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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Localization of DISC1 expression in the gut wall by immunofluorescence

Claudia Feldkamp

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :

Prof. dr. Werend BOESMANS

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Localization of DISC1 expression in the gut wall by immunofluorescence*

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*Running title: *DISC1 expression in the gut wall*

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ABSTRACT

Several mental disorders in which perturbed expression of the scaffold protein DISC1 plays a role like schizophrenia or autism spectrum disorder are frequently accompanied by gastrointestinal issues. The reason for this, however, remains to be elucidated. Preliminary data indicate that DISC1 is not exclusively expressed in cells of the central nervous system but also in the gut. Its expression here might explain why these mental disorders are linked to digestive issues. However, nothing was known about its exact location of expression and its functions in the gastrointestinal tract. To answer these questions, DISC1 expression levels were measured in wild-type mice and its expression was localized in the intestinal wall by means of immunofluorescence. DISC1 expression was observed in myenteric plexus-associated macrophages. These macrophages are located in close proximity to the enteric nervous system and can influence gastrointestinal functions via communication with enteric glia and enteric neurons. A perturbed expression of DISC1 as seen in some patients with mental disorders may thus not only be responsible for the mental problems seen in these patients but also for their digestive problems.

INTRODUCTION

Enteric nervous system - The enteric nervous system (ENS) is a network composed of millions of neurons and glial cells located in the wall of the gastrointestinal tract (1). Here, it plays an essential role in a variety of processes in the gut, such as digestion, absorption, passage of content, and gut homeostasis. The ENS influences these processes by regulating gut motility, secretion, and blood flow. Furthermore, it also interacts with the immune system, and with the gut-microbiota. Moreover, it is also involved in the microbiome-gut-brain axis (1-3). The ENS operates autonomously but also receives extrinsic inputs from the central nervous system (CNS) (1). By increasing motility and secretion to expel noxious agents and via neuro-epithelial and neuroimmune interactions, the ENS is also involved in host defense (1-3). Currently, many aspects of ENS function remain to be elucidated. Especially its interactions with the microbiome and immune system are not fully understood yet (1). The multiple functions of the ENS make it an essential part for intestinal homeostasis. Faults in wiring, such as seen in neurodevelopmental disorders can have serious consequences on gut functionality (1,4).

Organization of the ENS - Previously, neurons were thought to be the only active cells in the ENS which has led to a too neurocentric and inadequate understanding of enteric reflexes that control intestinal functions. However, more recent studies revealed that glia are also essential for the proper functioning of the ENS (5-8). The ENS is organized in enteric neural circuits consisting of intrinsic sensory neurons, excitatory and inhibitory interneurons, motor neurons, and enteric glia. These are organized in two ganglionated and interconnected plexus layers and form a wired network (1,9). One of them, the submucosal plexus, plays an essential role in controlling water and electrolyte secretion, and blood flow. This plexus is located beneath the mucosal epithelium lining the lumen. The myenteric plexus is located between

circular and longitudinal muscle layers and is mostly responsible for regulating motility (1,10). In the most basic enteric circuit, a sensory neuron either directly synapses onto an excitatory or inhibitory output motor neuron or synapses via an interneuron. However, glial cells form a fourth essential component of this circuit as they actively influence various gut functions such as intestinal motility and secretion as well (1,11,12). For instance, several developmental, degenerative, and inflammatory disorders of the nervous system have been associated with inadequate glial cell function which indicates that enteric glia are essential during embryogenesis and in postnatal life (5-8). Thus, enteric glia are a lacking component in the current models of regulatory mechanisms in the ENS as manipulation of enteric glia has been shown to significantly affect gut functions (9,13). Reflexes in the gut and the communication between extrinsic and intrinsic neurons are regulated by fast, bi-directional communication between enteric glia and enteric neurons (14-18). However, many important aspects of basic glial functions and how they are involved in regulatory mechanisms still remain to be elucidated (9).

Bi-directional communication between enteric glia and other cells - A fine-tuned crosstalk between enteric glia and enteric neurons is not only essential for the functioning of the ENS, but also for the communication between the ENS and other cell types in and outside the gastrointestinal tract (9,19,20). This bi-directional crosstalk of enteric glial cells is however not limited to neurons but occurs with all the different cell types that are present in the gut wall. For example, enteric glia seem to have a major impact on integrity and function of the intestinal epithelium (21). Studies revealed that enteric glial cells can influence cell adhesion, metabolism, and motility by modulating gene expression in epithelial cells (21,22). In addition, immune and inflammatory responses are regulated by the crosstalk of enteric glia with local and recruited immune cells (21,23-25). Lastly, studies suggest that during pathophysiological states, enteric glia contribute to a bi-directional communication between the periphery and the brain. The mechanisms regulating the glial crosstalk are, however, not yet understood (14).

Different subtypes of enteric glial cells - Enteric glia form a diverse population of cells in the gastrointestinal tract. Depending on their function and location within the intestinal wall enteric glia display a different phenotype that is altered by the physiological status of the ENS as well. Based on these features, enteric glia can be divided into four different subtypes that correspond to different locations within the plexus and extra-ganglionic spaces and display differences in the morphology of their processes (1,5). **Type I** enteric glia, also referred to as astrocyte-like glia due to similarities in morphology, display irregular, highly branched processes that extend to all directions and often terminate with "end-feet" like structures. These glia are located in the ganglia of the myenteric (type I_{MP}) and submucosal (type I_{SMP}) plexus and their processes often contact multiple neurons within the same ganglion (5,14). **Type II** enteric glia, located within or at the edge of interganglionic connectives, are characterized by lanky processes, mostly emerging from one side of the cell. Their processes run in parallel to each other and to the neuronal fibers of the interganglionic strands and contact most neural fibers (5,14). **Type III** enteric glia have four major plexuses with secondary branching that often wrap around small blood vessels. These glia can be found outside the ganglia and interganglionic connectives at the level of the myenteric and submucosal plexus, referred to as type $III_{MP/SMP}$. Besides this they can also be found in the lamina propria of the mucosa, then referred to as type III_{mucosa} (5,14). Lastly, **type IV** enteric glia are characterized by a bipolar morphology and can be found along nerve fibers within the circular and longitudinal smooth muscle layers (5,14,26,27). The three different subtypes localized in the myenteric plexus can furthermore be distinguished at the functional level as well, based on their different responses to adenosine triphosphate (5). Findings from previous studies indicate that all subtypes of enteric glia are most likely derived from a common progenitor which can generate different glial subtypes based on its final location and the physiological context (5). Moreover, it seems likely that glial cells cannot exclusively acquire different properties during embryonic or postnatal stages but keep this capability in adult stages. This indicates that the level of phenotypic plasticity of the ENS is higher than what was previously assumed (5). Enteric glia display various expression patterns of neurochemical markers such as glial fibrillary acidic protein (GFAP), S100 calcium-binding

protein β (S100β), and Sox10, however, there is no unique pattern that is specific to one of the four morphological glial subtypes (5). More recently, there has been evidence that enteric glia can be subdivided into seven distinct classes based on the results of single-cell sequencing studies (28). However, it is not clear yet how their morphological features, spatial distribution, and genetic profile influence function (1,28).

Plasticity - Enteric glia do not form a uniform population of cells but display an extensive heterogeneity and phenotypic plasticity. Depending on various factors in the microenvironment such as nutrition, microbiota, mechanical factors, or pathological factors enteric glia can adjust their phenotype and molecular characteristics. The majority of glia localized in the myenteric plexus express GFAP, S100β, and Sox10 while up to 80% of the glia localized outside of the myenteric ganglia do not express these markers. This is most likely due to dynamic gene regulation as lineage tracing experiments have revealed. All populations of enteric glia derive from Sox-10-expressing progenitors, moreover, expression of typical marker genes in enteric glia lineages can be very dynamic (5). It is suggested that the expression of unique combinations of markers in distinct subsets of enteric glia is due to differences in signals from their surrounding environment and/or due to functional specialization (5).

Development of the enteric nervous system - The ENS of vertebrates is majorly derived from the vagal crest which consists of two different subpopulations of ENS precursors. Cells bordering upon somites 1 and 2 form one of these subpopulations and are more relevant for the formation of autonomic ganglia in the esophagus and stomach. Cells bordering upon somites 3-7 represent the second subpopulation and contribute to the formation of sympathetic ganglia (14,29). Enteric glia share a common developmental pathway with enteric neurons. Both cells derive from enteric precursor cells expressing e.g., the transcription factor Sox10. They extensively proliferate while colonizing the gut from rostral to caudal (14,30,31). A changed expression of transcription factors leads to differentiation into neurons and glia (14). The migrating neural crest precursor cells consist of several subpopulations. Fate-restricted precursor cells for glia have a variable proliferative capacity, while fate-restricted precursors for neurons have a limited proliferative potential. Moreover, there are bipotential progenitors that can give rise to neurons as well as glia (14,32). Together, these precursor cells are capable of forming all existing subtypes of enteric glia and multiple subtypes of enteric neurons (14,33). The development of the ENS continues after birth and is influenced by the immune system and gut microbiota (14,34-36). In mice, initial colonization of the myenteric plexus happens around E12.5. At this stage, myenteric glia form and lead to the development of submucosal and mucosal glia (14,32). Colonization of the mucosa with enteric glia, for which the gut microbiota is essential, starts during suckling period and finishes after weaning (14,34). In contrast to this, gliogenesis in the myenteric plexus is low under steady conditions, however, it increases in certain circumstances such as inflammation, injury, or exercise (14,37,38).

