VU VRIJE UNIVERSITEIT AMSTERDAM

VU Research Portal

Development and modulation of mouse and human cortical circuitry

Kroon, T.

2019

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)
Kroon, T. (2019). *Development and modulation of mouse and human cortical circuitry*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam].

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:
vuresearchportal.ub@vu.nl

Download date: 16. Aug. 2024

6

Discussion

SYNOPSIS

Neurodevelopmental disorders (NDDs) can severely affect the quality of life of those who suffer from them. NDDs arise from a complex interplay between developmental processes and dysregulation of the expression of genes, or dysfunction of their gene products. It is therefore crucial to establish not only the phenotypes that arise from dysfunction of genes and proteins implicated in NDDs, and their basal functions, but also the time-courses according to which the genes are expressed, and, importantly, when their role is most critical during development. Accordingly, it is important to also establish their developmental context: the neurotypical functioning of the processes in which they are involved. In this thesis, I do this by providing a detailed description of the early postnatal development of pyramidal cells in the mouse (mPFC), as well as the impact of the deletion of NDD gene Oligophrenin-1 (*Ophn1*) on this.

In **chapters 2 and 3**, I describe the early postnatal development of pyramidal cells in layers 3 and 5 of mouse mPFC. I show that, with the exception of some small layer-specific differences, overall development of dendritic morphology and intrinsic membrane properties show the same progression in cells in both layers. Most parameters of dendritic morphology reach their final values by the end of the second postnatal week, as do many intrinsic physiological membrane properties. However, some properties of the membrane only reach mature values at the end of the fourth week. Although the development of morphology and intrinsic properties is similar between layers, the development of synaptic function is markedly different. The frequency of spontaneous excitatory currents increases faster in layer 3 neurons, while the frequency of inhibitory events increases faster in neurons of layer 5.

In **chapter 4**, I show that knockout of *Ophn1* leads to distinct age- and layer-dependent phenotypes that impact synaptic transmission. Surprisingly, the densities and morphology of dendritic spines was not heavily affected. *Ophn1* deletion – in contrast to many other NDD genes – does not impact the balance of excitation and inhibition.

Metabotropic glutamate receptors (mGluRs) have been implicated in several neurodevelopmental disorders in rodent, and are a prominent drug target in recent clinical trials. In **chapter 5**, I therefore shift my focus to the function of mGluRs in the human cortex. I show that mGluR-mediated depression is present in excitatory synapses onto pyramidal cells in human cortex. Furthermore, activation of mGluRs leads to spiking in Martinotti cells, which in turn causes widespread synaptic inhibitory inputs to all other cortical neuronal types measured. These results validate what has earlier been shown in rodents, and highlight the importance of the little-studied, but conserved action of mGluRs on cortical inhibition.

DISCUSSION

Development of dendritic morphology

In humans, the prefrontal cortex shows a delayed development relative to other regions of the cortex. In contrast, we show that the development of gross features of dendritic morphology in mouse mPFC is similar to what has previously been reported in other cortical areas. However, due to the methods used in the current study, there are some aspects of dendrite morphology development that we are not able to resolve. We imaged stained neurons in fixed brain slices from different animals at different ages. We find that the number of branch points of the apical tuft of layer 5 neurons is reduced in older animals. Because tuft dendrites in the younger age group show short, immature dendritic protrusions, we assume that it is these protrusions that are removed during development – a process known as pruning (**Riccomagno and Kolodkin, 2015**). However, we do not know whether this is the case. It may be that the immature protrusions seen at younger ages mature into fully grown dendritic branches, while other branches that seemed more mature are pruned instead. To resolve this, one could use *in vivo* approaches. *In vivo* two-photon imaging has been used to precisely monitor outgrowth and retraction of dendritic branches (**Gonçalves et al., 2016**), enabling a much more detailed study of the dynamics of dendrite development than is feasible with our methods. In addition, the pruning of neurites depends in part on neuronal activity (**Riccomagno and Kolodkin, 2015**). Using long-term *in vivo* imaging of neuronal morphology in combination with a measurement of physiological activity, such as voltage indicators, may show to what extent the electrical activity within a given branch determines whether this branch is pruned.

