying the onset of autism development in the Fmr1KO rat cerebral cortex based on network dynamics of local cortical microcircuits. The proposed scheme is based on electrophysiological recordings performed *in vitro*, in the acute brain slice preparation, in order to first establish a *phenotype*, namely describe when and where autism begins in the cortex and then investigate the underlying *mechanisms* responsible. The first could be based exclusively on extracellular recordings while the second would involve intracellular recordings and pharmacological experiments.

In order to pinpoint the onset of autism, spontaneous network activity of local cortical microcircuits could be used as a neurophysiological measure to trace cortical development. This would require *systematic* recordings from *multiple ages* composing a serial study from first postnatal days till adulthood and *multiple cortical sites* per each animal in both wild-type and transgenic rats. In addition, the validity of these comparisons (Wt vs FXS, among different cortices) would require recordings under *identical/comparable* experimental conditions. To this end I propose to take advantage of the cortex's intrinsic property of generating spontaneous activity in the total absence of external (sensory) inputs such as during quiescence *in vivo*, but also *in vitro* at the reduced level of the isolated, sensory-deprived brain slice. In particular spontaneous field potential (network) events (i.e. Up states) would be recorded *in vitro*, in rat brain slices preserved alive in the interface type of recordings chamber, as previously described (31, 32, 40, 56, 71-73, 115).

5.3. 1. Why record *in vitro*?

The brain slice has several advantages compared to the intact brain of anesthetized animals

