Rotavirus Disease Mechanisms

Diarrhea, Vomiting and Inflammation

-How and Why?

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List of papers

The papers included in this thesis are listed below.

- I **Rotavirus stimulates release of serotonin (5-HT) from human enterochromaffin cells and activates brain structures involved in nausea and vomiting.**

Hagbom M, Istrate C, Engblom D, Karlsson T, Rodriguez-Diaz J, Buesa J, Taylor JA, Loitto VM, Magnusson K-E, Ahlman H, Lundgren O and Svensson L.
PLOS Pathog. 2011 Jul;7(7):e1002115

- II **Rotavirus infection increases intestinal motility but not permeability at the onset of diarrhea.**

Istrate C, Hagbom M, Vikström E, Magnusson K-E and Svensson L.
J Virol. 2014 Mar;88(6):3161-9

- III **The cholinergic anti-inflammatory pathway contributes to the limited inflammatory response following rotavirus infection.**

Hagbom M, Nordgren J, Ge R, Lundin S, Wigzell H, Taylor J, Anderson U and Svensson L.

In manuscript

- IV **Intracellularly expressed rotavirus NSP4 rotavirus stimulates release of serotonin (5-HT) from human enterochromaffin cells.**

Bialowas S, Hagbom M, Karlsson T, Nordgren J, Sharma S, Magnusson K-E and Svensson L.

In manuscript

Populärvetenskaplig sammanfattning

Rotavirusinfektioner orsakar diarré och kraftiga kräkningar som kan leda till svår uttorkning. Trots att rotavirus upptäcktes redan år 1971 så är det inte klarlagt hur detta virus ger upphov till symptomen diarré och kräkningar. Infektionen ger en omfattande vävnadsskada med celdöd som följd, men trots det är det inflammatoriska svaret väldigt begränsat. Inflammation är ett sätt för vår kropp att bekämpa infektioner och främmande ämnen. Dock så kan inflammationer göra stor skada och ofta blir de långdragna. Vi vet inte hur det kommer sig hur rotavirusinfektion kan undkomma respons med inflammation, däremot så är infektionen kortvarig och självläkande.

Jag har studerat möjliga vägar för hur rotavirus orsakar sjukdomssymptomen diarré och kräkningar och hur det begränsar inflammation. En viktig upptäckt i denna avhandling är att rotavirusinfektion och det toxin som rotavirus producerar (NSP4), kan stimulera frisättning av serotonin från känselceller i tarmen. Serotonin, är ett ämne som kan aktivera nerver, och sedan tidigare känt för att kunna orsaka såväl diarré som kräkningar. Dessutom, svarade musungar som infekterades med rotavirus med nervaktivering i kräkcentrat, vilket visar på en kommunikation mellan tarm och hjärna. Mage/tarm och hjärna kan kommunicera genom att skicka signaler via vagusnerven, kroppens längsta nerv. Att rotavirusinfektion aktiverar hjärnan samt det faktum att det inflammatoriska svaret är så lågt, ledde oss till hypotesen att infektionen bromsar det inflammatoriska svaret via en så kallad anti-inflammatorisk reflex som går via vagus nerven. Vi fann bland annat att rotavirusinfekterade möss som saknar en intakt vagusnerv, liksom möss som saknar en specifik receptor i denna signaleringsväg, svarade med ett högre inflammatorisk svar.

Vi visar också att rotavirusinfekterade musungar har en ökad tarmmotorik vid uppkomsten av diarré, vilket kunde minskas med läkemedel som verkar på tarmens nervsystem. Dock så hade mössen inte någon ökad genomsläpplighet över epitelet, utan tvärt om så tätar tarmepitelet, vilket sannolikt är en skyddsmekanism för oss.

Sammanfattningsvis ger denna avhandling ny kunskap till hur rotavirus orsakar diarré, samt information om hur infektionen kommunicerar med hjärnan, framkallar kräkningar och minskar inflammation. Resultat från dessa studier stöder starkt vår hypotes att serotonin ger aktivering av hjärnan och tarmens nervsystem och bidrar därmed till såväl diarré, kräkningar som till att bromsa det inflammatoriska svaret vid rotavirussjukdom.

Abstract

Rotavirus infections cause diarrhea and vomiting that can lead to severe dehydration. Despite extensive tissue damage and cell death, the inflammatory response is very limited. The focus of this thesis was to study pathophysiological mechanisms behind diarrhea and vomiting during rotavirus infection and also to investigate the mechanism behind the limited inflammatory response.

An important discovery in this thesis was that rotavirus infection and the rotavirus toxin NSP4 stimulate release of the neurotransmitter serotonin from intestinal sensory enterochromaffin cells, *in vitro* and *ex vivo*. Interestingly, serotonin is known to be a mediator of both diarrhea and vomiting. Moreover, mice pups infected with rotavirus responded with central nervous system (CNS) activation in brain structures associated with vomiting, thus indicating a cross-talk between the gut and brain in rotavirus disease.

Our finding that rotavirus infection activates the CNS led us to address the hypothesis that rotavirus infection not only activates the vagus nerve to stimulate vomiting, but also suppresses the inflammatory response via the cholinergic anti-inflammatory pathway, both of which are mediated by activated vagal afferent nerve signals into the brain stem. We found that mice lacking an intact vagus nerve, and mice lacking the $\alpha 7$ nicotine acetylcholine receptor (nAChR), being involved in cytokine suppression from macrophages, responded with a higher inflammatory response. Moreover, stimulated cytokine release from macrophages, by the rotavirus toxin NSP4, could be attenuated by nicotine, an agonist of the $\alpha 7$ nAChR. Thus, it seems most reasonable that the cholinergic anti-inflammatory pathway contributes to the limited inflammatory response during rotavirus infection.

Moreover, rotavirus-infected mice displayed increased intestinal motility at the onset of diarrhea, which was not associated with increased intestinal permeability. The increased motility and diarrhea in infant mice could be attenuated by drugs acting on the enteric nervous system, indicating the importance and contribution of nerves in the rotavirus-mediated disease.

In conclusion, this thesis provides further insight into the pathophysiology of diarrhea and describe for the first time how rotavirus and host cross-talk to induce the vomiting reflex and limit inflammation. Results from these studies strongly support our hypothesis that serotonin and activation of the enteric nervous system and CNS contributes to diarrhea, vomiting and suppression of the inflammatory response in rotavirus disease.

Abbreviations

ACh	acetylcholine	ORS	oral rehydration solution
ANS	autonomic nervous system	ORT	oral rehydration therapy
Caco-2	human epithelial cell line from colorectal adenocarcinoma	PG	prostaglandins
CFS	cerebrospinal fluid	PLC	phospholipase C
CNS	central nervous system	PC	polymerase complex
CRP	C-reactive protein	PPR	pathogen recognition receptor
DLP	double-layered particle	RIG1	retinoic-acid-inducible protein 1
dsRNA	double-stranded RNA	RRV	rhesus rotavirus
EC cell	enterochromaffin cell	RV	rotavirus
EDIM	episodic diarrhea of infant mice	SEM	standard error of the mean
ELISA	enzyme-linked immunosorbent assay	SERT	serotonin reuptake transporter
ENS	enteric nervous system	Sf9	<i>Spodoptera frugiperda</i>
ER	endoplasmic reticulum	SGLT1	sodium glucose co-transporter 1
FITC	fluorescein isothiocyanate	siRNA	small interfering RNA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	TLP	triple-layered particle
GI	gastrointestinaltract	TLR	toll-like receptor
h p.i.	hour post infection	TNF- α	tumor necrosis factor alpha
5-HT	5-hydroxytryptamine, serotonin	VIP	vasoactive intestinal peptide
IL	interleukin	VP	virus protein
INF	interferon		
i.p	intra peritoneal		
IPANs	intrinsic primary afferent neurons		
IRF3	interferon regulatory factor 3		
MA104	rhesus monkey kidney cells		
MDA5	melanoma differentiation-associated protein 5		
MOI	multiplicity of infection		
mRNA	messenger RNA		
nAChR	nicotine acetylcholine receptor		
NF κ B	nuclear factor kappa beta		
NSP	non-structural protein		
NTS	<i>nucleus tractus solitarii</i>		

Introduction

Rotavirus gastroenteritis

Rotavirus is the major cause of acute gastroenteritis in young children, worldwide, and is responsible for 450,000 child deaths each year, mainly in developing countries¹. Although the introduction of rotavirus vaccines has decreased the mortality during the last decade, rotavirus infections are still of great clinical importance and disease mechanisms need to be defined². While rotavirus infections are global and occur regardless of socioeconomic status or environmental conditions, the outcome and consequences of the disease differ significantly between developed and developing countries³. Deaths occur mainly among children with poor access to medical care, and children die presumably due to dehydration and electrolyte imbalance. Despite reduced mortality in developed countries, it causes considerable morbidity and a substantial number of hospitalizations among children³.

The major clinical symptoms are severe diarrhea and vomiting, including fever. However, infections can also be asymptomatic, especially in neonates, older children and adults³. Cases of asymptomatic infections in older children and adults are probably due to active immunity. Usually all children have become infected several times during the 24 first months of life and by the time they reach 5 years of age most children have had repeated infections and developed a life-long lasting immunity to rotavirus disease³.