Crosstalk between macrophages and the ENS – Enteric macrophages are a highly heterogeneous population of phagocytes and have supportive functions during tissue development, homeostasis, and regeneration. Macrophages are located in multiple different layers of the gut wall and depending on their location perform niche-specific functions (39). Lamina propria intestinal macrophages, for example, are involved in barrier integrity and host defense as well as the maintenance of regulatory T-cells (39). Intestinal muscularis macrophages mainly accumulate in layers between the serosa and the longitudinal muscle layer, between longitudinal and circular muscles, as well as between outer and inner muscles (40). These muscularis macrophages are generally less defined than the macrophages situated in the mucosa. They are not only involved in phagocytosis and antigen-presentation but are also known to communicate with enteric neurons. This crosstalk is furthermore influenced by signals from the intestinal microbiota and can control gastrointestinal motility (40). Less is known about the communication between enteric glia and macrophages. However, macrophages situated in the muscularis layers are often compared to microglia in the CNS while enteric glia are frequently compared to CNS-astrocytes due to similarities in function and gene expression profile. For microglia and astrocytes, bi-directional communication is well-defined and determines the

functions of both cell types. Some studies suggest that enteric glia and muscularis macrophages communicate via similar mechanisms and by this, influence the phenotype of glia and macrophages as well as the function of enteric neurons (41). Myenteric plexus-associated macrophages form one of the macrophage populations located in the muscularis. These macrophages located in in close proximity to the ENS and appear to be involved in the crosstalk between mucosa-associated lymphatic gut tissue and the ENS (42).

Possible involvement of DISC1 in the formation of enteric neural circuits – In the CNS the scaffold protein Disrupted in Schizophrenia 1 (DISC1) which is expressed in neurons and glial cells is known to play a role in neurodevelopment (43). Moreover, it is involved in several different processes in the CNS such as neural migration, dendritic arborization, neurogenesis, and synaptic plasticity by interacting with numerous molecules including signaling proteins, RNA binding proteins, microtubule-associated molecules, and motor molecules (44). Here, disruption of DISC1 results in problems in neural circuit formation, perturbed proliferation, and impaired migration of cells. Furthermore, DISC1 disruption is associated with various mental disorders such as schizophrenia, autism spectrum disorder, depression, and bipolar disorder (43,44).

To this point, nothing is known about the potential expression and functions of DISC1 in the ENS, however, preliminary data indicate an expression of DISC1 in enteric glial cells (28). Questions regarding its functions here and possible consequences of DISC1 disruption in enteric glia remain to be elucidated. Astrocytes, which represent one type of glial cells in the CNS are involved in the formation of neural circuits and DISC1 has been found to be involved in this process (43). DISC1 in enteric glia may have similar functions as in astrocytes due to the resemblance of astrocytes and enteric glia. For example, DISC1 in astrocytes is involved in dendritic and synaptic maturation. This was revealed by coculturing wild-type primary neurons with DISC1 mutant astrocytes. Here, a decrease in elaborated dendritic arborization was observed. Under normal circumstances, DISC1 binds to serine racemase in astrocytes with the production of D-serine as a result. In astrocytes expressing mutant DISC1 this pathway was disrupted which caused the decrease in dendritic arborization and density of excitatory synapses (45). Due to the similarities of astrocytes and enteric glial cells expression of DISC1 in enteric glia may possibly play a role in the development of neural circuits in the ENS.

Interestingly, diseases associated with DISC1 disruption like schizophrenia or autism spectrum disorder are frequently accompanied by digestive problems such as constipation, diarrhea, or reflux, however, the reasons for this are not clear yet (46,47). DISC1 signaling in enteric glia might play a role in the formation of enteric neural circuits. A disruption could consequently lead to digestive problems and explain this association. The combination of this link and the involvement of DISC1 in neural circuit formation in the CNS makes it interesting to investigate the function of DISC1 in enteric glia.

Therefore, the long-term goal of this project is to gain better insight into regulatory mechanisms of ENS assembly, more specifically, the role of DISC1 in this process and how this affects gut functionality and potentially also brain function via the gut-brain-axis. We hypothesize that perturbing the function of DISC1 results in faulty enteric neuron wiring which subsequently leads to impaired gastrointestinal functions and an imbalanced gut microbiome. These imbalances may via the gut-brain axis additionally cause changes in the CNS and possibly contribute to the development of mental disorders. For this, DISC1 locus impairment mice, a model for schizophrenia, were used to assess the consequences on gut function and microbiome. As these goals and the hypothesis cannot be answered in the nine months of this internship, this thesis focuses on setting the first steps in this project, mainly studying the localization and the expression of DISC1 in the gut of wild-type mice by means of qPCR and immunofluorescence labeling.

EXPERIMENTAL PROCEDURES

Animals – For all experiments wild-type C57BL/6J mice were used. For qPCR experiments, these mice were aged 11 to 17 weeks. For immunofluorescence experiments including submucosa and mucosa, as well for immunofluorescence experiments using cryosections 7-week-old mice were used. Mice that were used for immunofluorescence experiments of

LMMP samples were aged 5-21 weeks. All mice were maintained in the animal facility of the Biomedical Research Institute (BIOMED) of Hasselt University according to the European Council guidelines. Animals were kept in standard cages with a constant room temperature of 22°C, a regular day/night cycle, and food and water supply.

qPCR sample preparation – For quantitative polymerase chain reaction (qPCR) tissue was either analyzed with mucosa and submucosa or after removing these layers. For analysis of gut tissue including the mucosa and submucosa gut tissue was cleaned with a syringe filled with 1x-phosphate-buffered saline (1xPBS) and was subsequently cut into 3 cm long pieces (3 cm of proximal small intestine, distal small intestine, proximal colon, and distal colon). These pieces were snap-frozen in liquid nitrogen and stored at - 80°C. For qPCR analysis of LMMP samples gut tissue was placed in ice-cold 1xPBS in Sylgard dishes (Sylgard 184 Elastomer, Dow Corning) and then pinned with pins (0.2 mm, FST). The tissue was opened along the mesenteric border and subsequently stretched and cleaned with 1xPBS. Then, the mucosa and submucosa were removed. Afterwards, the tissue was cut into 3 cm long pieces (3 cm of proximal small intestine, distal small intestine, proximal colon, and distal colon), snap-frozen in liquid nitrogen, and stored at -80°C.

qPCR – Samples were prepared as described above. RNA was extracted from snap-frozen samples using the RNeasy Mini Kit (Qiagen) and subsequently used for cDNA production, using the qScript cDNA SuperMix (Quantabio). Next, mRNA expression was measured by qPCR. cDNA was diluted to a concentration of 5 ng/µl. A master-mix (per sample 5µl SYBR Green, 1.9 µl RNase free water, 0.3 µl forward primer, and 0.3 µl reverse primer) was prepared. 7.5 µl master-mix and 2.5 µl cDNA (final concentration: 12.5 ng cDNA) were added to each well on a 96 well plate. Reactions were performed using an Applied Biosystems® StepOne™ Real-Time PCR System (Thermo Fisher Scientific). Each sample was measured in duplicate.

Immunofluorescence sample preparation – For analysis by immunofluorescence gut tissue was pinned (0.2 mm, FST) onto Sylgard dishes (Sylgard 184 Elastomer, Dow Corning) in ice-cold 1xPBS. An incision was made along the mesenteric border and the tissue was stretched and cleaned with 1xPBS. Then, the mucosa and submucosa were removed to isolate longitudinal muscle with adherent myenteric plexus. This step was skipped for analysis of samples with mucosa and submucosa. Subsequently, the tissue was lifted. Tissues were then washed in 1xPBS three times and fixed in 4% paraformaldehyde (PFA) in 1xPBS for 2h at room temperature (RT) on a shaker. After fixation, tissues were washed in 1xPBS three times for 10 minutes on a shaker.

Immunofluorescence - For immunostaining, samples of the small intestine and colon of adolescent WT mice aged 7 weeks were prepared as described above. First, tissue sections were washed 3x 10 minutes on a shaker. Tissue sections were then incubated in blocking solution (1% Triton-X PBS and serum) for 2h at RT, on a shaker. Per tissue 100 µl of blocking solution was used. After blocking, tissues were incubated on a shaker with primary antibodies (Table 1) diluted in blocking medium for two nights at 4°C on a shaker. Afterwards, tissues were washed in 1xPBS three times for 10 minutes and incubated in appropriate secondary antibodies (goat or donkey hosted Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647, Invitrogen and Jackson ImmunoResearch) diluted in blocking medium (1:1000) for 2h at 4°C on a shaker. Subsequently, tissues were washed three times on a shaker for 10 minutes in 1xPBS. Lastly, the tissue sections were mounted on glass slides with mounting medium (Immu-Mount, Thermo Scientific). Images were acquired with a fluorescence microscope (Leica Microsystems) and microscopy slides were stored at 4°C in the dark. Of each prepared tissue, images were taken in five different locations.

Table 1: primary antibodies used for immunofluorescence labelling.

Antigen	**Host**	**Dilution**	**Source**
S100β	Rabbit	1:10	Abcam
HuCD	Human	1:20,000	ThermoFisher Scientific
DISC1	Mouse	1:200 and 1:300	Santa Cruz Biotechnology
Iba1	Goat	6 µl/ml	Abcam

Cryosections for immunofluorescence – For immunostaining using cryosections, seven-week-old WT mice were used. First, the intestine was isolated from the mice and the outer fat line was

removed. Next, the gut was cleaned with ice-cold 1xPBS by using a syringe. Subsequently, the intestine was pinned onto Sylgard dishes (Sylgard 184 Elastomer, Dow Corning) in ice-cold 1xPBS with pins (0.2 mm, FST). Then, the tissue was fixed in 4% PFA in 1xPBS for 3-5h. After fixation, PFA was removed, and the tissue was washed in 1xPBS three times for 10 minutes. Subsequently, tissues were placed in a 30% sucrose solution overnight at 4°C on a shaker. The next day, tissues were washed three times for 10 minutes in 1x PBS and subsequently cut into short segments (approximately 1,5cm) and embedded in tissue glue (FSC 22 Clear, Leica microsystems). Embedded segments were snap-frozen in liquid nitrogen and stored at -80°C. Cryostat (CM3050 S, Lecia Microsystems) was used to cut slices into 50µm thick sections which were then placed on glass slides. After drying for at least 30 minutes the glass slides were stored at -80°C. For staining, tissue sections were surrounded using a PAP pen (Merck) to keep the solutions in their desired locations. Then, blocking solution (1% Triton-X PBS and serum) was pipetted onto the prepared slides and incubated for 2h at 4°C. Subsequently, blocking solution was removed with a pipette and primary antibodies were added to the slide (see Table 1). After 2 nights of incubation at 4°C, antibodies were removed with a pipette, and samples were washed 4x by pipetting 1xPBS onto the slides. Next, samples were incubated in appropriate secondary antibodies for 2-4 hours at room temperature. Afterwards, samples were washed 4x again and mounted. Images were acquired with a fluorescence microscope (Leica Microsystems) and microscopy slides were stored at 4°C in the dark. Of each prepared tissue, images were taken in five different locations.

