Dissecting the role of Gpr158 in the brain

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CHAPTER 6

General discussion

Summary and scope of the discussion

Gpr158 is an orphan G protein-coupled receptor that has been implicated in cognition, depression, ocular hypertension and cancer progression[18,20,22,194,230]. The expression of Gpr158 throughout the body and its unusual extracellular N-terminal protein structure make it an interesting candidate for functional exploration in different fields. In this thesis, we focused on the role of *Gpr158* in neuronal development, synapse formation, synaptic transmission, learning and memory. Furthermore, I investigated the interactome profile of Gpr158 in specific brain regions and the synaptic proteome changes in *Gpr158* KO mice. Moreover, I have also put great emphasis on the deorphanization process of Gpr158.

In chapter 2, I used *Gpr158* KO mice as a model to investigated the role of *Gpr158* in learning and memory using multiple behavioral paradigms, including the Morris water maze, fear conditioning and passive avoidance. Electrophysical analyses were carried out to assess the physiology of CA1 pyramidal neurons in the context of the observed learning and memory deficits in absence of *Gpr158*. I also explored the role of *Gpr158* on neuronal morphology both *ex vivo* and *in vitro*. In chapter 3, I report on the interactome profile of Gpr158 in hippocampus and striatum regions and the proteome changes in hippocampal synaptosomes of *Gpr158* KO mice. Furthermore, I investigated the involvement of *Gpr158* in reversal learning. In chapter 4, I analyzed the role of *Gpr158* on synapse formation and density in primary hippocampal neurons. In chapter 5, I performed a ligand screening analysis aimed at deorphanization of Gpr158.

In this chapter, I will discuss the implication of these findings in light of previously reported studies and I will consider future research directions for Gpr158.

***Gpr158* in neuronal development and synapse formation**

Knock-down & knock-out of Gpr158 *induces alteration of dendritic architecture in primary hippocampal neurons and CA1 pyramidal neurons*

Proper dendritic development is key to proper signal transmission in neuronal circuitry. Hence, disrupted dendritic morphology has been reported in several neurological and neurodevelopmental disorders and is also found correlated with reduced cognitive ability[30,31,112]. Many adhesion GPCRs (aGPCRs) have been identified as important players in dendritic development (chapter 1). In line with this, the N-terminal protein domain of Gpr158, characteristic for aGPCRs, pointed out a possible role of *Gpr158* in neuronal development. Therefore, in chapter 2 we investigated whether *Gpr158* is involved in dendritic development *in vitro* and *in vivo*. Interestingly, *Gpr158* KD in the early stage of primary hippocampal neuronal outgrowth (at DIV1) led to an extreme reduction of neuron viability. Similar results were obtained using primary hippocampal neurons from *Gpr158* KO mice, indicating that Gpr158 has a role early-on in neuronal development *in vitro*. At a later stage of development

in vitro, *Gpr158* KD showed a more limited reduction in neuronal survival. Morphological analysis of surviving neurons revealed that *Gpr158* KD induced impairment in dendritic development. In parallel to these *in vitro* observations, we found similar results *in vivo* after morphological reconstruction analysis. *Gpr158* KO mice showed impaired branching in the apical part of pyramidal neurons in the CA1 area of the hippocampus. Interestingly, using a quantitative proteomic approach we found support for the role of *Gpr158* in neuronal development, as we observed the dysregulation of neurodevelopment-related proteins in the synaptosome fractions of *Gpr158* KO mice (chapter 3). Recently, it was shown that Gpr158 regulates spine size and density in the CA3 area of the hippocampus[23], however it is unknown if Gpr158 has similar effects in the CA1 area as well. Currently, the precise molecular mechanism of the observed dendritic impairment in absence of *Gpr158* remains to be determined. Since BDNF levels are found reduced in *Gpr158* KO hippocampus[18] and BDNF is an important factor in dendritic development[231,232], BDNF might play a role in the Gpr158-affected dendritic development. In our screening assays in HEK293T cells, we found that Gpr158 can couple with $G\alpha_{q/11}$ protein and induce NFAT RE activation (chapter 5). Considering that NFAT plays a role in neuronal survival and axonal growth[204,233] and BDNF can regulate NFAT activation in cultured hippocampal neurons[208], it is well possible that *Gpr158* and BDNF might work via NFAT signaling and lead to the observed morphological alterations.