Rotavirus is spread through the fecal-oral route by contaminated hands, water or food². The amount of rotavirus shed in faeces has been shown to be 10^{10} virus particles/gram of stool⁴. There are few studies of infectivity but those indicate that only 10 or less particles are needed for an infection^{4,5}. Probably, as the very infectious norovirus, causing “the winter vomiting disease”, rotavirus may also be spread by aerosol through vomits, since droplet-spread of aerosolized rotavirus has been shown experimentally, using a mice model⁶.

Structure and classification

Human rotavirus was discovered in 1973 by Bishop and colleagues⁷. The name rotavirus comes from the wheel-like shape as seen in electron microscopy.

Rotavirus is a triple-layered segmented double-stranded RNA (dsRNA) virus and belongs to the family *Reoviridae*⁸. The size including the spikes is around 100nm. The viral genome has

11 gene segments, which code for 6 structural and 5 or 6 non-structural proteins (NSPs), depending on species (Table 1).

Rotaviruses are classified in groups, subgroups and serotypes⁹. According to the serological reactivity and genetic variability of the inner capsid protein VP6, 8 different groups have been defined (A-H)¹⁰, named RVA, RVB, RVC etc. A group can be further classified into subgroups based on the specificity of epitopes that are present on VP6.

Humans can be infected by the groups A, B and C, of which A is most common. Genetic reassortment occurs only among viruses within the same group⁸.

Rotavirus are classified serologically in different **serogroups**, based on the virus protein (VP) 6 reactivity, and within each serogroup there are multiple **serotypes** based on the outer capsid shell proteins VP7 (a glycoprotein, G types) and VP4 (protease sensitive, P serotypes)⁸. Both VP7 and VP4 induce a neutralizing response that is serotype specific as well as cross reactive, and is important for protective immunity. The classification of **genotypes** is determined by sequence analysis of VP4 and VP7. Within serotypes as well as genotypes, viruses are identified by their G- and P-type. G-types are based on the glycoprotein VP7 antigens and P-types on the protease sensitive VP4 antigens⁸.

Protein functions

The structural proteins build up the viral particle (Figure 1) and the NSPs have function either in the viral replication cycle or interaction with host proteins to influence the pathogenesis or immune response⁴.

Proteins contributing to the structure of the virion (Figure 1)

The innermost layer, the core shell, surrounds the viral dsRNA genome and is composed of 120 copies of **VP2**, formed by dimers¹¹.

The core of the virion is formed by the two minor proteins **VP1** and **VP3** and the 11 segments of genomic dsRNA, all encapsidated by the VP2¹². VP1 and VP3 appear to form a complex located on the inner surface of the VP2 layer and these two proteins have affinity for ssRNA, and play a role in the processes of RNA transcription and replication.

The intermediate layer is made up of 260 trimers of **VP6**¹¹. VP6 is extremely stable and contains epitopes that are conserved in many virus strains, which makes it the most commonly used antigen in diagnostic assays.

The outer capsid proteins, which are critical for attachment and entry into a host cell are built up of 260 trimers of the glycoprotein **VP7**, sitting directly on top of the VP6 trimers and form a continuous, perforated shell¹¹. VP7 trimers are dependent on bound calcium ions for their stability; two calcium ions are held at each subunit interface, requiring six bound ions in each trimer. Protruding through the VP7 layer on the rotavirus virion are 60 trimeric spikes, formed by the viral attachment protein **VP4**. Because of their key roles in infectivity, antibodies generated against VP7 and VP4 together with cleavage products VP5 and VP8 effectively neutralize rotavirus.

Newly assembled rotavirus virions are not fully infectious, so for membrane penetration the VP4 spike must be proteolytically cleaved into **VP5** and **VP8**, by trypsin-like proteases of the host gastrointestinal (GI) tract.

Proteins involved in replication, pathogenesis and immune response

Viruses interact with the host at all stages of replication, from cell entry to cell exit¹³. These interactions are crucial not only for producing new viruses, but also enable the host to recognize the presence of an infectious agent. Although hosts have evolved mechanisms to defend it self against pathogens, viruses have in turn evolved strategies to avoid the host immune response.

VP1 is an RNA-dependent RNA polymerase and is responsible for RNA and dsRNA synthesis. Positive-sense viral RNAs (+RNAs) are selectively packaged into assembling VP2 cores and replicated by the help of VP1 into the dsRNA genome.

VP3 is a capping enzyme, responsible for viral m-RNA capping¹¹.

NSP1 has been shown to have RNA-binding activity at 5' end of viral mRNAs and to enhance NSP3 inhibition of cellular mRNA translation¹⁴. Moreover, NSP1 seems to interact with the cellular transcription factor interferon regulatory factor 3 (IRF3) and targets it for degradation by the proteasome, thus acting to avoid the host antiviral defense by the blocking INF production¹⁴⁻¹⁸. However, NSP1 seems not to be necessary for rotavirus replication *in vitro* and its role is not fully clear¹⁹.

NSP2 and **NSP5** are responsible for the formation of inclusion bodies termed viroplasms, and are thought to co-localize around transcribing double-layered virus particles (DLPs). NSP5

has been shown to self-associate and interact with RNA and NSP2, suggesting that viroplasms may form large, semi-regular networks designed to sequester viral RNAs and capsid proteins for assembly in to nascent virions.

NSP3 has been shown to interact with the host translational machinery and to have viral RNA binding activity, enabling it to block cellular host protein synthesis and enhance viral mRNA translation¹⁴. NSP3 has also been associated with systemic spread of rotavirus^{20, 21}.

NSP4 is essential for rotavirus replication, transcription and morphogenesis. It is required for the outer capsid assembly and is a transmembrane glycoprotein that accumulates in the endoplasmic reticulum (ER), near the cytosolic viroplasms, electron dense structures where the replication takes place¹¹. Through an unknown mechanism, VP7 is also retained in the ER. The mechanism for the release of DLPs from the viroplasms is not known, nor the assembly of the outer capsid. The current model for outer capsid assembly is that NSP4 recruits both DLPs from nearby viroplasms and VP4 to the cytosolic face of the ER membrane. Interaction of the DLP with NSP4 tetramers results in ER membrane deformation and budding of the DLP/VP4/NSP4 complex into the ER. Thereafter the ER membrane and NSP4 are removed and VP7 assembles onto the particle¹¹. Moreover, NSP4 has been shown to have viroporin properties and to induce release of calcium from ER^{19, 22, 23}. It is not clear how intracellular NSP4 releases calcium from the ER, but this is presumably by a phospholipase C (PLC)-dependent mechanism^{22, 24}. NSP4 can by itself induce diarrhea in mice pups when given intra peritoneal (i.p), and the protein has thus been considered as an enterotoxin²⁵.

Moreover, it has been shown that NSP4 and a cleavage fragment thereof is secreted from infected cells²⁶⁻²⁸ and that NSP4 can trigger pro-inflammatory cytokines from macrophages via Toll-like receptor-2²⁹ and stimulate serotonin (5-hydroxytryptamine, 5-HT) release from human EC cells³⁰. Still, there are reports in the literature not being consistent with NSP4 being an important factor in the pathophysiology of rotavirus. As pointed out by Angel et al³¹, amino acids 131-140 of NSP4 is hyper-variable both in human and murine rotavirus, and there is no distinct correlation between amino acid sequence and virulence. Similar observations have been made in human studies^{32, 33}.

NSP6 is not encoded by all rotavirus strains, but when present, it is encoded by segment 11 as the NSP5. The exact role of NSP6 is still unclear but it seem to localize to the viroplasm and have binding affinities for ssRNA and dsRNA¹⁹.

Protein	dsRNA segment No	Location in virus capsid	Function	Numbers of molecules/virion
VP1	1	Core	dsRNA synthesis (RNA-dependent RNA polymerase)	12
VP2	2	Core	Inner shell protein	120
VP3	3	Core	Capping enzyme	12
VP4 (Cleaved to VP5 and VP8)	4	Outer Capsid	Viral attachment, P-type neutralization antigen	120
VP6	6	Inner Capsid	Middle shell protein	780
VP7	9	Outer Capsid	G-type neutralization antigen	780
NSP1	5		INF antagonist	
NSP2	8		Viroplasm formation	
NSP3	7		Enhance viral mRNA synthesis, Associated with systemic spread	
NSP4	10		Outer capsid assembly, Regulate calcium homeostasis, enterotoxin	
NSP5	11		Viroplasm formation	
NSP6	11		Viroplasm formation	

Table 1. Rotavirus proteins. Structural (shaded in pink) and non-structural (shaded in green) proteins; their function, genome- and structure localization.