In vivo two-photon imaging has also shown that dendrite pruning is a homeostatically regulated response to dendrite outgrowth, and that experimentally increasing dendritic branching leads to increased dendrite pruning (**Gonçalves et al., 2016**). We assessed dendritic morphology in the *Ophn1* KO and find only very minor phenotypes. However, it could be that OPHN1 does affect dendrite dynamics, but that this effect is counteracted by homeostatic mechanisms, and therefore does not show up in our snapshot measurements. This issue may be resolved by using either an *in vivo* approach, or by time-lapse imaging of neurons in organotypic cultures. The same may apply to our results concerning dendritic spines, which are known to be structurally highly dynamic (**Bhatt et al., 2009; Ebrahimi and Okabe, 2014; Fischer et al., 1998**).

Dendritic spine phenotypes in neurodevelopmental disorders

One of the earliest known functions of OPHN1 is the maintenance of dendritic spines. Interestingly, the nature of the effects of removal of OPHN1 from neurons on dendritic spines varies wildly from one study to the next. Initially, OPHN1 was found to reduce spine length, without affecting the density of spines or filopodia (**Govek et al., 2004**). Subsequent

studies have reported a decrease in both spine size and density (**Nadif Kasri et al., 2009**); a reduction in either spine length or density, depending on whether the measured dendrites was apical or basal (**Khelfaoui et al., 2007**); a reduction in mushroom spines with no effect on filopodia (**Powell et al., 2012**); and an increase in filopodium density, with no effect on other spine types (**Meziane et al., 2016**). We now find a decrease in thick (mushroom/stubby) spines, but only at 4 weeks in layer 3 pyramidal neurons.

How to solve these discrepancies? One important consideration is that the method of removal of OPHN1 differs between studies. The studies performed by Govek et al. and Nadif Kasri et al. use knockdown methods to reduce levels of *Ophn1*, whereas Khelfaoui et al., Powell et al., Meziane et al., and we use constitutive *Ophn1* knockouts. It seems that studies where *Ophn1* was knocked down tend to find a more consistent reduction in spine length. It is possible that spine length is restored later during development via some homeostatic mechanism, as appears to be the case for basal excitatory transmission (**Nadif Kasri et al., 2009**) and mGluR-mediated LTD (**Khelfaoui et al., 2007; Nadif Kasri et al., 2011**).

Similarly, different studies use animals of different ages. Meziane et al. assessed spine densities in 5-month-old animals. They find only an increase in filopodia, which they also attribute to compensatory mechanisms. The strongest evidence that the effect of OPHN1 on dendritic spines is dependent on age comes from the 2007 study by Khelfaoui et al., who find that while spine densities were reduced at 12 weeks, the densities of all types of spines were actually increased during *in vitro* differentiation of neurons from *Ophn1* KO animals. The spine phenotype we find here is also age-dependent, providing further evidence that OPHN1 function is dependent on context.

Lastly, there are differences in the brain regions used in these studies. While most studies use hippocampal tissue, the studies performed using knockdown methods are performed in organotypic cultures, while those performed in KO animals typically use acute slices. However, even within the hippocampus, some studies assess cells in CA1, while other use dentate gyrus. Our results show that, even within the same part of the cortex, cells from different layers show different phenotypes upon deletion of *Ophn1*. The influences of age and area on studying OPHN1 function are further discussed below.

There has been debate in recent years regarding the validity of much of the research done on dendritic spines using conventional light microscopy. For example, a recent investigation into dendritic spines in the *Fmr1* knockout mouse model for Fragile X Syndrome (FXS) using stimulated emission depletion (STED) microscopy indicates that dendritic spine phenotypes in this model are more nuanced than it was previously thought to be (**Wijetunge et al., 2014**). This may be due in part to the fact that non-superresolution microscopy methods often lead to errors in measurement of spine dimensions and misclassification of spine types (**Tønnesen et al., 2014**). Because the current study, as well as those mentioned previously, all use non-superresolution methods, extra care must be taken when interpreting their results.

E/I balance

Disruption of the balance of excitation and inhibition (E/I balance) is thought to be a major pathophysiological feature of many neurodevelopmental disorders (**Nelson and Valakh, 2015; Rubenstein and Merzenich, 2003; Selten et al., 2018**). We do not find any changes in E/I balance in our *Ophn1*$^{-/y}$ data. However, E/I balance can be measured in several ways. We measure either spontaneous events or responses to regulated extracellular stimulation in an acute slice. Using these methods, excitation and inhibition cannot be measured simultaneously. Another method by which E/I balance can be measured is to extracellularly stimulate a neuron while clamping it at several potentials. This way, the excitatory and inhibitory conductances in response to the same stimulation can be measured (**Cruikshank et al., 2007**). This gives a clearer image of the excitatory and inhibitory inputs onto the cell elicited by the same stimulation, which more closely resembles the neuron's response to, for example, a sensory stimulus in *in vivo*.