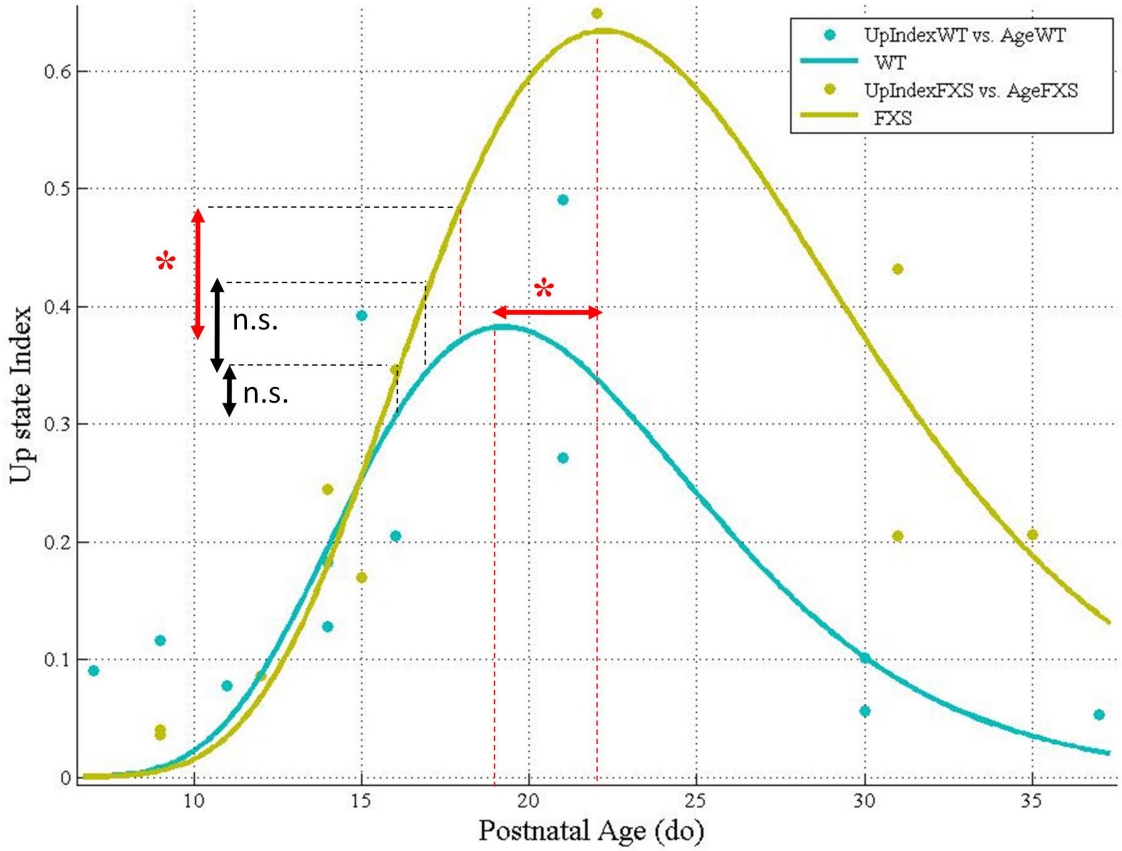

Figure 3. Comparing developmental trajectories of the WT and FXS cortices. Cortical Up state index values, i.e. a combined measure of incidence and size (Up sComparing developmental trajectories of the WT and FXS cortices. Cortical Up state index values, i.e. a combined measure of incidence and size (Up state index= occurrence * rectified area, see Figure 1) of the normal (WT, blue) and FXS (green) mouse were plotted as a continuum from early postnatal age till the end of the fifth postnatal week of life. Data points, each representing the mean Up state index at a given age, were fitted by peak functions (WT cortex (N_{slices}=12, $N_{animals}$=10): Up State Index = 0.3.8* exp(−0.5.* (ln(Age /19.2.1)/0.2.7)^2, curve-fit R^2 = 0.6.1, p < 0.0.5, FXS cortex (N_{slices}=10, $N_{animals}$=8): Up State Index = 0.6.3* exp(−0.5.* (ln(Age/22.2.2)/0.2.9)^2, curve fit R^2 = 0.8.9; p < 0.0.5). Red asterisks indicate statistically significant differences, while n.s. stands for non-significant. Developmental trajectories show a delayed peak in FXS cortex and that deviation from normal begins at P15 i.e. at the onset of cortical sensory processing and becomes statistically significant by P18 which corresponds to the age of a human toddler. Reproduced with permission from (116).

for the proposed research. In particular: (a) slices from both Wt and Fmr1KO rats can be placed and recorded *side by side* under identical experimental conditions. This is an essential prerequisite for the validity of the genotype-dependent comparisons. (b) The slow oscillation is extensively synchronized *in vivo*, in the intact brain, making comparisons between different cortical areas very difficult. On the contrary, distinct types of cortex are separated in different slices which will allow us to document the *intrinsic* properties and development of local network dynamics. And (c) experiments in anesthetized animals are curtailed by the fact that anesthesia affects the slow oscillation (34). This is an important point, since the proposed developmental study would involve animals of very different ages which may require different doses of anesthetics or may respond/be affected differently to the same dose of anesthesia. In addition, recording spontaneous events *in vitro* has several advantages: (a) events are easily detectable with clear onsets and offsets which eases their further analysis, (b) cortical areas are easily accessible for recordings and/or interventions and (c) the slice provides a steady and highly-controllable environment for prolonged combined extracellular and intracellular recordings with pharmacological protocols paving the way for understanding underlying mechanisms at the cellular and molecular level. On the contrary, *in vivo* it is difficult to dissociate the underlying components and perform proper pharmacological analysis.

5.3.2. Why record in the interface chamber? The interface vs the submerged chamber

There are two types of recording chambers *in vitro*: the submerged and the interface chamber. In the submerged type of chamber brain slices are placed (submerged) in a solution within which neurons can then be visualized at high magnification using a water-immersion objective allowing high spatial

resolution intracellular recordings. On the other hand, in the interface chamber slices are kept alive lying at the interface of a liquid and gas phase which restricts intracellular recordings to be made blindly. Although spontaneous Up states from cortical slices can be recorded both in the submerged and the interface type of chambers, the interface chamber is more appropriate for the proposed research for two main reasons:

5.3.2.1. The submerged chamber pertains to intracellular rather than extracellular recordings

Since the proposed research requires sampling at multiple ages and cortical areas, intracellular recordings could not be the technique of choice for this project. On the contrary, field potential (extracellular) recordings opt for multiple recordings in a single experimental day. In addition, it is essential to study cellular and synaptic changes that occur during normal and abnormal brain development in the light of field potential recordings of cortical network activity, not only as an emergent functional property of underlying cells and synapses (and ultimately, genes and molecules) but also as a measure that can be paralleled to the clinically relevant EEG recordings, an essential link for biomedical translational research. Although field potentials of spontaneous Up states can be recorded in cortical slices even in the submerged chamber this is achieved by using high rates of superfusion and relatively low temperatures in order to overcome the low diffusion of oxygen inherent to the submerged condition at low oxygenation rates and higher temperatures (56, 115). Importantly, even under optimal conditions Up states in the submerged slice occur at significantly lower incidences and are much more irregular compared to those recorded in the interface chamber.

5.3.2.2. Space limitations of the submerged chamber

The submerged chamber has been designed to record cells from a single brain slice at a time. In recent work of ours we adjusted the submerged chamber and managed to fit and record field potentials simultaneously from four (4) mouse brain slices increasing the yield of our experiment in terms of the total number of recorded slices (56, 115). However, these numbers still remain significantly lower compare to commercially available interface chambers which can fit up to ten (10) mouse brain slices.

5.3.3. Why developmental trajectories?

The proposed act would aim at describing and comparing cortical development in the FXS and Wt rat by outlining developmental trajectories based on parameters of network dynamics. In particular, we have recently shown that Up-states based developmental trajectories can mathematically describe cortical development in terms of a peak-function (115). Therefore spontaneous activity could be sampled at systematic intervals throughout development and at ages that are biologically meaningful for cortical development (elaborated below). Although the suggested ages don't consist a continuum in time they are sufficient to support a peak function that will predict developmental changes of network dynamics at the missing intervals therefore forming a *continuous* line of development (developmental trajectory) as we've shown ((115) and Figure 3). In turn, developmental trajectories allow us to *predict* values of network activity even at ages that have not been experimentally sampled, this way (a) reducing the need for sampling all ages (i.e. day by day) during development, without (b) necessarily restricting the onset of autism at the somewhat arbitrarily chosen sampled ages. The latter is a very important point for the objectives of the proposed research as it ensures a *"bottom-up"* definition of autism onset.

5.3.4. The choice of ages

Brain development goes through stages or phases, some of which (known as "*critical periods*") are crucial for the development of some of the cortex's features. Many of these developmental milestones belong to the first three weeks of the rat's life. For example postnatal days 3-4 (P3-4) and P5-7 are critical for the establishment of the anatomy in the somatosensory cortex (141); during the second postnatal week (P7-10) tonic inhibition is significantly reduced in the neocortex (151); around P10 GABAergic synaptic neurotransmission in the hippocampus is reversed from excitatory to inhibitory (the"GABA switch") (152); P10-14 and P14-16 are critical periods for synaptic plasticity of connections towards and within cortical layers II-III (141); on P14 rats open their eyes and begin to actively move their whiskers defining the onset of sensory processing in the cortex (153); on P21 juvenile are weaned from their mother (154); and rats become sexually mature (puberty) by their sixth postnatal week (P42) (154-156). The 7-10 days period before puberty (i.e. P30-40) is important for the social development of these animals characterized by significant changes in the brain's catecholaminergic systems (157). This pre-puberty period is also of special interest in regards with autism research since deficiencies in social interaction is the disorder's hallmark. Adolescence by definition is the gradual transition from childhood to adulthood which in rats practically covers their second month of life (~P30-P60) (154, 158, 159), while by three months old the rat is considered a young adult (154). In an attempt to see how rat brain development corresponds to that in humans, it is worth noting that rats are born "prematurely" compared to humans. Therefore, one week old neonatal rats (P0-7) correspond to human