Figure 1. The left panel shows a cryo-electron micrograph-image reconstruction of a mature rotavirus triple-layered particle (TLP) at 9.5Å resolution. The smooth external surface is made up of the VP7 glycoprotein (yellow) and is embedded with the VP4 spike attachment protein (red). The intermediate VP6 layer is shown in blue and the thin VP2 core shell is shown in green. Ordered portions of viral dsRNA that line the VP2 shell are shown in gold. Polymerase complex (PC) components, VP1 (the viral polymerase) and VP3 (the viral capping enzyme), are not visualized in this reconstruction, but are attached to the inner surface of VP2. The right panel shows a schematic cartoon of a rotavirus TLP with proteins and dsRNA colored according to the legend. Reprinted with permission from the publisher Elsevier, Trends in Microbiology.

Replication

Rotavirus replication takes place in the cytoplasm of infected cells, in viroplasms being electron dense structures near the nucleus and ER⁸. Newly made viruses budded out from viroplasms into ER, through binding to the tail of the ER transmembrane viral glycoprotein NSP4. Although the virus replication process includes synthesis and transport of glycoproteins, the Golgi apparatus is not involved in rotavirus replication. Instead rotavirus replication, morphogenesis and pathogenesis are regulated by intracellular calcium concentrations²⁷. Many *in vitro* studies on rotavirus replication have been done with the MA104 cell line and the rhesus rotavirus strain (RRV). *In vitro* rotavirus replication in non-polarized MA104 show maximal replication after 10 to 12 hours at 37 °C, when cells are infected at a high multiple of infection (MOI) of at least 10 infectious viral particle per cell⁸. Rotavirus replication differs however, depending on cell type, and in polarized human intestinal cells (Caco-2) replication was slower with a maximum viral yield at the apical side at 20 to 24 hours after infection⁸. The rotavirus toxin NSP4 has been shown to be released very early during an infection, first as an cleavage product including the toxic region released from infected cells, starting at 4 hours post infection²⁶ and later during infection as fully glycosylated NSP4²⁷.

The general steps of rotavirus replication, based on cell culture studies, are as follows^{8, 11}:

1. **Virus attachment** to cell surface by VP4 or the cleavage product VP8. The conformational change is protease-dependent, where VP4 is cleaved into VP8 and VP5. Rotavirus has tropism for mature enterocytes but the exact receptor for viral binding *in vivo* has not yet been identified, although sialic acid³⁴, integrins³⁵, histo-blood group antigens^{36, 37} and toll-like receptors (TLR) have been suggested^{29, 38}.
2. **Cell entry**, by receptor-mediated endocytosis occurs via VP5, thus indicating that cleavage of VP4 into VP5 and VP8 is required. Calcium dependent endocytosis has also been shown³⁹. Non-clathrin, non-caveolin –dependent endocytosis delivers the virion to the early endosome. It has also been suggested that rotavirus can enter the cell by direct entry or fusion⁴⁰.

3. **Uncoating of the TLP.** Reduced calcium concentrations in the endosome are thought to trigger the uncoating of VP7 and loss of the outer capsid (VP7, VP5 and VP8). Double-layered particles (DLP) (core proteins and inner capsid VP6) are released into the cytosol.
4. **Transcription and translation** takes place in the cytoplasm of the cell. The internal polymerase complex (PC) (VP1 and VP3) starts to transcribe capped (+)RNAs from each of the eleven dsRNA segments. (+)RNA serves either as mRNA for direct translation, synthesis of viral proteins by cellular ribosomes or as a template for (-)RNA synthesis of viral genome replication, taking place in viroplasms.
5. **Assembly.** The NSP2 and NSP5 interact to form viroplasms, where replication and sub-viral particle assembly takes place. DLPs are formed within the viroplasms. The assembly process of the outer capsid is not fully understood but it is thought that the transmembrane protein NSP4 recruits DLPs and the outer capsid protein VP4 to the cytosolic side of the ER membrane. The NSP4/VP4/DLP –complex then buds into ER. The removal of the ER membrane and NSP4 takes place in the ER through interaction with ER-resident VP7 and the final TLP is formed.
6. **Virus release** from the infected cell is through cell lysis or Golgi-independent non-classical vesicular transport. In the GI tract the virion will be exposed to trypsin-like proteases, which will cleave the protease-sensitive VP4 into VP5 and VP8, thus resulting in a fully infectious virion.

Animal models to study rotavirus infection

Our understanding of rotavirus pathogenesis is based primarily on animal studies⁸. The mouse model is the most commonly used animal model to study rotavirus immunity and pathophysiology⁴. Advantages comprise the animal's small size, availability, the existence of several virulent mouse rotavirus strains and the large number of immunological reagents. There are however limitations with this animal model. Mice are age-restricted to rotavirus-induced diarrhea and become resistant to diarrheal disease by 15 days of age⁴¹. Still, they remain susceptible to infection throughout their life, and shed virus in faeces without clinical symptoms. Rabbits, rats and pigs are also used since there are homologous infective rotavirus strains for these animals⁴². Rabbits have been used to study transmission and protection and rats to elucidate viremia kinetics and extraintestinal organ spread, since

several rat strains develop systemic disease. Also calves and lambs have homologous rotavirus strains and some immunological studies have been performed in these animals⁴². Moreover, rotavirus has also been detected in cats, horses, goats, chickens, turkeys and other avian species⁴³.

Today, there is no small-animal rotavirus model for vomiting studies. Mice, like all rodents lack the emetic reflex, but do have the signaling pathway to the brain⁴⁴. It is speculated that rodents possess a degenerated “emetic” response rather than lacking one⁴⁴. Furthermore, there are reports of “retching” in mice⁴⁴. Ferrets and dogs can vomit, but due to their big size, ethical aspects with dogs and since ferrets being very aggressive to handle, they are not commonly used. Moreover, there are no homologous rotavirus strains for these animals, although there are some reports of rotavirus detection in ferrets⁴⁵ and dogs⁴⁶. Most of the vomiting studies performed are focused on chemotherapy-induced vomiting and animals such as *Suncus murinus*⁴⁷ and *Cryptotis parva*⁴⁸ have been used in those studies. These animals are small in size and respond to a variety of stimuli, like chemotherapy, toxins and 5-HT. It is not known however, whether these animals become infected with rotavirus and there is no published data on GI virus infections of these animals.

Immunity

The mechanisms responsible for immunity to rotavirus infections are not completely understood. Animal models have been useful in elucidating the role of antibodies and in exploring the relative importance of systemic and local immunity⁴². In humans, rotavirus infection has been shown to induce a good humoral immune response and protection increases with each new infection and reduces the severity of the diarrhea⁴⁹. There seems to be a positive correlation between serum antibodies of IgG and IgA and reduced risk of rotavirus infections^{50, 51}, especially for IgA⁴².

It is important to remember that rotavirus immunity does not protect from infection, it only protects against disease. A clinical study with a two-year follow up of 200 newborns, showed that no child had moderate-to-severe diarrhea after two infections, irrespectively whether the previous infections were symptomatic or asymptomatic. Subsequent infections were significantly less severe than the first infection and the second one were more likely to be caused by another G type of rotavirus⁴⁹.

Since asymptomatic infections induce the same degree of protection as symptomatic ones⁴⁹,

⁵², it may reflect its importance for the long lasting protection of rotavirus disease. A study among children at day care centers found asymptomatic rotavirus infections in 3 to 4 times higher frequency than symptomatic⁵³.

During the first months of life, the baby receives maternal antibodies via placenta transfer and breastfeeding, which likely gives some protection against rotavirus infection⁵⁴. The peak incidence of diarrhea associated with infection occurs between 7 and 15 months of age and only 1 of 5 infected infants develop symptoms during their first two months of life⁵⁵. This suggests a protective effect by the maternal antibodies during the neonatal period.

General pathophysiology

The severity and localization of rotavirus infection vary among animal species and between studies, but pathological changes are almost exclusively limited to the small intestine.

Rotavirus infects the mature non-dividing enterocytes in the middle and top parts of the villi in the small intestine³. At the cellular level, the infection is characterized by vacuolization (Figure 2), blunting and shortening of the villi. Rotavirus also produces the enterotoxin NSP4, which is thought to play an important role in the pathophysiology and clinical symptom of rotavirus disease^{25, 29, 56}. The incubation time is 24 to 48 hours and illness usually last from 3 to 5 days, longer in immunocompromised individuals⁸.

There are few pathology studies of the duodenal mucosa of infants infected with rotavirus⁵⁷,⁵⁸. Biopsies have displayed shortening and atrophy of villi, distended endoplasmic reticulum, mononuclear cell infiltration, mitochondrial swelling and loss of microvilli^{57, 58}.

Systemic spread of rotavirus has been reported but is very rare and its clinical importance remains unclear²¹. In a few cases rotavirus RNA has been detected in cerebrospinal fluid (CSF)⁵⁹⁻⁶¹, possibly associated with meningitis⁶², encephalopathy^{63, 64} and encephalitis^{65, 66}. Several recent studies have demonstrated that antigenemia, viremia and limited systemic replication seems to occur frequently in different body sites, but there is little evidence that this systemic spread and replication is responsible for any specific pathologic findings in the host^{64, 67-70}. In severely immunocompromised infants it has been shown that rotavirus can replicate and cause abnormalities in the liver and other organs⁷¹.