Nevertheless, our data align with previous results. Zhang et al. (**2017**) show no differences in feed-forward inhibition between *Ophn1* KO and wild-type. Powell et al. (**2012**) show that, similar to our data regarding short-term plasticity of EPSCs upon high frequency firing, short-term depression is exaggerated in *Ophn1*$^{-/y}$ neurons. This is in accordance with our results, as E/I balance will be unaffected if the decrease in both excitation and inhibition is similar. Furthermore, E/I balance is regulated in a dynamic manner, and is at least partly dependent on activity in the postsynaptic cell (**Xue et al., 2014**). Homeostatic processes may thus act to decrease the influence of the synaptic deficit on the formation of the circuit.

However, interneurons, and PV+ basket cells in particular, fire at much higher frequencies than pyramidal cells *in vivo* (**Klausberger et al., 2003; Massi et al., 2012**). Hence, the impairment in neurotransmitter release upon high frequency firing may be far more relevant for inhibition than for excitation under *in vivo* conditions. It is therefore critical to assess the relative impairments of synaptic inhibition and excitation *in vivo* to determine whether E/I balance is disturbed during behaviour and during processing of sensory information.

Sensitive periods and neurodevelopmental disorders

The development of the brain is tightly regulated by various hardwired genetic cues. However, to ensure that the organism is able to respond to variations in its environment, external stimuli influence the development of neurons, as well as the way in which they are integrated into neuronal networks. While synaptic plasticity is an on-going process that is also evident in the adult brain (**Holtmaat and Svoboda, 2009; Verhoog et al., 2013**), there are periods during development during which the network is able to react particularly strongly to certain stimuli. These are known as sensitive (also *critical*) periods. The most well-studied of these is that of ocular dominance plasticity (**Hensch, 2005; Wiesel and Hubel, 1963**).

We have argued that sensitive periods are of particular importance within the context of neurodevelopmental disorders (**Kroon et al., 2013; Meredith et al., 2012**). The protein products of NDD genes often have functions at the synapse, and are involved in plasticity. The absence or aberrant function of these proteins may be more damaging during a sensitive period, while plasticity is heightened and the network is being formed, than at adult stages, when less plasticity is necessary. A second way in which the concept of the sensitive period can be applied to NDD genes is if the gene itself is only expressed for certain periods of time. In this case, the sensitive period is only applicable to the gene itself, and does not necessarily constitute a sensitive period at the network level. Phenotypes may then be restricted to periods during which the gene is expressed. However, the aberrant plasticity resulting from the incorrect function of the protein during the sensitive period may still lead to abnormal development of network and/or synapses, and therefore may also impact function even at times when the gene is not normally expressed.

Our results show the necessity of studying neurodevelopmental disorders in a developmental context. We also show that the function of OPHN1 further depends on the cell types within which it functions. One of the reasons for this is that in the case of OPHN1, an additional level of complexity is added by the diversity of proteins with which it interacts. This is exemplified by the fact that fasudil, a RhoA/Rho-kinase inhibitor that has been used to counter deleterious effects of *Ophn1* deletion, has different effects on dendritic spines, depending on whether OPHN1 is present or not (**Meziane et al., 2016**). We might therefore add another factor to the idea of sensitive periods: sensitive locations. The localisation of downstream effectors of RHOA, RAC1, and CDC42, of which there are many (**Iden and Collard, 2008**), is strictly regulated. They in turn can have many interactions with each other, depending on which effector proteins are present at their current location, and which ones are active and free to bind to. Consequently, the effect of deletion of one particular protein can have wide-ranging consequences at different moments, in different cell types, and conceivably, in different subcellular compartments within the same cell at the same time.

This necessitates a much more detailed study of how proteins with a diverse array of functions such as OPHN1 perform those functions at specific times and locations. In recent years, there has been progress in single-molecule imaging of processes in live cells (**Gebhardt et al., 2013; Wang et al., 2015**). Such approaches may in future be useful to disentangle the exact function of proteins like OPHN1 at specific times in specific cell types and locations.