Taken together, we identified *Gpr158* as an important player in dendritic development using both *in vitro* and *in vivo* approaches. We found a correlation between reduced architecture of CA1 pyramidal neurons and MWM learning deficit (chapter 2). In future studies, it needs to be determined whether the role of *Gpr158* on dendrite development is similar in the different subregions of hippocampus and what mechanism is behind this. Understanding the mechanisms of how morphological alterations in dendrites of different hippocampal subregions come about may help to better understand the learning and memory impairments in *Gpr58* KO.

Gpr158 modulates synapse formation in primary hippocampal neurons

Synapse formation is a complex process that needs presynaptic transmitter release and formation of the postsynaptic density to form functional synapses. Various types of cell adhesion molecules (CAMs) have been identified as players in this process[234,235]. To investigate the role of CAMs in synapse formation, artificial synapse formation assays have been used. However, these assays provide information on whether the protein of interest can mediate synapse formation rather than identifying the precise function of the proteins in the synapse formation process[178]. The interaction of Gpr158 with its newly identified interactor GPC4 was recently reported to regulate the presynaptic differentiation in primary hippocampal

neurons and shown to require the presynaptic LAR as a co-receptor[23]. In addition, these authors also showed that synapse density was increased, and the area of synapse density was reduced in mossy fiber MF-CA3 synapses in the absence of Gpr158[23]. We confirmed the function of Gpr158 on synapse formation using a similar co-culture assay. In addition, we investigated synapse density in primary culture upon *Gpr158* KD, and found a reduced density of post-synaptic protein 95 (PSD-95) puncta per neurite length leading to reduced synapse density (with required co-occurrence of pre-and post-synapse) in hippocampal neurons. This observation implies that in addition to the presynaptic LAR requirement, the inability to properly initiate post-synaptic development due to absence of or reduced signaling in the post-synaptic compartment might play a role in the full functional role of Gpr58 (see chapter 4). Together, these findings indicate that Gpr158 takes a specific role in synapse formation in addition to that in dendritic development.

Gpr158 in synaptic transmission

Normal basal synaptic transmission and increased excitability in CA1 pyramidal neurons of Gpr158 KO mice

Gpr158 has been implicated in synaptic transmission in the CA3 hippocampal area and the layer 2/3 of mPFC[18,20,23]. In the CA3 area, it was shown that sEPSC were reduced in *Gpr158* KO mice[23]. These authors also reported an increase in spine density on the apical dendrites of the SL CA3 region but not on the SR dendrites, indicating that input-related changes occur specifically in CA3 apical dendrites in *Gpr158* KO[23]. Furthermore, impaired paired pulse ratio (PPR) and LTP were observed in the MF to CA3 pathway in *Gpr158* KO[18,23]. In the mPFC L2/3, increased spine density and sEPSC frequency have been reported in *Gpr158* KO mice[20]. The absence of *Gpr158* did not affect PPR but induced synaptic strength through an increase in the AMPAR/NMDAR ratio[20].

Given the robust changes in neuronal architecture in CA1 pyramidal cells, we performed electrophysiological measurements of these neurons to explore possible effects of *Gpr158* on cell excitability and synaptic transmission (chapter 2). In parallel with the observed morphological alterations in dendritic arbors, we found increased action potential frequency and input resistance while having a more depolarized resting membrane potential. Increased action potential frequency and input resistance have been previously found to correlate with disrupted function of pyramidal dendrites[112]. Although, we detected increased excitability in CA1 pyramidal cells, basal sEPSC and sIPSC were not affected in absence of *Gpr158*. However, in the CA3 to CA1 connection, we observed a reduced Schaffer collateral (SC) drive onto CA1 pyramidal neurons in *Gpr158* KO without a change in the presynaptic release machinery.