Intussusception, a process in which a segment of the intestine invaginates, folds into another section of the intestine, has been associated with rotavirus infections and vaccine. It can result in bowel obstruction and infarction, which may require surgery. The first licensed

rotavirus vaccine RotaShield® was withdrawn in the US following reports of intussusception among vaccinated children^{72, 73}.

Several mechanisms have been proposed to account for the watery diarrhea associated with rotavirus infection. These include osmotic diarrhea due to loss of epithelial absorptive function, effects of the rotavirus toxin NSP4 and an active role of the ENS and 5-HT^{25, 30, 74-77}.

The mechanism for how rotavirus induces vomiting has only recently begun to be investigated, despite its importance in contributing to dehydration. The observation that rotavirus communicate with CNS and activate brain structures of the vomiting center, were published by us in year 2011³⁰.

Figure 2. Histopathological changes in rotavirus infected intestinal epithelium of mice. (A) Vacuolization of cells in the upper part of the villi, as can be seen more easily in the enlarged picture. (B) The arrow shows an infected enterocyte with a vacuole, nearby an EC cell; stars indicate EC cells, stained by the neuroendocrine marker chromogranin. Hagbom et al, PLoS Pathog, 2011.

Clinical symptoms

Rotavirus infections induce a variety of symptoms that can vary from mild to severe and also be asymptomatic^{3, 4, 77}. Symptoms include vomiting, diarrhea, fever and a general sickness feeling, including tiredness and weakness⁸. Delayed gastric emptying has also been observed⁷⁸. The histopathological changes cannot solely explain the clinical symptoms as symptoms sometimes occur before histological changes are apparent⁸. Most studies have been focused on how rotavirus induce diarrhea, although vomiting contributes to the dehydration and complicates the standard treatment with oral rehydration therapy (ORT). Both diarrhea and vomiting are evolutionary protection mechanisms assumed to help the host to get rid of ingested subjects, for instance toxins and pathogens. Even fever⁷⁹ and the feeling of sickness have been suggested as protection mechanisms, focusing the energy to fight an infection⁸⁰.

Sickness behavior

There is evidence that the vagus nerve contributes to the feelings associated with infections as loss of appetite, tiredness and the induction of sickness behavior⁸¹.

The acute phase response, the feeling of “sickness”, is an initial response of the innate immune system to a broad range of potentially infectious agents. It comprises a systemic inflammatory reaction mediated by proinflammatory factors such as the cytokines IL-1, IL-6 and TNF- α ⁸². The behaviors of sick animals and humans are not regarded as maladaptive response or the effect of debilitation, but rather an organized, evolved behavioral strategy to facilitate the role of fever in combating viral and bacterial infections⁸⁰. Sickness behavior *per se* has not been studied during rotavirus infection, although the feeling of sickness and classical symptoms are usually present in symptomatic infections.

Vomiting

Nausea and vomiting can be induced by a wide variety of stimuli, such as pregnancy, space travel, raised intracranial pressure, radiation, cytotoxic drugs⁸³, foul odors, strong emotions and travel sickness⁸¹. This indicates that several different afferent links to the vomiting reflex center do exist⁸¹. Activation by noxious stimuli, like chemotherapeutic drugs or toxins can stimulate EC cells to release 5-HT, which then activates 5-HT₃ receptors on both extrinsic and intrinsic afferents⁸³. Neurons from area postrema project into the NTS and may this way initiate the vomiting response⁸¹. Moreover, substances in the bloodstream can cause vomiting by direct action at the area postrema of the medulla, which lacks the blood-brain barrier. This may result not only in hyper -secretory and -motor reflexes, but also in the distant activation of brain structures associated with nausea and vomiting, all aiming to expel the harmful contents out of the body^{81,83}. The emptying of the stomach is caused by coordinated contractions of the smooth muscles in the stomach wall and striated muscles in the diaphragm and abdominal wall, but laryngeal and pharynx muscles, the soft plate and tongue also participate⁸¹. These serial events suggest that the reflex center activates visceral (parasympathetic) afferent neurons and motor neurons at several levels of the brainstem and in the spinal cord in a well coordinate and specific order⁸¹. The vomiting reflex center is quite widely spread, but is mainly restricted to the medulla, including the NTS⁸¹. From the vomiting reflex center, signals pass on from the medulla to the motor nuclei via synaptic interruption in the reticular formation and reticulospinal fibers, still there is also a direct spinal projection from the NTS to the motor neurons of the diaphragm and abdominal wall⁸¹.

As an evolutionary aspect on vomiting, animals possess an arsenal of special abilities for survival and many of these are used for consumption of foods, since food intake is a risky behavior leading to the exposure of internal organs to possible food-related disease, including viral and bacterial infection, allergies and food intolerance⁸⁴. As protective system, vomiting cannot afford to make mistakes, and must thus have a low threshold for activation⁸⁴. Viral infections as norovirus and rotavirus seems to be more associated with severe vomiting compared to bacterial infections^{85, 86}. Bacterial infections are on the other hand more commonly associated with prolonged inflammation and bloody diarrhea but less vomiting as for *Salmonella*, *Shigella* and *Yersinia*⁸⁶. Mechanism to how vomiting occurs has mainly been investigated due to chemotherapy, radiation and post-surgery^{87, 88}. Radiation and chemotherapy may result in release of 5-HT from EC cells in the small intestinal mucosa, and 5-HT subsequently activates 5-HT₃ receptors on vagal abdominal afferents to the NTS and area postrema and induce the vomiting reflex⁸⁹, as discussed earlier. Moreover, in the US and Canada, gastroenteritis-induced vomiting is now commonly treated by 5-HT₃ receptor antagonists⁹⁰, but no etiology is assessed. Vomiting is a hallmark of rotavirus disease and contributes not only to dehydration but also hampers the effectiveness of the ORT⁹¹. We have previously shown that rotavirus infection and the toxin NSP4 induce 5-HT release from human EC cells and that infection in mice induces activation of NTS and area postrema, structures of the vomiting center^{30, 77} (Figure 3). If rotavirus-induced vomiting could be attenuated this would favor ORT, reduce need for intra venous rehydration, reduce hospitalization time and costs, result in faster recovery of children and prevent spread of virus, since rotavirus may be spread trough the vomits.

Figure 3. Proposed mechanism to rotavirus-induced vomiting. Rotavirus (RV) stimulates vagal afferents connected to the vomiting center in the CNS by 5-HT release from EC cells in the gut. 5-HT₃ receptor antagonists are used to attenuate vomiting in children with acute gastroenteritis.

Diarrhea

Virtually all of the fluids that enter the intestinal tract are absorbed by the body across the walls of the small intestine, through osmosis but also through active transport². The mucosa is lined of simple columnar epithelium, on wrinkles or folds called *plicae circulares*, where finger-like projections “villi” are projected. The individual epithelial cells also have many microvilli. The functions of *plicae circulares*, villi and microvilli are to increase the amount of surface area available for absorption, but also for digestion of foods since the microvilli membrane express disaccharidases and peptidases⁹². Thus, proteins, carbohydrates and lipids are digested and absorbed during the passage of the small intestine.

Intestinal epithelial cells originate from stem cells in the base of crypts and there is a continuous renewal of cells, which mature on their way to the top of villi². The lifetime of an intestinal epithelial cell is 4-7 days, but during pathological conditions the turn over may be enhanced². The small intestine is divided into 3 parts based on structural and histological differences, the duodenum, jejunum and ileum. Although most of the absorption takes place in jejunum, all segments of the intestine from duodenum to distal colon is assumed to have mechanisms for both absorbing and secreting water and electrolytes.

The mechanisms by which rotavirus induce diarrhea are not well defined but are thought to be multifactorial (Figure 4), resulting from direct effects of virus infection and by indirect effects of the infection and the host response²¹.

Figure 4. Mechanisms to rotavirus diarrhea. Decreased absorption, increased secretion, altered motility and permeability are the 4 main mechanisms proposed in rotavirus diarrhea.

Figure modified from Viral Gastroenteritis, First edition, 2003, Editors Desselberger and Grey.

Mechanisms proposed to explain rotavirus diarrhea:

- i) Malabsorption due to reduced capacity to digest and absorb nutrients and electrolytes that will result in **osmotic driven diarrhea** through such undigested food constituents reaching the colon. Malabsorptive diarrhea due to villus ischemia has also been proposed⁹³.
- ii) **Increased paracellular permeability** due to effect on tight junctions and the cytoskeleton.
- iii) **Increased intestinal motility.**
- iv) Increased active chloride secretion resulting in **secretory driven diarrhea**.

Malabsorption

The mechanism of malabsorption-associated diarrhea may involve loss of absorptive cell surface, villus ischemia, decreased digestive enzyme expression at microvilli of the apical

membrane of cells which reduces nutrient absorption and by general inhibition of Na⁺/solute co-transporter system, mainly the sodium-glucose co-transporter-1 (SGLT1)². The rotavirus effect on SGLT-1 seems to be mediated by NSP4⁹⁴.

The fact that treatment with oral rehydration solution (ORS) works well to rehydrate children, indicate enough absorption capacity of the epithelium and argue against the hypothesis of malabsorption and inhibition of fluid absorptive mechanisms.