Statistical analysis – For quantification of macrophages and DISC1 expressing cells, cells were counted with a minimum intensity of 40 arbitrary fluorescence units (AFU) and a maximum intensity of 200 AFU in Image J. Subsequently, based on the scale bar the total size of the image was measured in Image J and the number of cells per 10cm^2 was calculated. Statistical analysis was performed using JMP Pro 14 software. Data are represented as the median including the first and third quartile. Numbers of cells counted on immunofluorescent stainings were analyzed with the Mann-Whitney test. For qPCR results, the Kruskal-Wallis test was used. A p-value <0.05 was considered significant.

RESULTS

Quantification of DISC1 expression in the gut – In order to measure the mRNA expression level of DISC1 in the gut, qPCR experiments were carried out. For this, CT values in proximal and distal regions of the small intestine and colon of 11–17-week-old adult WT mice were compared. The rather high CT values indicate a rather low expression of DISC1 in the gut. Due to the low expression, in the next step the same experiments were repeated but now the mucosal and submucosal layers were removed during sample preparation to investigate whether this results in higher DISC1 mRNA levels. The rationale for this was possible enzymatic degradation of DISC1 by enzymes located in the mucosa and submucosa of the gut that are released during the RNA extraction processes. Also, the relative contribution of mRNA from myenteric neurons and glia would be increased. However, this unexpectedly resulted in an even lower expression of DISC1 as measured CT values are slightly increased compared to measurements including the mucosa and submucosa, though not at a statistically significant level (Fig. 1).

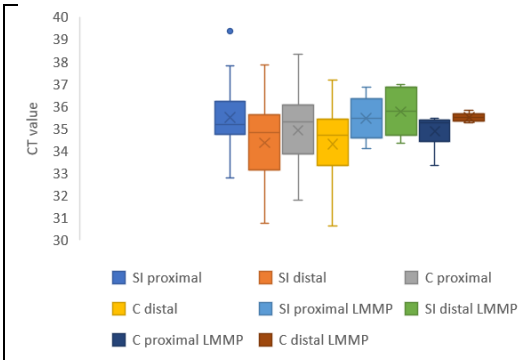

Fig. 1 – DISC1 is expressed in the small intestine and colon of wild type mice. Gene expression of DISC1 in different regions of the small intestine and colon was compared in 11–17-week-old wild-type mice with and without removal of the mucosa and submucosa. Measured CT values are shown. The line indicates the median, the box represents the 25th to 75th percentile, the maximum and minimum values are shown by the bars, and outliers are indicated as dots. Data were analyzed by Kruskal-Wallis test. SI: small intestine; C: colon

Localization of DISC1 expression – Since relative gene expression of DISC1 turned out lower after removing the mucosa and submucosa we next aimed to study whether expression of DISC1 can be visualized in these layers and to generally localize the expression of DISC1 by means of immunofluorescence labeling.

To first get a general picture of the ENS and test whether the staining methods are working, enteric glia and enteric neurons were labeled using antibodies for S100β and HuCD, respectively, in combination with DAPI to label the nuclei. As can be seen in Fig. 2, enteric neurons are surrounded by enteric glia that form multiple connections to each other and are in close contact with nerve cell bodies. Subsequently, enteric neurons, enteric glia, and DISC1 were stained in the small intestine (Fig. 3) and colon (data not shown) of adult wild-type mice. As can be seen on the image (Fig. 3e) DISC1 staining was not optimal since there was a high background signal which made it hard to identify DISC1 expressing cells. Thus, adjustments regarding the concentration of primary DISC1 antibody and incubation time are needed. Unexpectedly, when merging the images, DISC1 expression could neither be seen in enteric glia nor in enteric neurons. Instead, signals for DISC1 were detected in other cells close to the myenteric ganglia and within the smooth muscle layers (Fig. 3a).

As these cells had morphology reminiscent to that of intestinal macrophages, subsequently macrophages in adult wild-type mice were stained using an antibody for Iba1 in combination with a DISC1 staining (Fig. 4). Merging these images revealed that DISC1 is indeed expressed by macrophages as these two stainings were overlapping, both in the small intestine (Fig. 4a) and in the colon (data not shown). In order to increase the specificity of the DISC1 immunoreactivity, the concentration of primary antibody was lowered (from 1:200 to 1:300) and incubation time was increased to 48 hours instead of 24 hours. Moreover, the time of washing was increased. This resulted in a better signal for DISC1 and a reduction in background (Fig. 4c). Here, most but not all macrophages appear to express DISC1 as the number of Iba1 positive macrophages was slightly higher than the number of counted DISC1 positive cells, however, not statistically significant (Fig 4e).

Then, the relationship of DISC1 expressing macrophages with ENS cells was visualized in the small intestine (Fig. 5) and colon (Fig. 6). Stainings for DISC1 and macrophages largely overlap (Fig. 5a and Fig. 6a), confirming the expression of DISC1 by enteric macrophages. Moreover, enteric glia (Fig. 5b and Fig. 6b) were visualized as well. Merging these three stainings shows that the DISC1 expressing macrophages are located in close proximity to the enteric glia. Here, the number of DISC1 expressing cells was observed to be higher than the number of Iba1 positive macrophages indicating that DISC1 is not exclusively expressed in macrophages (Fig. 6e). However, this higher expression was not statistically significant.

As macrophages are not exclusively expressed in the muscularis externa, which was studied by removing the mucosa and submucosa, the previous experiments were repeated in tissue samples where these layers were not removed. In this way, DISC1 expression in other layers of the gut wall could be studied. First, DISC1 was visualized in combination with markers for enteric glia and neurons in small intestinal samples (Fig. 7 and Fig. 8). Here, DISC1 immunoreactivity was observed in different layers (Fig 7c). Focusing on myenteric or submucosal plexus enteric neurons (Fig. 7b) and enteric glia (Fig. 7d) and overlaying the different channels shows that the DISC1 expressing macrophages are located in close proximity to the cells of the ENS (Fig. 7a) but also in the other layers of the gut wall (Fig. 8). Similar results were obtained in colon samples (Fig. 9)

Subsequently, macrophages and enteric glia in the small intestine (Fig. 10) and colon (not shown) were stained in combination with a DISC1 staining. Here enteric glia are difficult to see as the mucosa is located above the myenteric plexus and by this blocking the signal (Fig. 10b). Merging these images shows that DISC1 (Fig. 10c) is expressed in macrophages (Fig. 10d), however, this overlap is not present in all cells (Fig. 10a). Here the number of DISC1 expressing cells was higher than the number of macrophages in the small intestine (figure 10e) while in the colon this was vice versa (figure 10f). These differences were, however, not statistically significant.

As immunofluorescence results without removing the mucosa and submucosa have shown that DISC1

is not only expressed in macrophages associated with the myenteric plexus, but also in other layers of the gut, the same experiments were performed using intestinal cryosections. Here the tissue samples were cut radially to get an overview of all different layers of the gut wall. This should allow localization of DISC1 expressing cells across the different layers of the gut wall. First, the relation of DISC1 expressing macrophages to the ENS was studied (Fig. 11). Here, DISC1 immunoreactivity appeared prominent in the myenteric plexus and the submucosal plexus, as well as in the mucosal villi (Fig. 11c). However, in the villi, DISC1 immunoreactivity is only present in the first half located closer to the submucosa and not in the more luminal parts of the villi. Macrophages are also present close to the myenteric and submucosal plexus, however, to a higher degree in the mucosal villi (Fig. 11d). Merging these images shows that the macrophages located near the myenteric plexus, submucosal plexus, and first half of the mucosal villi are expressing DISC1 while macrophages in the more luminal parts of the villi seem to not express DISC1 (Fig. 11a). Based on these immunostainings more DISC1 positive cells than Iba1 positive macrophages were counted, though not at a statistically significant level (fig 11e).

Moreover, the relation of DISC1 expression to ENS cells was studied as well (Fig. 12). Here, enteric neurons as well as DISC1 can be seen in the myenteric and submucosal plexus, as well as in the mucosa (Fig. 12c and 12d). Expression of DISC1 occurs close to the neurons (Fig. 12a). Enteric glia were also stained, however, no typical enteric glia structures could be seen here (Fig. 12b).