Clinical implications of age-dependent phenotypes

Neurodevelopmental disorders pose a complex problem. Although the age at which most NDDs are diagnosed can differ significantly (**Marín, 2016**), many may have their origins at much earlier ages, before symptoms appear. To make matters more complicated, as

suggested by our data, homeostatic mechanisms may work to counteract the initial perturbation. However, homeostatic mechanisms by themselves may not be able to fully rescue the phenotypes. And even if they do rescue the phenotype at the synaptic level, the damage may already be done when it comes to the formation of the neuronal circuitry. Thus, it is important to take the timing of the intervention into account when devising therapeutic strategies. For one thing, drugs that might potentially be used as therapeutics may have different effects depending on the age of the subject. Even the outcome genetic reinstatement of the affected gene in the case of a single-gene mutation may be severely affected by the timing of the intervention. For example, reinstatement of maternal *Ube3a* in a model for Angelman Syndrome is effective when done in the embryo, but only partially rescues certain phenotypes when performed in juvenile animals, while it is ineffective in the adult (**Silva-Santos et al., 2015**).

In the case of Oligophrenin-1, there have been two drugs that have been used in preclinical studies to rescue certain phenotypes in the mouse model. These are Y27632, which inhibits Rho-associated protein kinase (ROCK), a downstream target of RHOA, and fasudil, which inhibits RHOA and ROCK. In acute slices, short application of Y27632 have been used to rescue the deficiencies in neurotransmitter release in *Ophn1*$^{-/y}$ neurons. However, short application did not rescue dendritic spine phenotypes (**Powell et al., 2012**). Fasudil has been used *in vivo* to rescue recognition memory and spatial learning deficiencies in adult mice (**Meziane et al., 2016**). These results are encouraging and imply that treatment in adult patients may alleviate symptoms. One must keep in mind, though, that the cognitive tasks for which performance was improved by fasudil are relatively simple. They are also tasks that the mice were trained to perform as adults, and therefore apply to the capacity for learning and plasticity in the adult brain. They do not represent any capabilities that are learned during development.

Translation to the human condition may therefore not be straightforward. Administration of fasudil in adult patients may alleviate some symptoms, but may not rectify the more complex traits affected by the disorder, such as language and social skills (**Santos-Rebouças et al., 2014**). Language acquisition in particular has been shown to undergo sensitive periods (**Kuhl, 2010**). It would be interesting to investigate certain forms of behaviour that are dependent on proper network formation during development, to assess whether models for NDDs exhibit phenotypes in those behaviours, and to test whether therapeutic intervention in the adult can rescue the symptoms, or if intervention during development is necessary.

Recent evidence would suggest that the latter might be the case. Preclinical studies that include interventions have shown that early interventions are often more successful than those performed at later ages. For example, administration of bumetanide at an early age effectively prevented structural brain damage and normalised adult behaviour in a genetic model for epilepsy (**Marguet et al., 2015**). In a separate study, administration of

mGluR5 antagonist MPEP was more effective during development than in adult mice in rescuing the dendritic spine phenotype in *Fmr1* knockout mice (**Su et al., 2011**). This latter study is particularly interesting, as mGluR5 is an important therapeutic target in Fragile X Syndrome (**Gross et al., 2012**).

Recently, clinical trials were performed in FXS patients, using drugs that target mGluR5 function. Unfortunately, these have so far proved ineffective (**Berry-Kravis et al., 2016; Jeste and Geschwind, 2016**). One explanation for the disappointing results of these clinical trials may be that they were conducted mainly in adolescent or adult patients. According to the theoretical framework put forth in this thesis, the intervention may simply come too late. Thus, while some symptoms may be alleviated by therapeutics at this time, the damage may already be done at this age when it comes to important developmental milestones of circuit development. Therefore, the major dysfunctions apparent in the disorder would not be affected by treatment that comes too late. Earlier intervention may provide better treatment outcome in future trials, as has been suggested elsewhere (**Jacquemont et al., 2014; Marín, 2016**). Indeed, a clinical trial in children aged 3 to 6 is currently underway (see https://clincaltrials.gov/, identifier number NCT02920892; "AFQ056 for language learning in children with FXS").