Paracellular permeability

The control of paracellular transport and integrity of tight junctions are crucial for the epithelial function as a protective barrier. Most of rotavirus permeability studies have been performed *in vitro*, on cell monolayers, which showed a disruption of tight junctions, decrease of transepithelial resistance^{95, 96} and increased transepithelial permeability to macromolecules⁹⁶. The effect of paracellular leakage might be induced by NSP4⁹⁷. *In vivo* studies of intestinal permeability in rotavirus-infected children has been done, showing the opposite results with less permeability of polyethylene glycol molecules in urine of rotavirus infected children compared to their uninfected state⁹⁸. The same observation, no increase in permeability, has been found in animal studies⁹⁹. This phenomenon may be explained by the role of nerves in controlling the gut barrier, and demonstrates the risks with conclusion from cell culture systems, lacking the nerve innervation. The vagus nerve has also been shown to affect the intestinal permeability^{100, 101}. Indeed, vagus nerve stimulation has been shown to attenuate disruption of tight junction in intestinal epithelium in endotoxemic mice, by a mechanism that seem to involve $\alpha 7$ nicotine acetylcholine receptor ($\alpha 7$ nAChRs)¹⁰². Tightening of the epithelium may be a response to protect the host from invading pathogens.

Increased motility

The ENS as well as the vagus nerve are responsible for intestinal motility¹⁰³. Traveller's diarrhea, due to different infectious agents or toxins are usually treated with drugs that reduce the intestinal motility, and the delayed passage of intestinal content will increase the time for absorption¹⁰⁴. Loperamide is such as a drug, reducing intestinal motility by acting on opioid receptors on the myenteric plexa. However, there are contradictive studies whether loperamide reduces rotavirus diarrhea^{105, 106}. Increased motility has been observed in rotavirus infected mice⁹⁹ which was reduced by loperamide⁹⁹.

Active secretion

Direct stimulation of crypt cells⁵⁶ or indirect by the activation of ENS⁷⁶ are likely contributors to rotavirus diarrhea (Figure 5). Today there are increasing evidence for the involvement of the ENS in rotavirus diarrhea^{76,99}. The secretory component of rotavirus diarrhea was first proposed 1991, after discovery that rotavirus affect the calcium homeostasis in infected cells¹⁰⁷. Later studies showed that the rotavirus NSP4 was able to induce increase of intracellular calcium¹⁰⁸ and lead to the discovery of NSP4 as an enterotoxin, since it was shown to be able to induce diarrhea in mice⁵⁶. NSP4 and rotavirus have also been shown to induce release of 5-HT from EC cells³⁰. Since 5-HT is a potent transmitter within the ENS and also to CNS, 5-HT and its effects may be a contributor factor to rotavirus diarrhea. Additionally, prostaglandins (PGs), which can be released from enterocytes may be involved in induction of rotavirus diarrhea, since children had higher levels of PGE2 and PGF2 in stool and serum during rotavirus infection compared to their own control sera and stool¹⁰⁵. PGs and 5-HT can act on intrinsic nerves to induce release of vasoactive intestinal peptide (VIP) from nerve endings, which then stimulate active secretion by crypt cells in the base of villi¹⁰³. Pharmacological blockade of 5-HT and VIP did reduce rotavirus diarrhea in mice, supporting their participation in rotavirus diarrhea⁷⁵. Moreover, oral treatment with aspirin, which blocks PG synthesis, was shown to attenuate rotavirus diarrhea in children¹⁰⁵.

Figure 5. Hypothesis for rotavirus-induced diarrhea. Infection of EC cells and extra cellular NSP4 stimulate release of 5-HT from EC cells and activate afferent nerves. Prostaglandin (PG) formation is presumably stimulated and released from infected enterocytes and EC cells. It is shown that aspirin can attenuate rotavirus (RV) diarrhea by inhibiting PG synthesis. The submucosal plexus stimulate water and electrolyte secretion and the myenteric plexus mainly intestinal motility. Released vasoactive intestinal peptide (VIP) from nerve endings binds to crypt cell receptors and increase cAMP, leading to chloride ion and water secretion. Racecadotril, an enkephalinase (Enk) inhibitor presumably reduces intracellular cAMP and thus water secretion (diarrhea). Loperamide, an opioid-receptor agonist acts on receptors in the myenteric plexus and presumably attenuate rotavirus-induced intestinal motility and thus diarrhea.

Fever

Fever is a part of the acute-phase response by the immune system⁸¹ and is the most ancient and widely known hallmark of disease⁷⁹. The definition of fever is often referred to any increase in body temperature, but such a definition can be confusing to distinguish between true fever and hyperthermia (e.g. due to exercise, stress response)⁷⁹.

Fever is usually accompanied by sickness behavior, which includes inactivity, tiredness, depression and reduced intake of food and water^{80, 82}. It has been shown that fever enhance the immune response through increased mobility and activity of white blood cells, stimulation of IFN production and function, activation of T lymphocytes and induction of hypoferremia which has suppressive effect on the pathogens growth⁷⁹.

IL-1 β , TNF- α and IL-6 have been shown to be responsible cytokines in the induction of fever, and where IL-1 β seems to be dependent on IL-6 to induce fever^{81, 109}. Blood borne cytokines may act directly at sites lacking the blood-brain barrier, they might induce secondary cytokine production in hypothalamus or might be actively transported in there⁸¹. There is also evidence that peripheral impulses in the afferent vagus nerve can induce fever^{81, 110}.

Rotavirus infection is commonly associated with fever, although less elevated compared to bacterial infections with salmonella and shigella, which are associated with very high fever^{85, 111}. Children with rotavirus have been shown to have elevated levels of cytokines in serum and those with fever had significantly higher levels of IL-6¹¹². Although fever is common, the infection is not generally associated with signs of inflammation^{4, 21, 76, 113}. Initially cytokine response from immune cells, stimulated by pathogens, may activate the vagus nerve to induce fever and the cholinergic anti-inflammatory pathway to suppress inflammation¹¹⁴. Fever as a response to rotavirus has not been elucidated and needs to be investigated.

Inflammatory response to rotavirus infection

The inflammatory response in vertebrate organisms is a protective mechanism that limits the replication and spread of infectious agents in the host. In certain cases, where regulatory mechanisms are uncontrolled, inflammation may be deleterious to the host, resulting in toxicity and death¹¹⁵.

Rotavirus infection gives in both humans and animals a most modest inflammatory response, locally and systemically, although there is extensive tissue damage^{4, 21, 76, 113}. In contrast to many invasive bacterial gastrointestinal infections, C-reactive protein (CRP) and calprotectin, clinical biomarkers of inflammation, are not elevated during RV infection^{4, 116}.

¹¹⁷. Furthermore, abdominal pain and bloody diarrhoea are more common in intestinal bacterial infections than in RV infections¹¹⁷. How rotavirus keeps a low inflammatory response, although with extensive pathology is a key question in the research field.

The immune system is divided into the innate (non-specific) and adapted/acquired (specific) response. The adapted immune response is only found in vertebrates, and includes B-cell responses with antibody production and immunity¹¹⁸. The innate immune system is an evolutionary old defense strategy, found in very primitive organisms, indicating high importance for survival¹¹⁸. Upon a viral infection the innate immune system will be activated as a first line of defense.

Viral components are recognized by different pathogen recognition receptors (PPRs)¹¹⁹. Double-stranded RNA, which is synthesized during the replication of many viruses, is recognized by TLR-3 and retinoic-acid-inducible protein 1 (RIG-I)/melanoma differentiation-associated protein 5 (MDA-5) in a cell-type- and pathogen-type-specific manner¹¹⁹. After rotavirus infection and replication, dsRNA activates the cytoplasmatic PPR RIG-1 and the MDA-5, which in turn activate IRF3 and NF- κ B, transcription factors for interferons (IFNs)¹²⁰. Production of IFNs is important for host cell protection, by the ability to block viral replication¹³. Some rotavirus strains have however overcome the anti-viral IFNs by an antagonizing effect to IRF3 and NF- κ B by NSP1¹²⁰. Due to the importance of NF- κ B activation in cytokine induction, many viruses, have evolved strategies to actively limit NF- κ B activation¹³. Although there are many advantages for the virus to have a mechanism that prevents host-cell gene expression, there are also disadvantages as the cell will not produce IFNs or other cytokines and limit replication time since it will also suppress expression of anti-apoptotic genes and result in a more rapid cell death¹³. Furthermore, virus cannot establish a persistent or latent infection in cells with an inhibited cellular protein synthesis¹³. The full extent of NF- κ B inhibition by rotavirus and its impact upon cytokine expression requires further investigation¹²¹.

While IFN induction and signaling are required to reduce viral loads, the absence of IFNs signaling does not increase the time for viral clearance¹²²⁻¹²⁴.

Recently, the rotavirus NSP4 has been shown to be involved in the innate inflammatory response, by stimulation of the pro-inflammatory cytokines TNF- α and IL-6 from macrophages, by the PPR TLR-2²⁹.