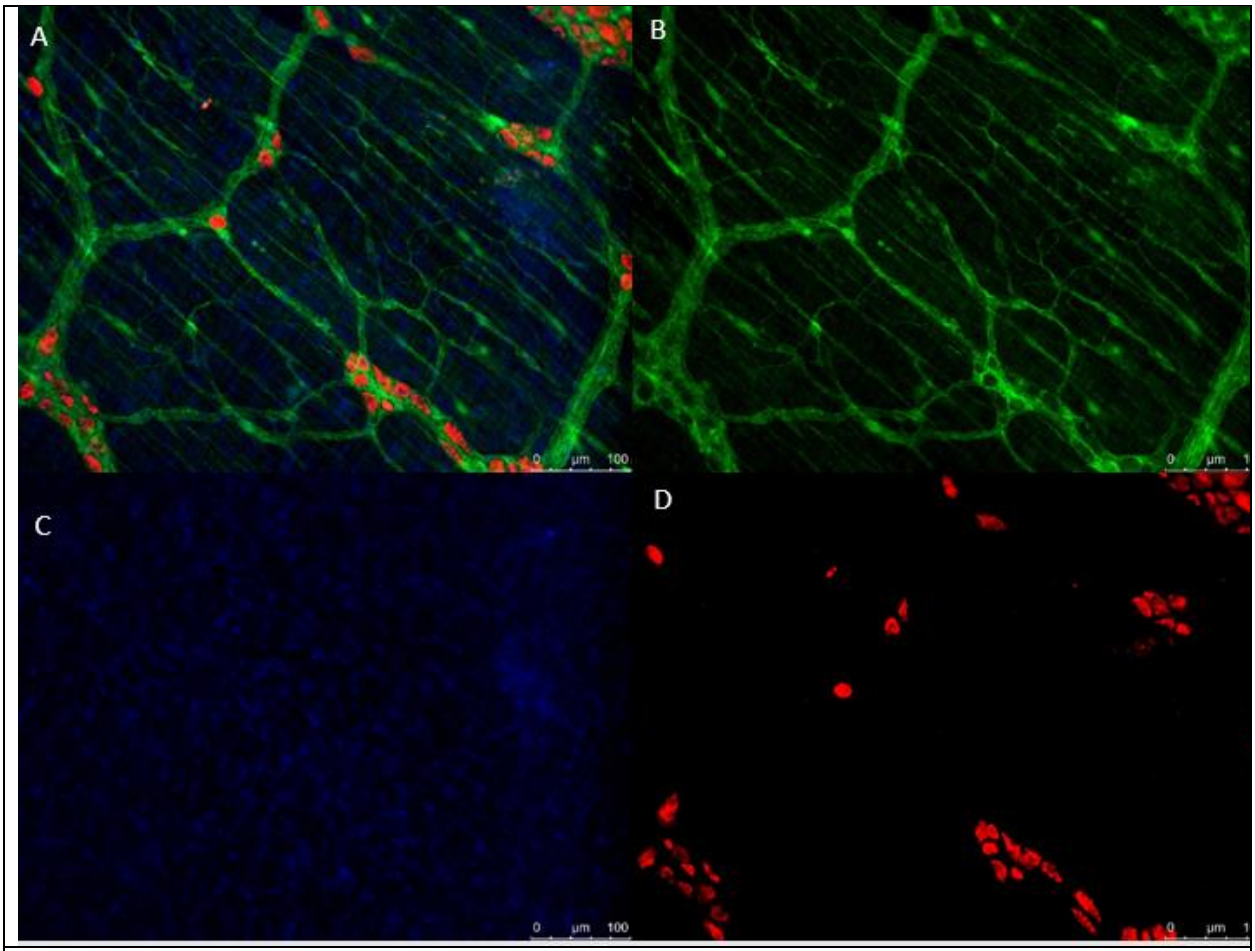

Fig 2. – Visualization of the murine enteric nervous system. To test whether the staining of enteric glia and neurons works or needs to be optimized, the small intestine of adult wild-type mice was used. S100β (1:10) – GαRb 488 was used to stain enteric glia (B), DAPI was used for staining of the nucleus (C), and enteric neurons are stained with HuCD (1:20.000) – GαHu 555 (D). Merging these three images shows the structural organization of the enteric nervous system in mice and the connections between neurons and enteric glial cells (A).

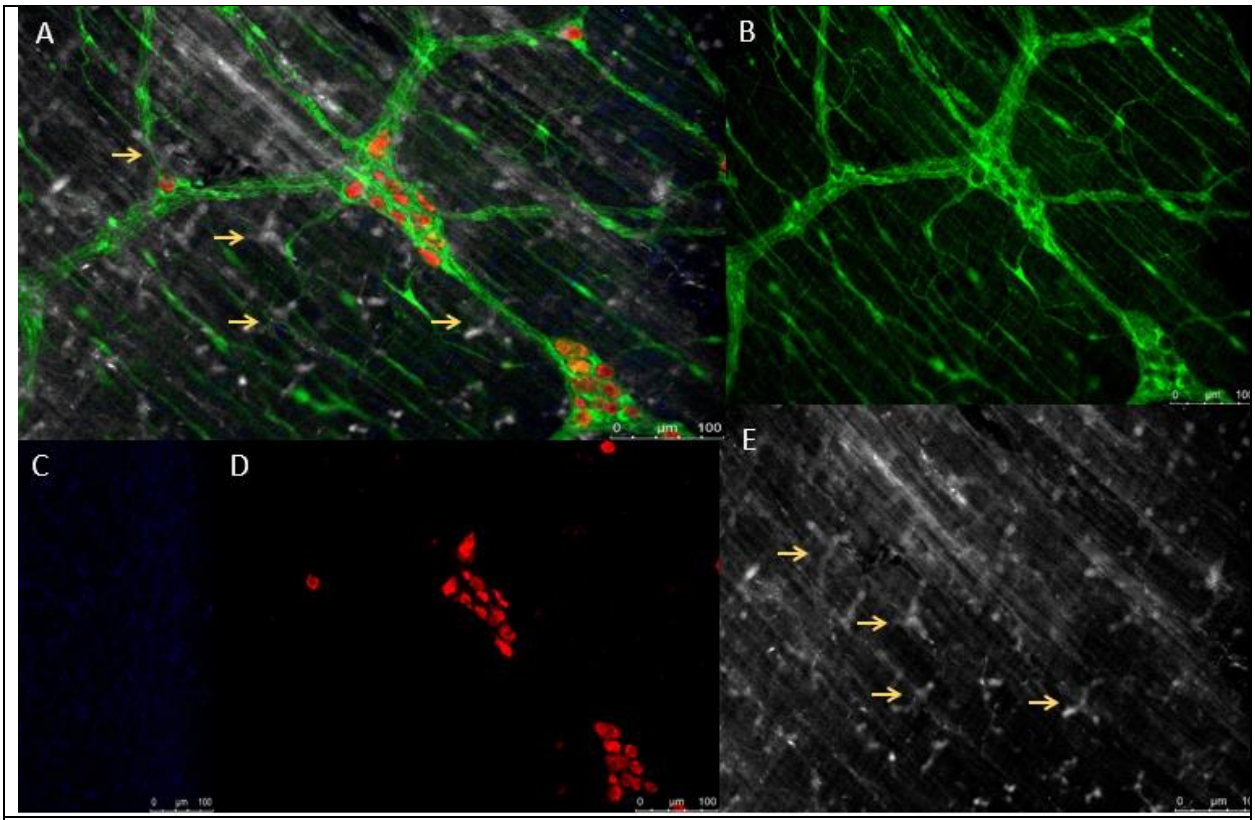

Fig 3. – Localization of DISC1 expression in the enteric nervous system. Small intestine tissue of adult wild-type mice was used to determine the location of DISC1 expression. Enteric glia are stained by using S100β (1:10) – GαRb 488 (B), the nuclei are visualized with DAPI (C), for enteric neurons HuCD (1:20.000) – GαHu 555 was used (D) and DISC1 is stained with DISC1 (1:200) – GαM568 (E). DISC1 expression is not seen in neurons or glial cells but close to the cells of the ENS (A, arrows). Arrows indicate DISC1

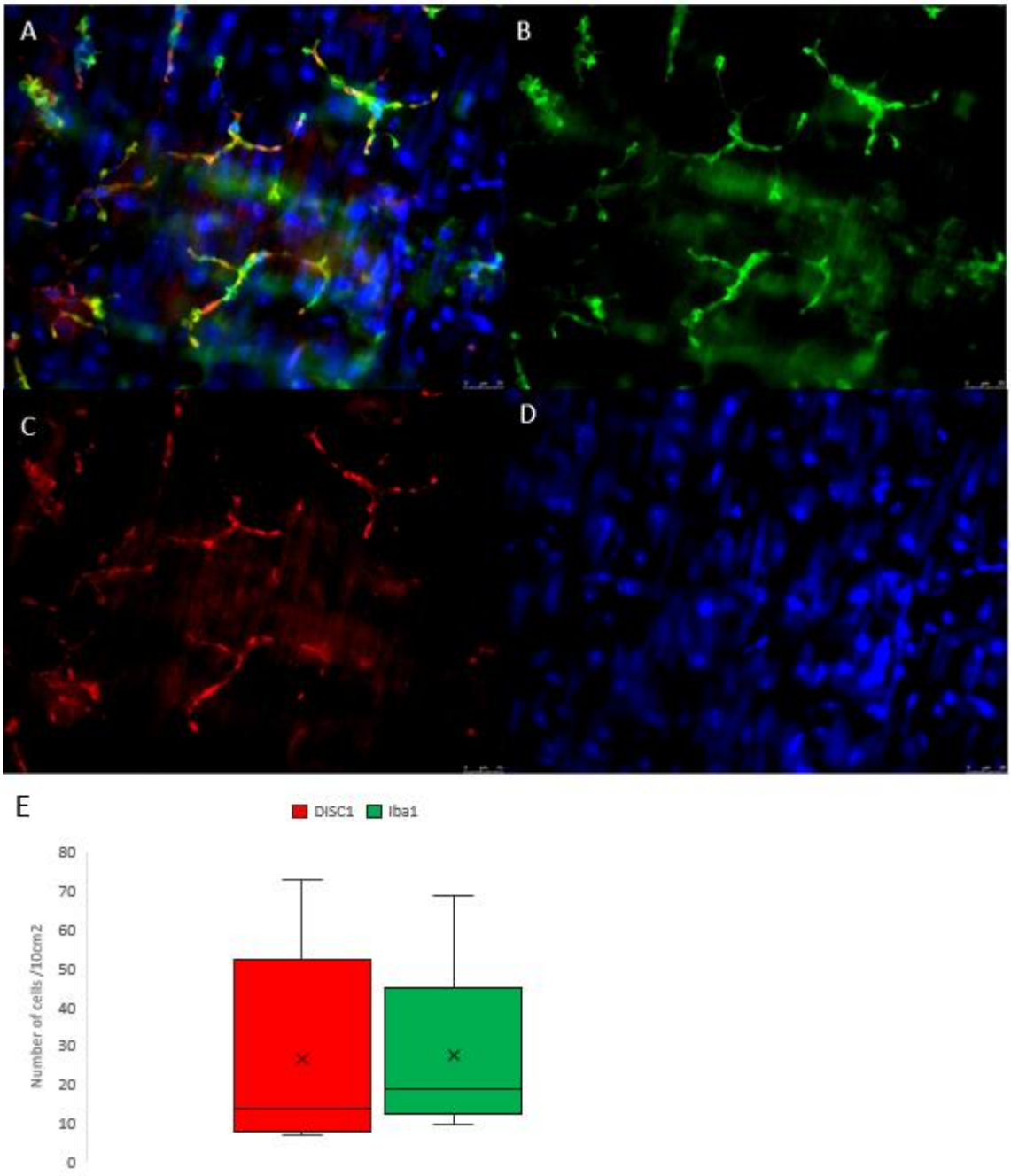

Fig 4. – DISC1 is expressed in macrophages of the ENS in adult wild-type mice. Macrophages in small intestine tissue are stained using Iba1 (6µL/mL) – DαG488 (B), and DISC1 is visualized using DISC1 (1:300) – DαM 555 (C). DAPI was used to stain the nuclei (D). Merging these images confirms that DISC1 is expressed in the macrophages as these stainings are overlapping. Quantification of Iba1 and DISC1 positive cells is shown in E. The median including the first and third quartile is given, and data were analyzed with the Mann-Whitney test.