embryos of the third trimester, and it is only by the middle of their second postnatal week (P8-10) that rat neonates correspond to full-term human newborn (160-165). Juvenile rats 10-17 days old correspond to human babies and toddlers (163, 165); an age of special interest for the ontogeny of autism. Finally rats at P18-30 can be paralleled to prepubertal children (9-10 years old) (163, 165, 166). Based on this information network dynamics in the neocortex could be sampled for example from at a total of thirteen age groups covering the entire development of the rat brain from the first postanatal days till young adulthood, namely: P4±1, P7±1, P10±1, P13±1, P16±1, P20±1, P25±1, P30±1, P35±1, P40±2, P50±2, P60±2 και P90±2. Such a systematic and detailed work would allow us to pinpoint the developmental ages at which autism begins in the cerebral cortex.

5.3.5. The choice of cortices

An important element in the proposed research scheme would be to record in parallel distinct cortical areas *in isolation*. This is an important condition for the validity of the inter-cortical comparisons during development in order to test whether the ontogeny of autism involves the entire cortex or not (the issue of *heterochronicity*). Importantly, the cortex is composed of anatomically distinct areas, which differ functionally, cytoarchitecturally and phylogenetically. The primary somatosensory cortex of the whiskers (S1BF), the primary motor cortex (M1) and the prefrontal cortex (PFC) are examples of functionally distinct areas in the neocortex. Autistic children show a developmental delay of motor skills and excessive responses to sensory stimuli (1-4), facts that justify the choice of a sensory and motor cortical area to study. In regard with the sensory cortex, the S1BF cortex consists an established model for the study of cortical sensory processing; it has a stereotyped and well-characterized development (167); and changes in network dynamics (Up states) of this cortex have been described in animal models of autism (144, 145). In addition, S1BF and M1 cortices are known to be different in several ways, in regard with their cytoarchitecture: S1BF has a well-developed layer IV and belongs to the granular type of cortex, while M1 whose layer V is expanded at the expense of layer IV belongs to the agranular type of cortex (168); the plasticity of their intrinsic synaptic networks (169, 170); the dynamics of their thalamocortical inputs (171); their paroxysmal oscillatory activity (71, 72, 172) and their development (115). Finally, the prefrontal cortex consists an established model to study cortical malfunction in a number of psychiatric disorders (173); it controls social behavioral which is primarily distorted in autism (174-176); it has been clinically associated with the ontogeny of autism (15); and electrophysiological changes have been reported for this cortical area in animal models of autism (137, 139). Therefore, these cortices could be valid choices for the proposed research scheme and their intercortical differences may consist a neurobiological context to test the idea whether the developmental deviations associated with autism involve the entire cortex or rather changes in some areas are earlier and/or more intense than others.

6. CONCLUSION

Spontaneous neuronal network activity may help us understand not only *how* local microcircuits of the cortex may be affected in autism, but also *when*, therefore shifting the research paradigm of autism from the time of symptoms to the time of onset. Moreover, *when* during development and *where* in the brain/cortex autism attacks are clinically highly relevant questions, which, however, are very difficult to be addressed in humans. To this end, appropriate animal models of autism would be instrumental to study the neurobiological mechanisms underlying the onset of this devastating disease placing studies in place and in context, i.e. at developmental periods and cortical areas relevant to the ontogeny of the disorder. The spontaneously active brain slice could provide us the means to study the neurobiology of autism in a behaviour independent manner in appropriate animal models of the disorder, in which, however, it is often very difficult to replicate and link complex human behaviours. In addition, spontaneously active slices provide a functional read out of network dynamics which would integrate the effect of single molecules and cells on the ontogeny of the disease. Therefore, understanding when and where in the brain development goes awry in autism will place the respective research of underlying neurobiological mechanisms in the context of disease-relevant critical periods and brain areas.

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