Moreover, TLR-3 has been associated with rotavirus susceptibility, viral shedding and histological damage upregulation of epithelial TLR3 expression during infancy might

contribute to the age-dependent susceptibility to rotavirus infection³⁸. Adult mice deficient in TLR-3 or the adaptor molecule had significantly higher viral shedding and decreased epithelial expression of proinflammatory and antiviral genes as compared to wild-type animals. In contrast, neonatal mice with the same deficiencies did not display these features, but increase of TLR-3 expression was observed over time and may be related to the age-dependent susceptibility to rotavirus infection³⁸.

The pro-inflammatory response to rotavirus infection has only been briefly investigated. Although some animal^{125, 126} and human studies^{112, 127} have demonstrated an increase of some pro-inflammatory cytokines, the cytokine levels in rotavirus infection seems to be significantly lower compared to bacterial infections⁸⁵. Cytokines are not always inducing a lasting inflammatory response, they can appear initially early upon an injury/infection and activate the “cholinergic anti-inflammatory pathway”, a host protective reflex that will suppress further cytokine release and contribute to resolution and tissue repair^{114, 115, 128}.

The cholinergic anti-inflammatory pathway

The newly discovered cholinergic anti-inflammatory pathway can detect and regulate the inflammatory response, with function as "immunosensing" and "immunosuppressing"^{114, 115, 129}. When a tissue injury or inflammation occurs, cytokines are released from immune cells at the injury site. These cytokines, the pathogen itself or other mediators e.g. ischemia or stress can activate vagal afferents to the brain, where a protective feedback is initiated via outgoing efferents and release of acetylcholine from vagal nerve endings. Acetylcholine then acts on nicotinic receptors on immune cells and brake inflammation by reducing cytokine release.

This most interesting neuro-immune circuit was discovered by Kevin Tracey in the beginning of 20th century¹³⁰. Vagus nerve stimulation and activation of $\alpha 7$ nAChRs on immune cells act as a brake to suppress inflammatory responses. Previous studies have shown that vagus nerve stimulation can prevent systemic inflammation in sepsis^{130, 131}, rheumatoid arthritis¹³², prevent gut barrier failure after severe burn injury¹³³ and affect bacterial clearance in *Escherichia coli* peritonitis¹³⁴. Systemic cholinergic anti-inflammatory effects have been shown to be dependent on the spleen, ACh-producing T-cells and splenic macrophages¹³⁵. However, the circuit of local intestinal inflammation seems to differ and has been shown to be regulated independently of the spleen and T-cells¹³⁶, most likely by vagal cholinergic enteric neurons that release ACh that acts on $\alpha 7$ nAChRs on intestinal resident

macrophages¹³⁶.

A contribution of the cholinergic anti-inflammatory pathway in virus-induced inflammation and infection was until my study not reported (manuscript, paper III). Giebelen and coworkers have found that a deficiency of the $\alpha 7$ nAChR facilitates clearance of *E. coli* after i.p infection by increasing the recruitment of neutrophils¹³⁴. They suggested that the $\alpha 7$ nAChR is important in the very early response to infection.

We have recently shown that rotavirus infection in mice causes an activation of vagal afferents (vagus nerve) and activation of brain areas related to nausea and vomiting³⁰. Our hypothesis, that rotavirus stimulates the vagus nerve and activate the cholinergic anti-inflammatory system, thus down regulates the inflammatory response was investigated in different mice models *in vivo* and *in vitro* macrophages (submitted manuscript, paper III). My study presents the first evidence for participation of the vagus nerve and the $\alpha 7$ nAChR in regulating the pro-inflammatory response to a viral infection (manuscript, paper III) (Figure 6).

Figure 6. Hypothesis to how rotavirus may suppress inflammation.

Inflammatory products produced in rotavirus-infected damaged tissues (red dots) may activate afferent nerves to the NTS and dorsal motor nucleus in the brain. Vagal efferent nerves then respond by releasing acetylcholine (ACh) near gut resident macrophages. ACh interacts with $\alpha 7$ nACh receptors ($\alpha 7$ nAChR) and acts as a brake to suppress cytokine release from macrophages thus resulting in tissue repair/ limitation of tissue injury. This circuit, named the cholinergic anti-inflammatory pathway may contribute to attenuate rotavirus inflammation.

Gut-brain communication

The ENS is explained as the brain of the gut, which is controlled and protected by influence of the CNS, through the bodies longest nerve, the vagus nerve¹³⁷. Quite recent work demonstrates that gut-brain communication occurs via neural, immunological and hormonal routes⁸³.

The vagus nerve

The vagus nerve is the tenth of the twelve cranial nerves. It extends from the brain stem to the abdomen, with branches in the neck, thorax and abdomen⁸¹. The vagus nerve is a part of the autonomic nervous system (ANS), has efferent fibers, ascending signals from the brain to peripheral organs as well as sensory afferent fibers, sending information from peripheral tissue to the brain¹³⁸. The ANS consists of three components: the sympathetic (noradrenergic) and parasympathetic (cholinergic) systems, which originate in the CNS (with cell bodies in the brainstem and spinal cord) and the ENS¹³⁸. The vagus nerve is a part in the parasympathetic system but is influenced by the sympathetic nervous system, which has the opposite action, like a "fight or flight reaction"¹³⁸. Functionally, parasympathetic signals in the efferent vagus nerve reduce the heart rate, constrict the bronchi whereas the peristaltic movements and secretions are increased in the stomach and intestine, as well as from the pancreas. In the abdomen the vagus sends efferent fibers to the stomach, the small intestine and the first half of the large intestine. The visceral afferent fibers are most likely involved in sensing hunger, satiety and the general sense of physiological condition of the body, as e.g. the sickness feeling⁸¹.

In mid to late 1900 there was emerging evidence that cytokines could communicate with the brain by stimulating afferent terminals of peripheral nerves, including the vagus nerve^{139, 140}. This neural-immune interaction can directly modulate the systemic response to pathogenic invasion. Thus, activation of vagus and parasympathetic efferents during systemic stress response is a protective advantage to the host by restraining the magnitude of a potentially lethal peripheral immune response. Incidentally, 70–80% of the body's immune cells are contained within the gut-associated lymphoid tissue, reflecting the unique challenge for this part of the immune system to maintain a homeostatic balance between tolerance, inflammation and immunity in the intestine¹⁴¹. Subsets of vagal afferent nerve terminals are in close proximity to mucosal immune cells and contain receptors for signaling molecules released from these cells¹⁴¹. The role of the vagus nerve in rotavirus infection has recently been investigated (manuscript, paper III). Together with our previous findings, that rotavirus diarrhea involves the activation of the ENS^{76, 99}, stimulation of 5-HT release from EC cells and activation of the vomiting center in CNS via the vagus nerve^{30, 77}, collectively suggests a close, integrated communication within the gut-brain axis for rotavirus-induced diarrhea, vomiting and inflammation.

The enteric nervous system

In humans the ENS contains 500 million neurons¹⁰³ which regulate motility, secretion and blood supply in the GI tract. There are two major plexa, the myenteric and submucosal plexus. The myenteric plexus provides motor innervation to the circular muscle layer and the longitudinal muscle and regulates intestinal motility including peristalsis¹⁴². The principal role of submucosal plexus is to coordinate reflexes such as secretion and absorption as well as motor control of the smooth muscles. Neurons can be grouped by their functions as: intrinsic primary afferent neurons (IPANs), which are sensory neurons, motor neurons and interneurons that connect neurons between the two layers. The IPANs are located within both the submucosal and myenteric plexuses and can activate enteric reflexes that regulate motility, secretion and blood flow. Transmission from vagal input neurons to enteric neurons is mediated principally by acetylcholine acting on nicotinic cholinergic receptors, but several other transmitters are involved in these processes¹⁴².

The role of ENS in rotavirus diarrhea was first described by Lundgren et al in year 2000⁷⁶. They did perfusion in mice intestinal segments, with and without treatment with four different drugs blocking nerves within the ENS. The observation that all four drugs significantly attenuated the intestinal secretory response to rotavirus strongly suggests that the ENS participates in the rotavirus-induced electrolyte and fluid secretion. The involvement of the ENS may explain how comparatively few virus-infected cells at the villus tips can cause the intestinal crypt cells to increase their secretion of electrolytes and water. Over the years, it has become more and more evident that ENS is involved in rotavirus diarrhea^{30, 74-77}. By blocking enteric neurotransmitters, that can act to induce active secretion (e.g. VIP, 5-HT) and increase motility (e.g. 5-HT), rotavirus diarrhea was attenuated in mice pups⁷⁵. Moreover, loperamide a drug acting on myenteric neurons, and atropine, acting on muscarinic acetylcholine receptors on nerves and enteric muscle cells, both attenuated diarrhea of infant mice⁹⁹, further supporting the role of ENS in rotavirus diarrhea.

Enterochromaffin (EC) cells

EC cells are specialized sensory cells in the intestinal epithelium, located predominately in the lower part of villi. These cells are thought to “taste” and “sense” the intestinal lumen and can respond by releasing transmitters to underlying neurons¹⁴².