mGluRs in human networks

Another caveat of these clinical trials is that they are based entirely on the results of preclinical animal studies. The most prevalent theory regarding the pathophysiology of FXS is known as the mGluR theory of Fragile X. This theory states that loss of FMRP leads to exaggerated mGluR-dependent LTD. This in turn interferes with synapse development and maturation, which disrupts development of neuronal circuits (**Bear et al., 2004**). However, although a mechanism that is crucial to this theory – that activation of mGluRs leads to LTD of excitatory synapses onto pyramidal cells – has been extensively described in mice, it has not been replicated in human cortex. This is mainly due to the need for living human brain tissue, which is often not readily available. So far, only one type of excitatory synapse onto human basket cells has been shown to exhibit mGluR-mediated LTD (**Szegedi et al., 2016**).

We show here that excitatory synapses onto pyramidal cells undergo mGluR-LTD. This is a first step in validating the mGluR theory of Fragile X in humans. Whether or not mGluR-mediated LTD is affected in FXS patients as it is in the mouse model remains an open question, as it is currently not feasible to obtain cortical tissue from FXS patients on which electrophysiological experiments can be performed. One option is to use induced pluripotent stem cell (iPSC) technology to culture induced neurons from FXS patients (**Halevy et al., 2014; Telias et al., 2015**). These can then be used to assess whether FXS patients show changes in mGluR function compared to healthy controls. This technique is not perfect, as the neurons obtained this way are grown in culture. It therefore disregards

the developmental aspects of the disorder. Nevertheless, it is a useful tool for further validation of the effect of FXS on mGluR function in human neurons.

An aspect of mGluR function that is often overlooked is its effect on synaptic inhibition. mGluRs can have various effects on synaptic signalling. Notably, activation of group I mGluRs increases the frequency of inhibitory events in the cortex of rodents (**Chu and Hablitz, 1998; Zhou and Hablitz, 1997**). This is due to direct activation of certain types of interneurons, particularly O-LM neurons in the hippocampus (**McBain et al., 1994; Van Hooft et al., 2000**), and their counterparts, Martinotti cells, in the cerebral cortex (**Beierlein et al., 2000; Cosgrove and Maccaferri, 2012**).

Here, we show that a similar mechanism operates in the adult human cortex. Martinotti cells are depolarised directly by group I mGluR activation in the absence of excitatory neurotransmission, and subsequently provide increased inhibition to neighbouring pyramidal cells and fast-spiking basket cells.

Dysfunction of inhibitory signalling in FXS has not been the topic of much research and has only recently begun to attract attention (**Cea-Del Rio and Huntsman, 2014**). Interestingly, the activation of Martinotti cells by group I mGluRs and subsequent increase in synaptic inhibition has been shown to be reduced in the *Fmr1* knockout (**Paluszkiewicz et al., 2011b**). The presence of this effect of mGluRs in human cortex highlights the need for more research into the role of inhibitory signalling in FXS. It also implies that drugs that affect mGluR function may have unintended consequences for inhibitory transmission, in addition to their intended effect on excitation. It is therefore crucial to also take GABAergic inhibition into account when designing therapeutic strategies.

CONCLUSION

In this thesis, I have advanced several concepts relating to development of the cerebral cortex and aberrant development in neurodevelopmental disorders. First, a detailed overview is provided of the development of cellular morphology, intrinsic physiology, and synaptic connectivity of pyramidal cells in layers 3 and 5 of the mouse medial prefrontal cortex. These data show that pyramidal neurons in both cortical layers show largely concurrent development of morphological and intrinsic physiological properties, but are differentially integrated into the cortical circuitry. Next, the effect of *Ophn1* deficiency on the development of the medial prefrontal cortex was investigated. Absence of OPHN1 caused only minor alterations in cellular morphology, but affected both excitatory and inhibitory synaptic transmission in a manner that was dependent on both age and cortical layer. These data have important clinical ramifications, and suggest that temporary synaptic phenotypes may disrupt network formation, leading to behavioural deficits later in life. Finally, we show that activation of group 1 mGluRs not only causes LTD in human excitatory synapses, but also elicits strong synaptic inhibition throughout the network in human cortex. This provides experimental validation of current therapeutic strategies in humans that are based on mGluR-mediated LTD. On the other hand, our results provide a caution that when developing therapeutics targeting mGluRs, one should take into account their potential effect on synaptic inhibition.