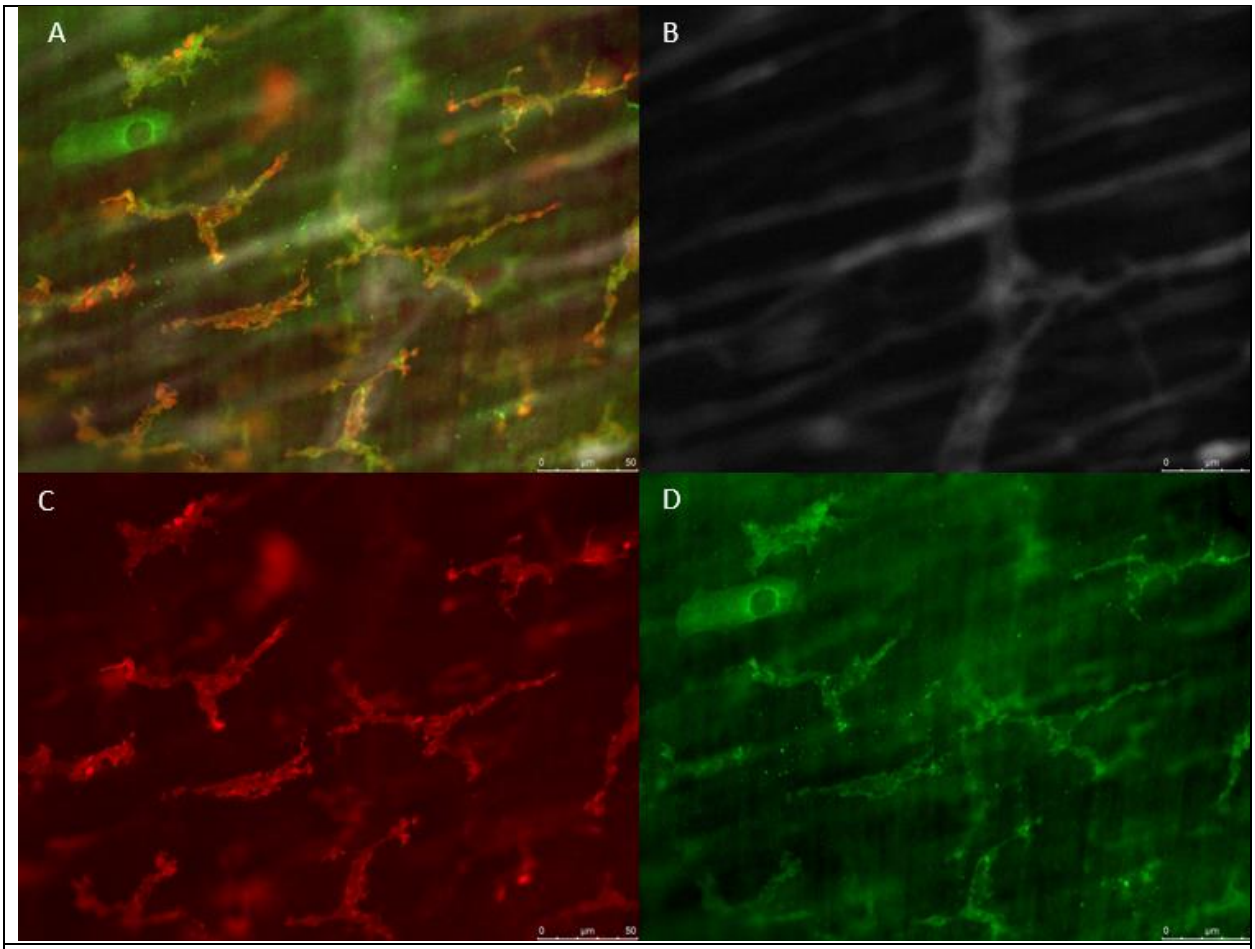

Fig 5. – DISC1 is expressed in macrophages of the gut wall in adult wild-type mice. Enteric glia in tissue sections of the small intestine are stained using S100β (1:10) – DαRb647. Macrophages are stained with Iba1 (6µL/mL) – DαG555 (C), and DISC1 is visualized using DISC1 (1:300) – DαM 488 (D). Merging these images shows the expression of DISC in the enteric macrophages (A).

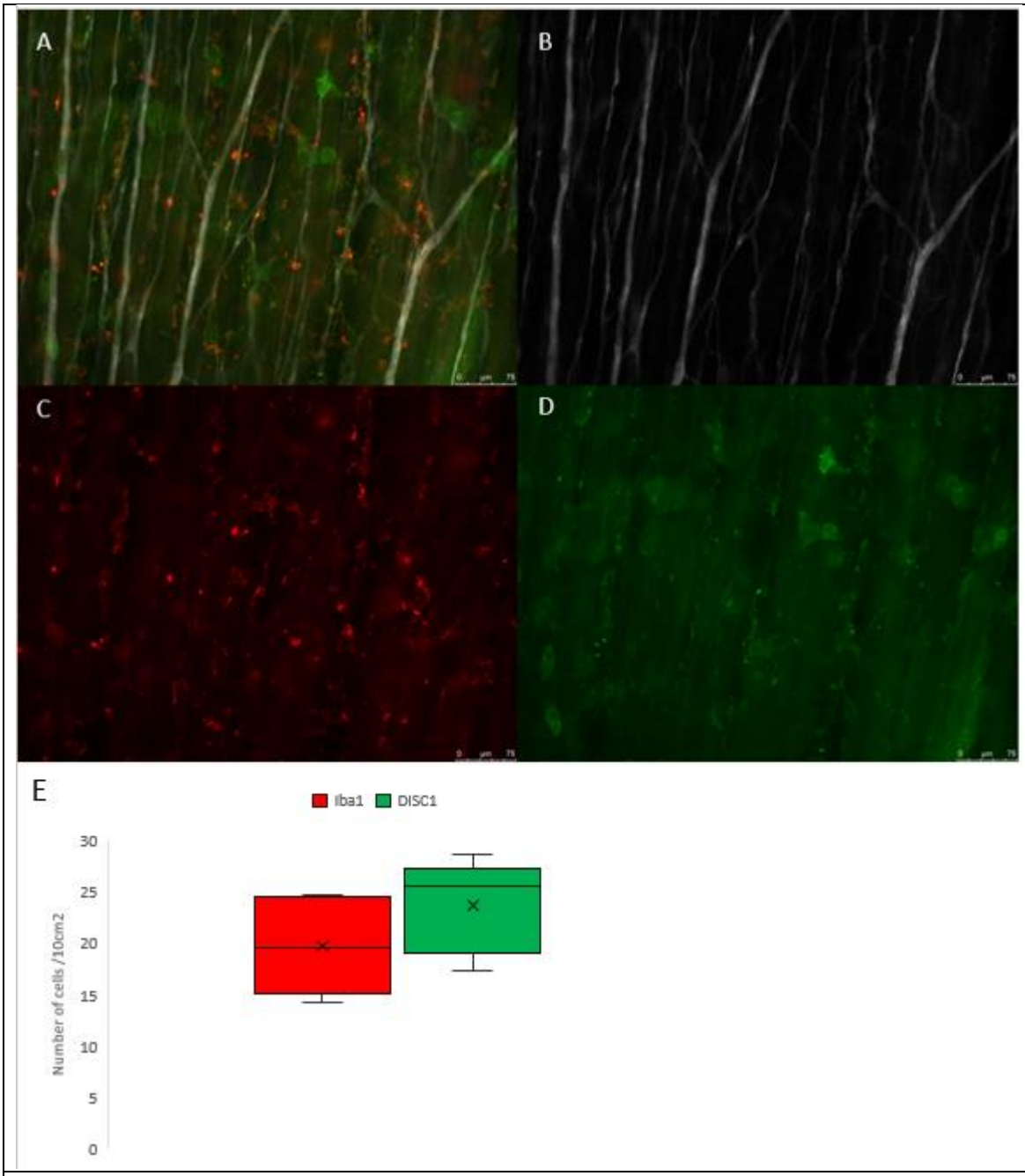

Fig 6. – DISC1 is expressed in macrophages of the gut wall in adult wild-type mice. Enteric glia in colon samples are stained using S100β (1:10) – DαRb647. Macrophages are stained with Iba1 (6µL/mL) – DαG555 (C), DISC1 is visualized using DISC1 (1:300) – DαM 488 (D). Merging these images shows that expression of DISC is present in the enteric macrophages (A). Quantification of Iba1 and DISC1 positive cells is shown in E. The median including the first and third quartile is given, and data were analyzed with the Mann-Whitney test.