Hence, both our findings and those in literature show that *Gpr158* plays a role in synaptic transmission in an input- and a region-specific manner. The observed changes in *Gpr158* KO mice in the different brain regions and neuronal pathways (i.e. MF-CA3, SC-CA1) might be caused by impaired neuronal morphology (dendritic or spine morphology) and/or altered synaptic signaling mechanisms (including the synaptic function of *Gpr158*). The reduced sEPSCs in CA3 with no changes in CA1 in *Gpr158* KO mice raise the possibility of a compensation mechanism in the hippocampus circuitry for maintaining synaptic transmission. Since full *Gpr158* deletion has a different impact on the mPFC region, studies with a conditional knock-out of *Gpr158* in brain regions of interest may be performed to clarify the specific roles of *Gpr158* on synaptic transmission. Finally, the *Gpr158* KO effect on spine morphology and LTP may well be in accordance with the observed learning and memory deficits in these mice.

Gpr158 in learning and memory

Spatial learning and memory, reversal learning and extinction of safety learning are impaired in Gpr158 KO mice

Considering the role of *Gpr158* in neuronal morphology and synaptic transmission in hippocampus, we tested *Gpr158* KO mice in hippocampal-dependent behavioral paradigms to explore its impact on learning and memory (chapter 2). The learning deficiency of *Gpr158* KO mice in the MWM task was previously reported by Khrimian *et al*., however without assessing the LTM with a probe trial[18]. In our study, *Gpr158* KO mice showed LTM impairment when assessed using the probe trial. Furthermore, we found a correlation between reduced dendritic arborization of CA1 pyramidal neurons and MWM learning deficits. We also reported on memory performance in *Gpr158* heterozygous mice in the MWM task and showed absence of an acquisition deficit in the presence of a probe-deficit. The milder phenotype of heterozygous mice compared with homozygous *Gpr158* KO mice implies that reducing Gpr158 expression levels affects spatial memory.

In our study, we also measured contextual memory acquisition and expression using cFC and PA tests. We did not find context-specific memory deficits in *Gpr158* KO mice neither in cFC nor PA. However, we found a decrease in acquisition of a safe memory for *Gpr158* KO mice in PA. It has been reported that *Gpr158* knockdown using shRNA targeting *Gpr158* in the dorsal hippocampus induces context-specific memory deficits in mice[18]. These findings raise the question whether conditional absence of *Gpr158* has a different impact on context-specific memory. The observed impaired synaptic transmission in the CA3 area and reduced SC-mediated response on CA1, together with the alterations in dendrite morphology suggest that disturbed information flow in the hippocampal circuity might cause the spatial learning deficiency and impaired safety learning in *Gpr158* KO mice.

Since a robust anxiety phenotype has been found previously for *Gpr158* KO mice[18], we measured the anxiety level of *Gpr158* KO mice in the open field test, as well as in the fear conditioning task prior to conditioning. In contrast to what was previously reported[18], we did not observe an anxiety-like phenotype for *Gpr158* KO. Such differences could be related to the genetic background of the mice used (chapter 2).

Gpr158 has been associated to depression-like behavior where increased *Gpr158* expression in the PFC was observed after physical stress in mice[20]. Given that a considerable level of *Gpr158* expression exists in striatum and PFC[15,18,20] and these brain regions are involved in behavioral flexibility[143], we measured reversal learning (RL) performance of *Gpr158* KO mice using a reward-based discrimination task and reported RL deficits in *Gpr158* KO mice (chapter 4). We concluded that *Gpr158* plays a role in this form of learning in addition to being involved in spatial learning and memory. However, this potential role of Gpr158 in behavioral flexibility should be further investigated in different types of reversal learning tests and supported by molecular measurements of Gpr158 expression in different brain regions.