EC cells are widely distributed along the GI tract and are found in the mucosa of the gastric antrum, duodenum, jejunum, ileum, appendix, colon and rectum¹⁴³. The cytoplasm of the EC

cells contains a large number of secretory granules, which are storage sites of secretory products. The main secretory product of EC cells is 5-HT and the EC cells account for more than 90% of all 5-HT synthesized in the body. Minor amounts of peptide hormones, e.g. tachykinins, enkephalins, motilin¹⁴³ and PGs¹⁴² may also originate from EC cells. 5-HT is synthesized from the amino acid tryptophan and transported into granules¹⁴³. Following stimulation by diverse agents e.g. hyperosmolarity, carbohydrates, mechanical distortion of the mucosa, cytostatic drugs, cholera toxin^{144, 145} and rotavirus³⁰, EC cells mobilize intracellular calcium and release 5-HT¹⁴⁶. 5-HT is involved in the regulation of gut motility, intestinal secretion, blood flow and several GI disorders¹⁴⁷⁻¹⁵¹ including rotavirus diarrhea³⁰, chemotherapy-induced nausea and vomiting^{152, 153} and *Staphylococcal* enterotoxin-induced vomiting¹⁵⁴. Rotavirus infection and the rotavirus toxin NSP4 have been shown to stimulate human EC cells *in vitro* and *ex vivo*, to release of 5-HT³⁰. Intracellularly expressed NSP4 can stimulate 5-HT release from EC cells, an effect that was attenuated by the intracellular calcium chelator BAPTA/AM (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) (manuscript, paper IV). Moreover, rotavirus has been shown to be able to infect EC cells in mice small intestine³⁰.

Treatment and prevention

The golden standard for treatment of rotavirus gastroenteritis is ORT, with a sugar-salt solution, recommended by WHO¹⁵⁵. Furthermore, WHO recommend that children should simultaneously with ORT, also receive zinc treatment which reduces the duration and severity of diarrhea episodes, stool volume and the need for advanced medical care. Zinc inhibits the cAMP mediated Cl⁻ secretion by blocking ion channels at the basolateral side of the epithelial cells^{156, 157}. Moreover, WHO states that breast milk is an excellent rehydration fluid and breastfeeding should be continued along with ORS therapy. Non-breastfeeding children with diarrhea should continue to be fed during the diarrhea, since food intake supports fluid absorption from the gut into the bloodstream, thus preventing dehydration and helps maintaining nutritional status and the ability to fight infection.

To prevent rotavirus infections and decrease mortality, WHO recommends that vaccines should be included in all national immunization programs and considered a priority particularly in countries with high rotavirus gastroenteritis-associated fatality rates, such as in south and south-east Asia and sub-Saharan Africa¹⁵⁵.

Live attenuated vaccines were tested in early 1980 and the first commercial vaccine

RotaShield®¹⁵⁸ was introduced in 1989, but was withdrawn due to its association with intussusception, a medical condition in which a part of the intestine folds into another section of intestine (invagination). In 2006, the two current rotavirus vaccines, Rotarix®, a live attenuated human single-strain vaccine and RotaTeq®, a live vaccine containing five bovine–human reassortant strains, were introduced. Both are oral vaccines and protects well against severe rotavirus disease. The efficacy has been shown to be high in European countries and USA (>90%), less protective in African countries (50-80%). Recently, in March 2015, an Indian oral vaccine Rotavac®, based on a strain isolated from a neonate from Dehli, who shed the virus but had none of the symptoms was licenced¹⁵⁹. This is the cheapest rotavirus vaccine and clinical trials have shown Rotavac® to have similar efficacy to Rotarix® and RotaTeq®¹⁵⁹. Rotavirus vaccines have however yielded different efficacy when tested in different populations, since population and environmental factors may account for these differences, study design characteristics should also be considered¹⁶⁰.

Today more than 20 countries have introduced rotavirus vaccination in their national immunization centers and the mortality has decreased from 850.000 to 450.000 deaths per year, many of which but not all could be attributed to the vaccination¹⁵⁸. In European countries the introduction of rotavirus vaccine in national immunization program is slow due to less mortality and low cost–benefit. A future alternative treatment strategy can be to prevent dehydration by reducing vomiting through use of 5-HT₃ receptor antagonists. In the US and Canada 5-HT₃ receptor antagonist are already used to treat vomiting in children with acute gastroenteritis, although no etiology is done⁹⁰.

Aims of thesis

The overall aim of this thesis was to study the pathophysiological mechanisms how rotavirus causes disease symptoms.

Specific aims:

- **Study the role of enterochromaffin cells in rotavirus diarrhea and vomiting.**
- **Study if increased intestinal motility and/or increased permeability are contributing factors of rotavirus-induced diarrhea.**
- **Study the role of the vagus nerve and cholinergic anti-inflammatory pathway in the inflammatory response to rotavirus infection.**

Results and discussion of the papers

Paper I

Rotavirus stimulates release of serotonin (5-HT) from human enterochromaffin cells and activates brain structures involved in nausea and vomiting.

Rotavirus can cause severe dehydration and death. While most deaths occur due to excessive loss of fluids and electrolytes through vomiting and diarrhea, the pathophysiological mechanisms that underlie vomiting remain to be clarified. It has been shown that drugs that inhibit the function of the ENS also could reduce symptoms of rotavirus disease in mice^{75, 76}.

In this study we have addressed the hypothesis that rotavirus infection triggers the release of 5-HT from EC cells in the small intestine, leading to activation of vagal afferent nerves connected to brain structures associated with vomiting, the NTS and area postrema in the brain stem. Our experiments showed that rotavirus can infect and replicate in human EC tumor cells *ex vivo* and *in vitro* and are localized to both EC cells and infected enterocytes in the close vicinity of EC cells in the jejunum of infected mice. Purified NSP4 evoked release of 5-HT within 60 minutes and increased the intracellular calcium concentration in a human midgut carcinoid EC cell line (GOT1) and *ex vivo* in human primary carcinoid EC cells concomitant with the release of 5-HT. When we administered 5-HT i.p to infant mice, all 7 mice pups responded with diarrhea within 60 minutes. Moreover, rotavirus infection in mice induced Fos expression in the NTS, similar as seen in animals that vomit after e.g. administration of chemotherapeutic drugs^{48, 161}.

The results of the present study strongly suggest that rotavirus per se and/or NSP4 released from adjacent virus-infected enterocytes increase intracellular calcium concentrations and induce 5-HT release from EC cells. We propose that released 5-HT activates both nerves within ENS and vagal afferent nerves located in close vicinity to EC cells. Activation of vagal afferents to the vomiting center triggers the emetic reflex. It is apparent that EC cells and nerves play an important role in rotavirus induced diarrhea and vomiting and the present

findings may be of more general importance for our understanding of pathophysiological mechanisms of many different types of infection-induced diarrhea and vomiting.

Our observations may be of clinical importance, since a possibility to reduce virus-induced vomiting by 5-HT₃ receptor antagonists will favor ORT, attenuate fluid loss, reduce the need for intra venous therapy, reduce hospitalizations and reduce the viral spread. In US and Canada, all kind of gastroenteritis-induced vomiting is treated with 5-HT₃ receptor antagonists, but no etiology is done⁹⁰.

Paper II

Rotavirus infection increases intestinal motility but not permeability at the onset of diarrhea.

Several disease mechanisms, such as malabsorption, altered permeability, motility disturbances, involvement of ENS and effect of NSP4 have been associated with fluid loss in rotavirus diarrhea^{25, 30, 74-77}. Some of these mechanisms may indeed occur after the onset of diarrhea rather than being the actual cause. Only a few studies have addressed if rotavirus induce intestinal motility and thereby if that contribute to diarrhoea.

In this study, we have investigated how intestinal motility and epithelial permeability are related to the onset of rotavirus diarrhea. These two important disease- associated mechanisms have not previously been studied together in the early phase of rotavirus diarrhea. Motility of the small intestine, as in all parts of the digestive tract, is controlled predominantly by excitatory and inhibitory signals from the myenteric plexus in ENS, however modulated by inputs from the CNS⁹⁹. It is well established that stimulating vagal efferent nerves to the gut increases intestinal motility.

To study motility, fluorescein isothiocyanate (FITC) -dextran 4kDa was given to rotavirus-infected and non-infected adult and infant mice. Mice were also treated with the opioid receptor agonist loperamide, a widely used antidiarrheal agent, attenuating intestinal contractions by acting on receptors in the myenteric plexus^{162, 163}. Atropine, a muscarinic receptor antagonist, can block this effect from nerve endings and was also used in this work. To investigate whether rotavirus increases permeability at the onset of diarrhea, two different sized markers of fluorescent 4- and 10- kDa dextran were given to infected and non-infected mice, and fluorescence intensity was measured in serum.

We found that rotavirus increased the motility in infant mice, detected at 24 h p.i. and persisted up to 72 h p.i. Moreover, both loperamide and atropine decreased the intestinal motility and attenuated diarrhea. Analysis of passage of fluorescent dextran from the intestine into serum indicated unaffected intestinal permeability at the onset of diarrhea (24 to 48 h p.i.). These results indicate that rotavirus-induced diarrhea is associated with

increased intestinal motility via activation of the myenteric nerve plexus, but the event is not associated with increased intestinal permeability.