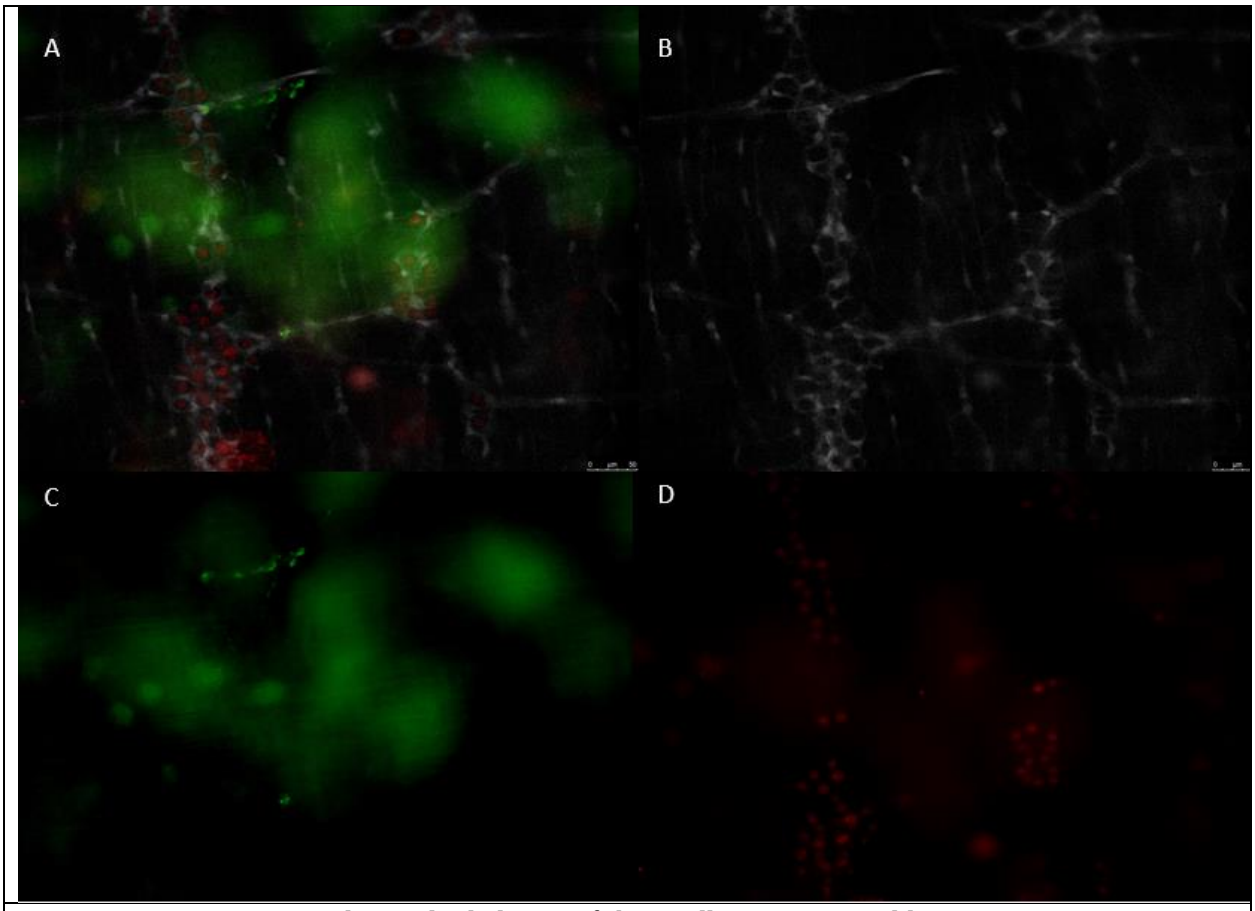

Fig 7. – DISC1 is expressed in multiple layers of the small intestine in wild-type mice. Tissue sections of the small intestine including the mucosa and submucosa were stained using S100β (1:10) – DαRb647 for enteric glia (B), DISC1 (1:300) DαM 488 for DISC1 (C), and HuCD (1:20.000) – GαHu 555 to visualize the enteric neurons (D). A merge is shown in A

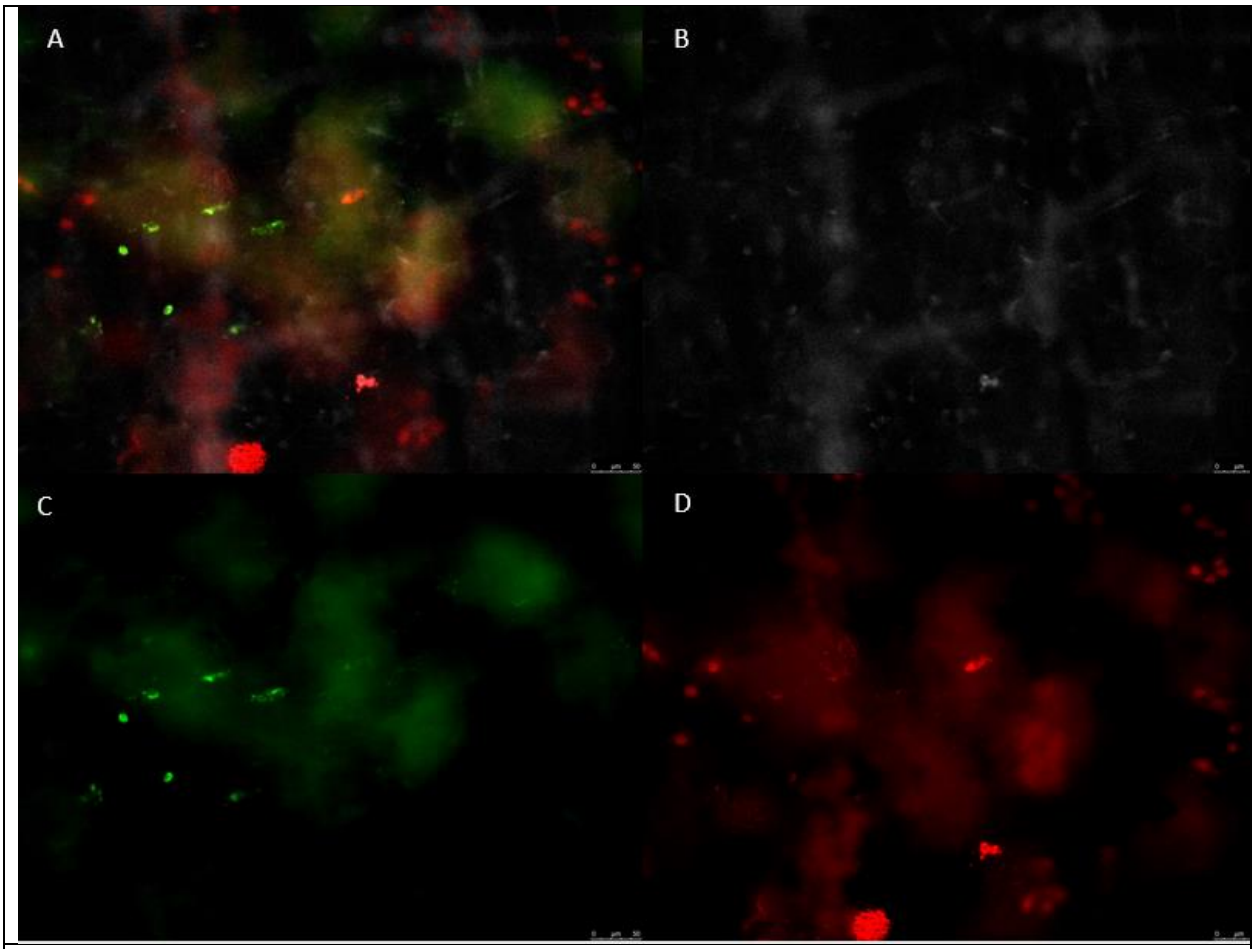

Fig. 8. – DISC1 is expressed in multiple layers of the small intestine in wild-type mice. Tissue sections of the small intestine including the mucosa and submucosa were stained using S100β (1:10) – DαRb647 for enteric glia (B), DISC1 (1:300) DαM 488 for DISC1 (C), and HuCD (1:20.000) – GαHu 555 to visualize the enteric neurons (D). A merge is shown in A. The same position is shown as in Fig. 9 but here the layer in which DISC1 is present is in focus.

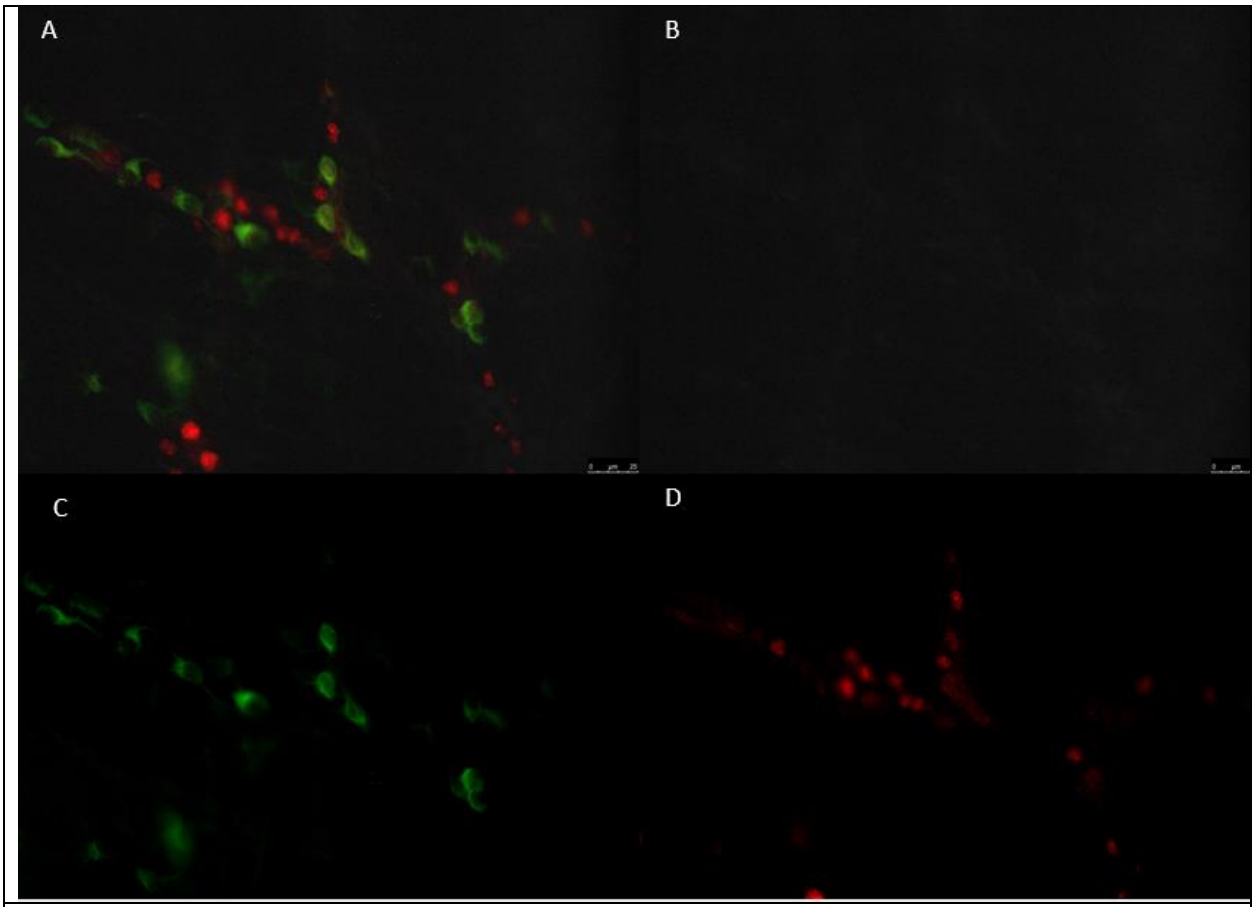

Fig 9. – DISC1 is expressed in multiple layers of the colon in wild-type mice. Tissue sections of the colon including the mucosa and submucosa were stained using S100β (1:10) – DαRb647 for enteric glia (B), DISC1 (1:300) DαM 488 for DISC1 (C), and HuCD (1:20.000) – GαHu 555 to visualize the enteric neurons (D). A merge is shown in A.