Although *Gpr158* has become an important player in cognition and depression, the contribution and the extent of the involvement of *Gpr158* in specific brain regions in the observed behavioral phenotypes remains to be determined more precisely. In future studies, the altered synaptic transmission and dendrite morphology and the correlation of these with cognitive functions might be examined using *Gpr158* conditional KD mice.

Gpr158 deorphanization

Gpr158 regulates G protein-related signaling in HEK293T cells

Gpr158 has been linked to osteocalcin (OCN) regulation in cognition[18]. In the same study, it has been shown that pull down of $G\alpha_q$ protein with biocytin-labelled OCN was reduced in the hippocampal membrane fraction of *Gpr158* KO mice[18]. This was the first evidence that pointed to the possibility of Gpr158 coupling with $G\alpha_q$ protein, however without any evidence for G-protein signaling. In chapter 5, we provided first evidence that Gpr158 indeed couples to $G\alpha_{q/11}$ protein and induces NFAT RE activation in HEK293T cells. This activation was induced in a non-classical manner and did not include IP_3 accumulation in the functional screening assay. Recent research has demonstrated that *Gpr158* is involved in stress-induced depression by modulating cAMP signaling together with RGS7 in a non-canonical mechanism in the mPFC, highlighting the link between Gpr158 and $G\alpha_{i/o}$ signaling and $G\beta\gamma$ non-canonical signaling pathways[20,22]. Consequently, Gpr158 might be involved in different G protein-related signaling pathways in a tissue- or cell-specific manner. Further functional screening is needed to determine the neuronal intracellular pathway changes and the possibility of crosstalk between G protein-signaling for Gpr158.

The potential ligand(s) of Gpr158 present in the brain

Until recently, molecular and functional characterization of Gpr158 has been investigated without a known endogenous ligand. Previously, OCN has been suggested as a ligand for Gpr158[18]. Recently, Gpr158 has been found as a binding partner for the heparan sulfate proteoglycan glypican 4 (GPC4). GPC4 is enriched on hippocampal granule cell axons. Gpr158 is post-synaptically expressed and restricted to the proximal segment of CA3 apical dendrites. Gpr158-induced presynaptic differentiation in contacting axons requires cell-surface GPC4 and the co-receptor LAR[23]. However, in both studies, the authors did not investigate the activation of Gpr158 at the level of G protein-related signaling.

In chapter 5, we investigated the endogenous ligand(s) of Gpr158 in mouse brain using a tissue-extract based reverse pharmacology approach, which is widely used to find previously non-identified ligands[13]. We used both proteomic- and lipidomic-based approaches for ligand preparation and a functional G-protein signaling assay for activity detection in the presence or absence of candidate ligands. Using both approaches, we suggested that Gpr158 might have multiple ligands (peptide and lipid-like) in the brain and induces NFAT RE activation. Although, we were able to detect NFAT RE activity in the presence of potential ligands, we were not able to identify neither peptide nor lipid-like candidates in existing proteomic and lipidomics databases. In addition, testing for Gpr158 activation by OCN did not yield a positive effect. This implied that the candidate ligands of Gpr158 were not a classical neurotransmitter or biological compound that has been identified previously.

The non-successful identification of Gpr158 ligands with MS-based approaches indicated that other approaches (e.g. NMR-based) could be useful. Also, alternative ligand preparation protocols, input amount and sample source (other species) might need to be considered. Based on chemical structure predictions derived from our lipidomic-based approach, we in the end suggested the modification of the DDM detergent –used for sample preparation– to activate Gpr158 in the functional assay. Given that the increased NFAT RE activity related with modified DDM was only detected in tissue included fractions, a proper screening for the endogenous ligand might need the use of another type of detergent in sample preparation and subsequent assays. Collectively, we provided information for the further investigation of the Gpr158 deorphanization process (i.e. ligand preparation protocol, G protein-related activity screening assay and the search of Gpr158 ligands) and points of consideration that have to be taken in account for new attempts. Of course, the question remains whether Gpr158 still has an unknown ligand or whether GPC4 and its co-receptor can explain all its synaptic signaling.