Our results are similar to other human studies of permeability during rotavirus infection. Stintzing *et al.* investigated intestinal permeability in young children with rotavirus infection using polyethylene glycols (PEG) of different molecular weight⁹⁸. PEG was given orally to 9 children having acute rotavirus diarrhoea and 6 h urine was collected and PEG concentrations were determined. The same children served as their own control 3–5 weeks later. A significantly low urinary recovery of PEG was noted in the urine during the acute phase of diarrhoea in comparison to high urinary recovery of PEG among the same children 3–5 weeks later. Similar observations have been made by Serrander *et al.*¹⁶⁴. Moreover, Johansen *et al.*¹⁶⁵ found that children with acute rotavirus infection excreted significantly less PEG of all sizes than control children and children with *enteropathogenic Escherichia coli* infections. Permeability studies performed *in vitro*, on cell monolayers, have however showed a disruption of tight junctions, decrease of transepithelial resistance^{95, 96} and increased transepithelial permeability to macromolecules⁹⁶. The significant discrepancies between *in vivo* and *in vitro* studies is most likely due to the absence of hormone regulation, nerves and authentic non-transformed cells in the *in vitro* systems.

Moreover, it is interesting that intestinal permeability have in several observations been found to be controlled by the vagus nerve^{101, 166}, further supporting participation of nerves in rotavirus disease.

Our observations from this study may contribute to a better understanding of the mechanisms involved in rotavirus diarrhea. This work was selected by the editors of *Journal of Virology* for inclusion in "Spotlight" a feature that highlights research articles of significant interest.

Paper III

The cholinergic anti-inflammatory pathway contributes to the limited inflammatory response following rotavirus infection.

While rotavirus infections is characterized by extensive lesions of the small intestinal epithelium, the inflammatory response is low^{4, 21, 76, 113}, with limited abdominal pain, no bloody diarrhea and no elevated levels of C-reactive protein (CRP) or calprotectin^{4, 116, 117}, clinical markers of inflammation. The question why the inflammatory response remains low during an extensive infection has frequently been raised but remain unresolved.

The role of the vagus nerve and $\alpha 7$ nAChRs in the inflammatory response to rotavirus infection were explored in $\alpha 7$ nAChR gene-deficient mice, vagotomized mice and wild-type mice treated with the $\alpha 7$ nAChR antagonist mecamylamine. Following oral inoculation with murine rotavirus, the levels of TNF- α , IL-1 β and IL-6 were measured in serum, spleen, duodenum, ileum and jejunum at 48 h p.i.. To determine if modulation of the inflammatory response affects virus shedding, the $\alpha 7$ nAChR was blocked with mecamylamine in infected and non-infected mice and virus was quantified in faeces. To investigate whether stimulation of the $\alpha 7$ nAChR pathway could attenuate the release of pro-inflammatory cytokines, mouse peritoneal macrophages and human blood monocyte-derived macrophages, were treated with nicotine before stimulation with rotavirus toxin NSP4.

Our results show that stimulation of the vagus nerve and $\alpha 7$ nAChR-mediated signaling attenuate the pro-inflammatory response during rotavirus infection and blockade of $\alpha 7$ nAChR reduce virus shedding in infected mice. The most prominent cytokine IL-6 was significantly increased in duodenum and serum of vagotomized mice and in jejunum and spleen of $\alpha 7$ nAChR knock out mice. Similarly, nicotine attenuated the release of TNF- α and IL-6 from macrophages stimulated by NSP4 *in vitro*.

Previous studies have shown that vagus nerve stimulation can prevent systemic inflammation in sepsis^{130, 131} and rheumatoid arthritis¹³², prevent gut barrier failure after severe burn injury¹³³ and affects bacterial clearance in *Escherichia coli* peritonitis¹³⁴. Moreover, De Jonge and coworkers have shown that vagus nerve stimulation prevented surgery-induced intestinal inflammation and improved postoperative ileus¹⁶⁷, suggesting

that maintenance of intestinal immune homeostasis involves participation of the vagus nerve through the ENS and that resident macrophages of the intestinal muscularis layer that express $\alpha 7$ nAChRs are the target of the gastrointestinal cholinergic anti-inflammatory pathway.

In conclusion, our study presents evidence for participation of the vagus nerve and the $\alpha 7$ nAChR in regulating the pro-inflammatory response to a viral infection. Our previous findings are that rotavirus diarrhea involves activation of the ENS, stimulation of 5-HT release from human EC cells and activation of the vomiting center in CNS via the vagus nerve. Collectively these observations suggest a close, integrated communication within the gut-brain axis in rotavirus-induced diarrhea, vomiting and inflammation.

Paper IV

Intracellularly expressed rotavirus NSP4 stimulates release of serotonin (5-HT) from human enterochromaffin cells.

In paper IV we studied which of the intracellularly expressed rotavirus protein(s) being responsible for the stimulation of the neurotransmitter 5-HT.

While rotavirus has been shown to infect and stimulate secretion of 5-HT from human EC cells and to infect EC cells in the small intestine of mice, the role of intracellular viral protein(s) responsible for this property remains to be identified. We have previously shown that extracellular NSP4 can stimulate release of 5-HT from human EC cells *in vitro*, but since rotavirus also can infect EC cells directly, the importance of intracellularly expressed NSP4 and/or contribution of other viral proteins was investigated in this study.

To address this issue, human EC cells were transfected with small interfering RNA (siRNA) targeting major structural (VP4, VP6 and VP7) and the non-structural (NSP4) viral proteins (VP) followed by infection with rhesus rotavirus and measuring of 5-HT response by ELISA. Moreover, to determine whether infection modulate expression of the serotonin reuptake transporter (SERT) or the synthesis of 5-HT, mRNA expression of SERT and the rate-limiting enzyme tryptophan hydroxylase-1 (TPH1) were determined in the small intestine of infected and uninfected infant mice. Moreover, confocal microscopy was used to determine if rotavirus infection re-organize 5-HT distribution in EC cells.

We showed that intracellularly expressed NSP4 stimulated 5-HT secretion from human EC cells *in vitro* and that NSP4 accounted for the majority of the 5-HT response, compared to the other investigated viral proteins. We also demonstrate a significant difference in the localization of 5-HT in rotavirus-infected cells compared to uninfected cells, with intense granular appearance in the periphery of the infected cells and a more diffuse 5-HT appearance in uninfected cells. We speculate that an infection of EC cells promotes translocation of 5-HT from the cytoplasm to secretory granules followed by release of 5-HT basolaterally.

Similar to Kerckhoffs and co-workers observation of no difference in *TPH1* mRNA expression in duodenum of IBS patients¹⁶⁸, we did not find any statistical difference in *TPH1* mRNA

expression levels in the small intestine of rotavirus-infected mice. The unaffected TPH1 mRNA expression in the investigated intestinal segments, suggests that the release of 5-HT primarily occurs from pre-made 5-HT rather than from newly synthesized 5-HT. Further support for this is the rapid release of 5-HT following rotavirus stimulation.

Moreover, down-regulation of SERT mRNA in ileum presumably resulted in reduced re-uptake of 5-HT by SERT to EC cells and thus increased extracellular 5-HT in the small intestine.

SERT regulates the bioavailability of 5-HT^{169, 170} and medical consequences of a deficient function may result in sensory (e.g. pain) and secretory (diarrhoea) symptoms. Two clinical studies have reported a reduction in 5-HT reuptake due to reduced levels of SERT in patients with IBS and ulcerative colitis^{147, 171}. In addition similar results have been shown in mice where decreased SERT expression was observed in the small intestine of mice infected with enteropathogenic *Escherichia coli*¹⁷².

Altogether our observations propose that intracellularly expressed NSP4 have neurotransmitter (5-HT) -stimulating properties and that rotavirus infection of EC cells results in down-regulation of SERT mRNA.

These new observations add additional information to understanding of rotavirus diarrhea, and a disease mechanism to rotavirus diarrhoea is proposed.

Concluding remarks

Focusing on rotavirus pathophysiological mechanisms my thesis work have shown that:

Rotavirus infection in mice induces a tightening of the epithelium at the time of diarrhea onset, indicating that infection-caused diarrhea is not necessary associated with increased permeability. Rotavirus infection in mice affects initially the intestinal permeability, but not at the onset of diarrhea, when there is rather a tightening of the mucosal barrier.

There is an increase of intestinal motility during rotavirus infection, which was attenuated by drugs acting on nerves in the myenteric plexus of ENS; supporting our hypothesis that rotavirus diarrhea involve activation of enteric nerves.

Rotavirus infection in mice activate brain structures involved in nausea and vomiting and both extracellularly released- and intracellularly expressed- NSP4 stimulate EC cells to release 5-HT, which is a potent activator of the vagus nerve and vomiting reflex in humans.

The limited inflammatory response to rotavirus infection might partly be due to activation of the cholinergic anti-inflammatory pathway, since vagotomy and lack of the $\alpha 7$ -nACh receptor results in increased inflammatory response during rotavirus infection. These results contribute to the understanding of the limited inflammatory response to rotavirus infection.

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