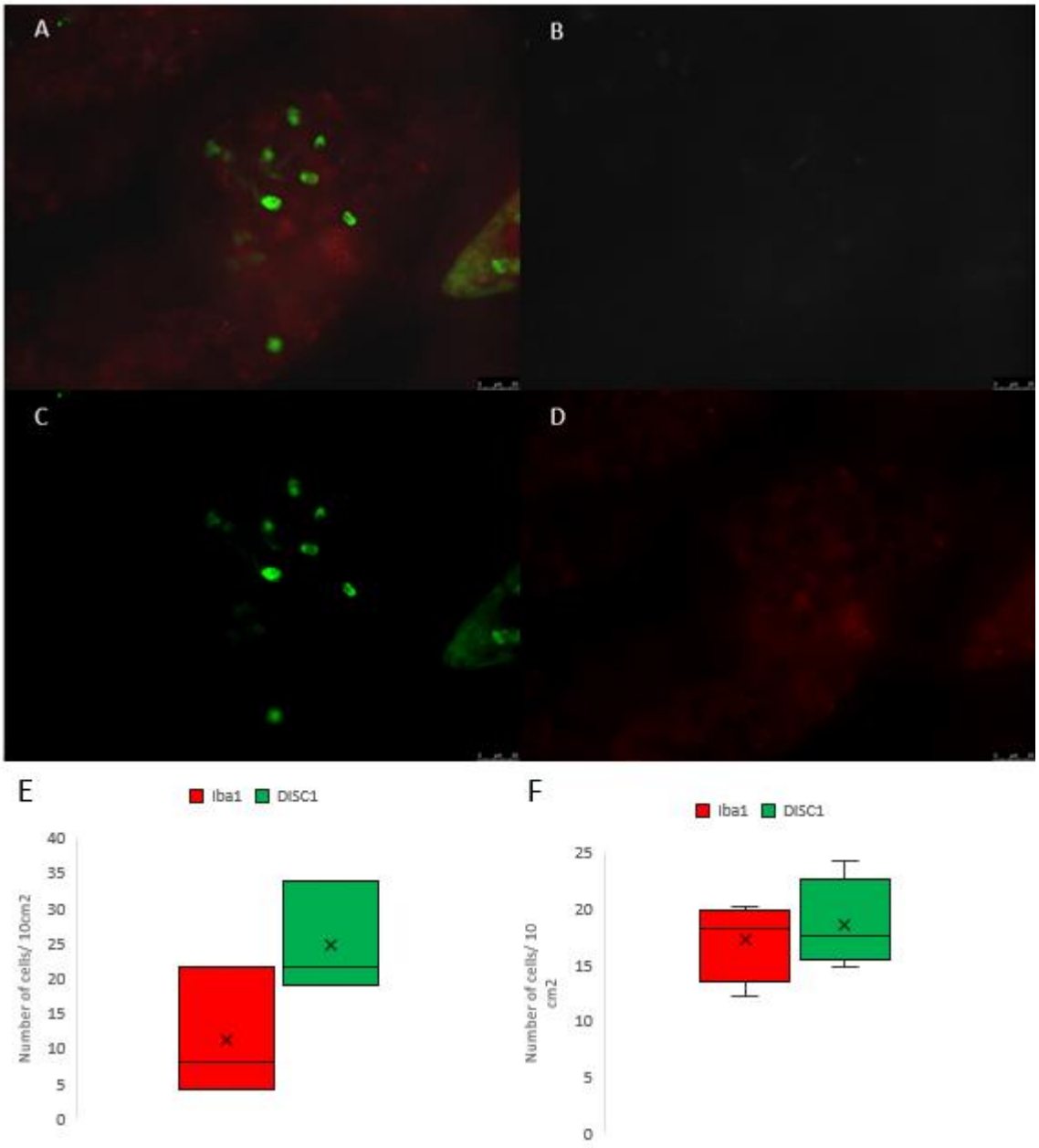

Fig 10. – DISC1 is expressed in macrophages of the small intestine in wild-type mice. Tissue sections of the small intestine including the mucosa and submucosa were stained using S100β (1:10) – DαRb647 for enteric glia (B), DISC1 (1:300) DαM 488 for DISC1 (C), and Iba (6µl/ml) – DαG 555 to visualize the enteric neurons (D). A merge is shown in A. Quantification of Iba1 and DISC1 positive cells is shown in the small intestine (E) and colon (F) are shown. Data are represented as median including the first and third quartile, and were analyzed using the Mann-Whitney test.

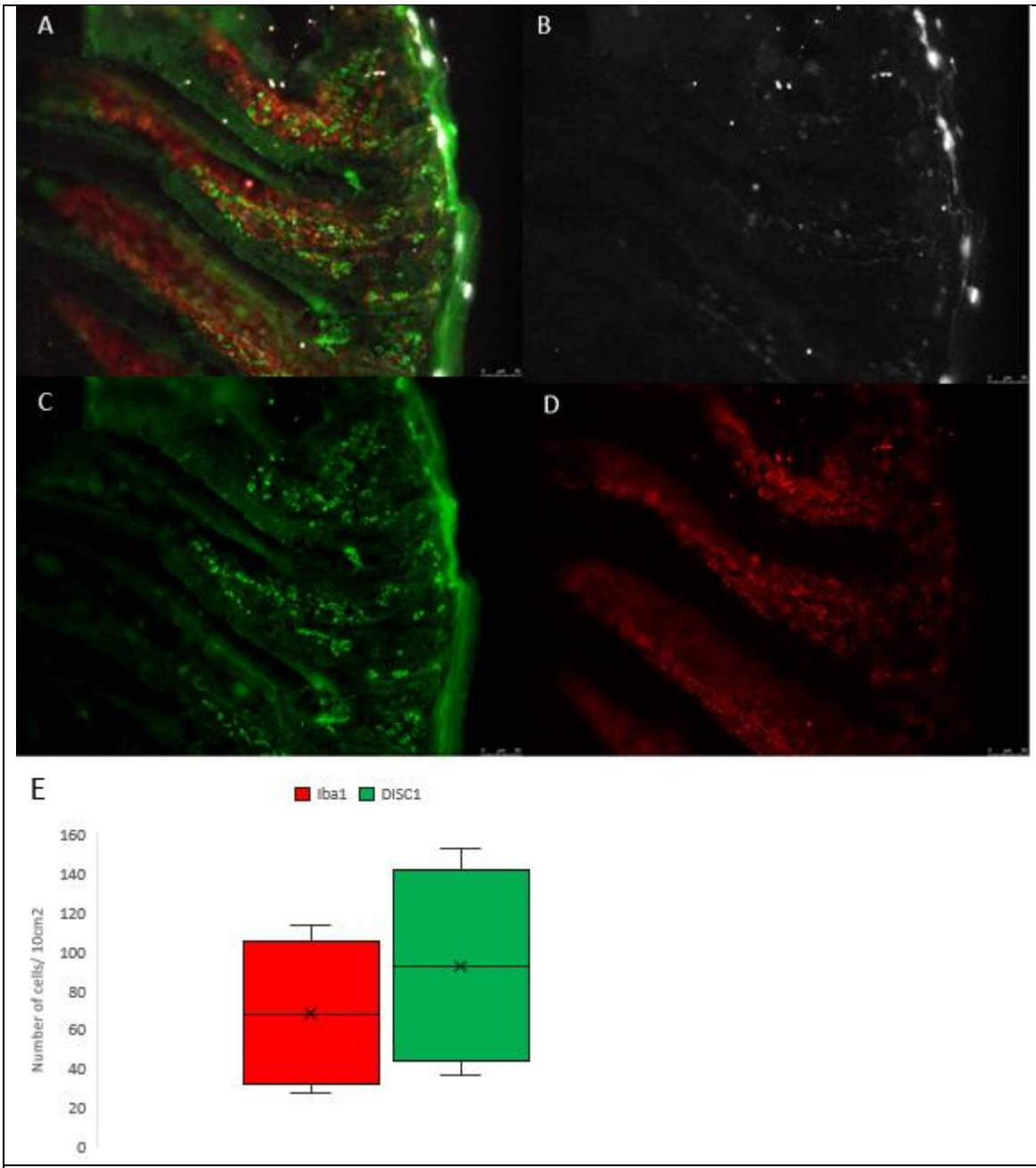

Fig. 11 – Expression of DISC1 in macrophages in the small intestine of wild-type mice. Cryosection samples were stained for enteric glia by using S100β (1:10) – DαRb647 (B), DISC1 was visualized with DISC1 (1:300) – DαM 488 (C), and macrophages are stained with Iba1 (6µl/ml) – DαG 555 (D). Merging these images shows the expression of DISC1 in macrophages in different layers of the small intestine (A). Quantification of Iba1 and DISC1 positive cells is shown in E. Data are represented as median including the first and third quartile, and were analyzed using the Mann-Whitney test.

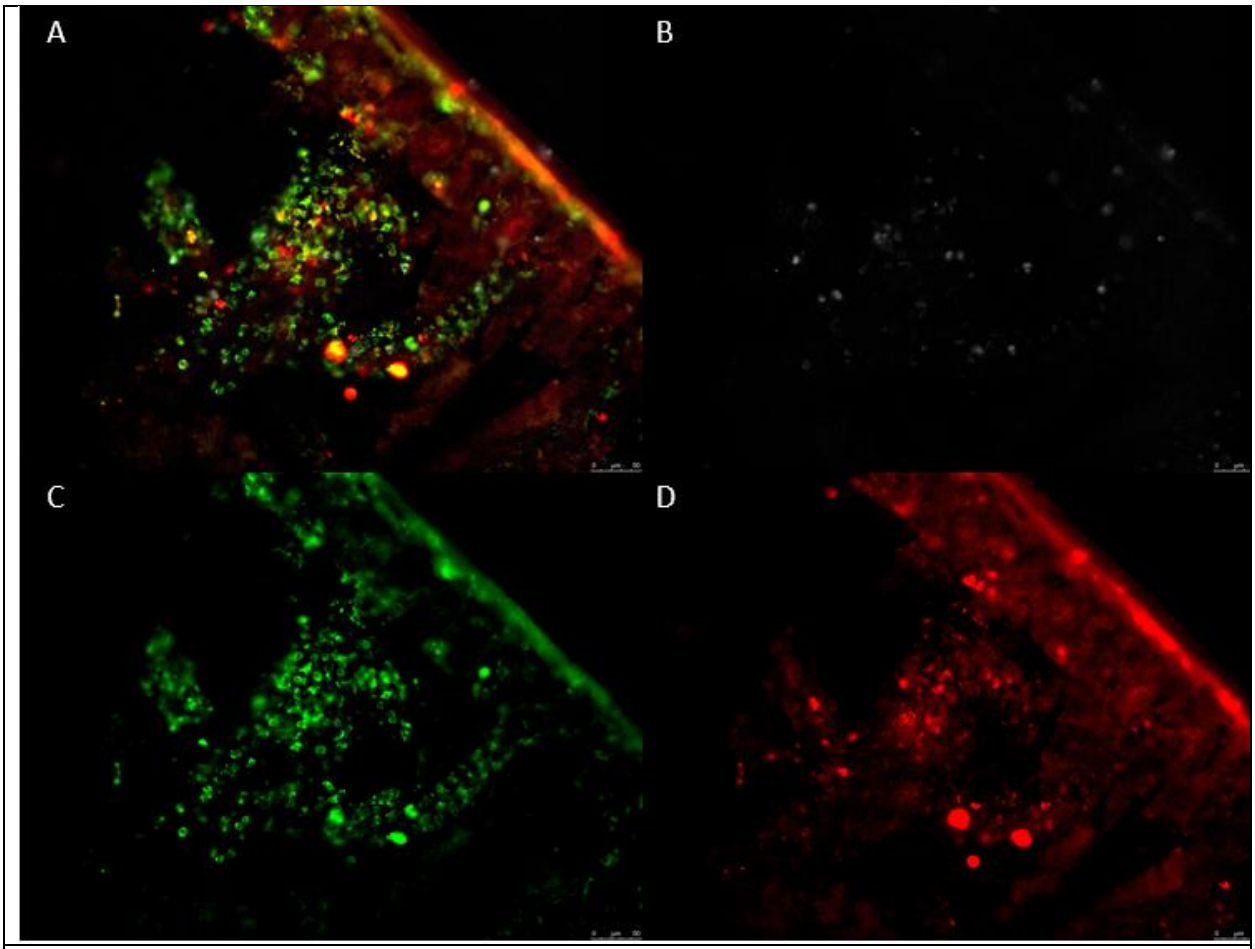

Fig. 12 – DISC1 is expressed in multiple layers of the small intestine in adult wild-type mice. Cryosection samples were stained for enteric glia by using S100β (1:10) – DαRb647 (B), DISC1 was visualized using DISC1 (1:300) – DαM 488 (C), and enteric neurons are stained with HuCD (1:20.000) – GαHu 555 (D). The overlay shows the relations of these cells (A).

DISCUSSION

Initially, we assumed that DISC1 is expressed in enteric glial cells of the ENS. Since gene expression was rather low and even decreased after removing the submucosa and mucosa of the gut prior to qPCR analysis, the question as to whether DISC1 is indeed expressed in enteric glial cells or in other cells in the gut was raised. Immunostaining was used to localize the expression of DISC1 in the gut. Here, indeed no overlap of DISC1 staining with the enteric glial cells was seen. Instead, DISC1 immunoreactivity was found in macrophages. Given that DISC1 is expressed by CNS microglia, which are considered as the macrophages of the brain, this was not completely unsurprising (48). While functions of DISC1 in neurons of the CNS have already been described, its role in microglia remains largely unknown (48). One study investigated the effect of mimicking the environmental as well as genetic background of schizophrenia (mutation of DISC1) on microglia. Here, a perturbation of microglia was found (49). The number of microglia was significantly increased and a significant alteration in morphological features such as area and cell spread were observed as well. This study proposed that this might contribute to the neuronal, glial, and circuit dysfunction involved in the development of mental disorders (49). Potentially, DISC1 expression in macrophages may be involved in the development of the gastrointestinal problems that are commonly seen in patients with mental disorders. DISC1 expression by intestinal macrophages is likely necessary for normal macrophage function, hence for normal gastrointestinal function and homeostasis. Disruption of DISC1, for example by a mutation as seen in schizophrenia, might result in abnormal macrophage function, and by this lead to gastrointestinal problems. Whether similar changes as in microglia in the brain can also be seen in the macrophages associated with the ENS is currently unknown. Together with the possible consequences on gut function, this needs to be investigated in future studies.

Generally, macrophages in the gastrointestinal tract are involved in tissue-support with specialized functions depending on their specific location. Macrophages are located in all different layers of the intestinal wall (39). Since the images of samples in which the mucosa was removed (Fig. 5 and Fig. 6) were all taken at the same focal depths, the macrophages that are visible here are the microglia-like macrophages located at the level of the myenteric plexus. They are located close to the ganglia containing enteric glial cells and enteric neurons. From literature, it is known that these macrophages play an important role in the maintenance of the ENS. They are believed to perform similar roles as microglia in the brain such as supporting ENS signaling and, synaptic pruning (41,42). In addition, muscularis macrophages have been demonstrated to communicate with enteric neurons in a bidirectional manner. While muscularis macrophages produce BMP2 and by this support enteric neurons, the neurons produce the macrophage-specific growth factor CSF-1 in order to promote macrophage homeostasis (40). Moreover, the crosstalk between muscularis macrophages and enteric neurons can be influenced by signals coming from the intestinal microbiota and thereby alter gastrointestinal motility (40). They are, furthermore, essential for maintenance of ENS homeostasis since they have been observed to phagocytose adult neuronal structures (50). Absence of these macrophages has been shown to lead to an increased number of myenteric neurons as well as a less organized architecture of the ENS (50). Moreover, macrophages also communicate with enteric glia which for example plays a role in chronic colitis (41). Thus, even though expression of DISC1 could not be found in the cells of the ENS (in the enteric glia and neurons), its expression in myenteric plexus-associated macrophages might influence the ENS via crosstalks between these cells. An impaired expression of DISC1 in these macrophages may potentially result in an abnormal architecture of the enteric nervous system which may cause problems in gastrointestinal functions such as motility. This might explain the link between mental disorders with involvement of DISC1, such as schizophrenia and autism spectrum disorder, and digestive problems like constipation or diarrhea.

Immunofluorescence stainings on gut tissue samples without removal of the mucosa and submucosa revealed that DISC1 is expressed in different layers of the gut wall as some cells expressing DISC1 are in focus while others are not focused (Fig. 7c). This is because the microscope can only focus on one chosen depth which explains

why cells located in a different layer and thus at a different depth are out of focus.
Additionally, immunofluorescence analysis using cryosections again showed that DISC1 is not exclusively expressed in macrophages located near the myenteric plexus but also in other layers of the gut wall such as the mucosal macrophages in the lamina propria. These macrophages are actively involved in barrier integrity and host defense and display a high phagocytotic activity. Furthermore, they play a role in the maintenance of FoxP3+ T regulatory cells by secreting interleukin (IL)-10 (39). A disturbed expression of DISC1 in these macrophages may potentially lead to abnormal barrier integrity and by this contribute to mental problems in disorders such as schizophrenia or autism spectrum disorder via the microbiome-gut-brain axis. Future studies with DISC1 locus impairment mice are needed to test whether a perturbed DISC1 expression indeed affects barrier integrity and whether this contributes to the mental problems seen in diseases related to problems in DISC1expression. Moreover, the effects of DISC1 locus impairment on intestinal motility need to be evaluated to assess the role of DISC1 in microglia-like macrophages.
Besides this, specificity tests of the DISC1 antibody are needed. Quantification of DISC1 positive and Iba1 positive cells showed that these numbers typically slightly differed. In some samples more DISC1 positive cells than macrophages were seen (Fig. 4, 6, 11, 10), while in other samples more Iba1 positive cells were counted (Fig. 10). Which cells apart from macrophages express DISC1 needs to be investigated in future studies. Looking at the shape of cells that were positive for DISC1 but not for Iba1 these cells might be serosal cells. Furthermore, future studies need to investigate whether DISC1 is only expressed in specific subtypes of macrophages as not all macrophages appeared to express DISC1.

CONCLUSION

In summary, DISC1 is expressed in macrophages located in multiple layers of the intestinal wall. Particularly its expression in myenteric plexus-associated macrophages is interesting as these macrophages influence the enteric glia and enteric neurons of the ENS via bidirectional communication. Whether an impaired expression of DISC1 in these myenteric plexus-associated macrophages affects the structural organization of the ENS and by this impairs gastrointestinal functions needs to be investigated in future studies. These findings may help in detecting a reason for the association of DISC1 associated mental disorders and the digestive issues seen in patients with these diseases and may potentially open up new possibilities for the development of therapies targeting processes that are impaired in these patients.

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