J Physiol 598.23 (2020) pp 5317–5332

The Journal of Physiology

Submucosal enteric neurons of the cavine distal colon are sensitive to hypoosmolar stimuli

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Edited by: Kim Barrett & Michel Neunlist

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Key points

- Neurons of the enteric submucous plexus are challenged by osmolar fluctuations during digestion and absorption of nutrients.
- Central neurons are very sensitive to changes in osmolality but knowledge on that issue related to enteric neurons is sparse.
- The present study focuses on investigation of osmosensitivity of submucosal neurons including potential molecular mediating mechanisms.
- Results show that submucosal neurons respond to hypoosmolar stimuli with increased activity which is partially mediated by the transient receptor potential vanilloid 4 channel.
- We provided important information on osmosensitive properties of enteric neurons. These data are fundamental to better explain the nerve-mediated control of the gastrointestinal functions during physiological and pathophysiological (diarrhoea) conditions.

Abstract Enteric neurons are located inside the gut wall, where they are confronted with changes in osmolality during (inter-) digestive periods. In particular, neurons of the submucous plexus (SMP), located between epithelial cells and blood vessels may sense and respond to osmotic shifts. The present study was conducted to investigate osmosensitivity of enteric submucosal neurons and the potential role of the transient receptor potential vanilloid 4 channel (TRPV4) as a mediator of enteric neuronal osmosensitivity. Therefore, freshly dissected submucosal preparations from guinea pig colon were investigated for osmosensitivity using voltage-sensitive dye and Ca^{2+} imaging. Acute hypoosmolar stimuli (final osmolality reached at ganglia of 94, 144 and 194 mOsm kg^{-1}) were applied to single ganglia using a local perfusion system. Expression of TRPV4 in the SMP was quantified using qRT-PCR, and GSK1016790A and HC-067047 were used to activate or

Dr Patrick Kollmann studied biology at the Technical University of Munich and during his PhD at the chair of human physiology investigated the osmosensitivity of enteric neurons using high-speed imaging techniques. As part of the research–training group GRK1482 he worked in an interdisciplinary environment focused in metabolic and inflammatory diseases. His passion for high-speed imaging techniques led him to Vetter Pharma-Fertigung GmbH, where he is responsible for the automated inspection of parenteral drugs. **Kristin Elfers, PhD** studied veterinary medicine at the University of Veterinary Medicine, Hanover, and prepared her PhD thesis on the effect of alimentary nitrogen reduction on mineral metabolism in goats, at the Institute of Physiology and Cell Biology. She started her postdoctoral career in the newly founded working group of Gemma Mazzuoli-Weber and learned imaging techniques to study enteric neuronal activity. In the future, she wants to address the question of how this activity is modulated by shift work.

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DOI: 10.1113/JP280309

block the receptor, respectively, revealing its relevance in enteric osmosensitivity. On average, 11.0 [7.0/17.0] % of submucosal neurons per ganglion responded to the hypoosmolar stimulus. The Ca^{2+} imaging experiments showed that glia responded to the hypoosmolar stimulus, but with a delay in comparison with neurons. mRNA expression of TRPV4 could be shown in the SMP and blockade of the receptor by HC-067047 significantly decreased the number of responding neurons (0.0 [0.0/6.3] %) while the TRPV4 agonist GSK1016790A caused action potential discharge in a subpopulation of osmosensitive enteric neurons. The results of the present study provide insight into the osmosensitivity of submucosal enteric neurons and strongly indicate the involvement of TRPV4 as an osmotransducer.

(Resubmitted 12 June 2020; accepted after revision 24 August 2020; first published online 3 September 2020)
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Introduction

The gastrointestinal tract (GIT) is the only organ containing an intrinsic nervous system able to mediate reflexes in the complete absence of central nervous system input. This is called the enteric nervous system (ENS). Neurons of the ENS are bundled in ganglia, which again are organized in the myenteric plexus (MP) and the submucosal plexus (SMP). Ganglia are connected to each other and to effector systems through fibre tracts. In addition to neurons, a similar number of enteric glia are present in the ENS, neighbouring the neurons. The main task of the MP, which is located between the circular and longitudinal muscle layers of the gut is the regulation of intestinal motility. The SMP instead is located beneath the mucosa and is largely responsible for the regulation of epithelial functions including secretion and absorption (Furness, 2000; Gwynne & Bornstein, 2007). Neurons of the ENS can be activated by various stimuli, detecting mechanical deformation as well as intraluminal chemical stimuli (Bertrand *et al.* 1997; Neunlist *et al.* 1999; Schemann *et al.* 2002; Mazzuoli & Schemann, 2009, 2012; Mazzuoli-Weber & Schemann, 2015a; Kugler *et al.* 2018). The simplified classification of enteric neurons into sensory neurons, motor neurons and interneurons has been revised over the last few years. The finding that not only intrinsic primary afferent neurons, but also motor and interneurons, show sensitivity to mechanical stimuli, has had a major impact on the field of enteric neuroscience, leading to the awareness of so-called multifunctional enteric neurons with more than one functional property (Mazzuoli & Schemann, 2009).

A critical parameter that may also influence the behaviour of enteric neurons but has not been studied so far, is the extracellular osmolality. Physiological values of plasma osmolality in mammals cluster around 300 mOsm kg^{-1} with physiological fluctuations of 1–3 % (Baylis, 1983; Bourque, 2008). Decreased extracellular osmolality leads to an inflow of water into the cytoplasm and therefore cell swelling until the point where the hydrostatic pressure equals the osmotic pressure. Excessive changes in cell volume interfere with the integrity of the cell membrane and the cytoskeletal architecture. In addition, even small changes in intracellular water content have profound influences on protein function and cellular performance due to disturbances in the complex intracellular communication network. This is caused by the dilution of messenger molecules and ions (Pasantes-Morales & Tuz, 2006; Lang, 2007). Therefore, in vertebrates, plasma osmolality is maintained within a narrow range. This is achieved by behavioural regulation of water intake and control of renal water and electrolyte excretion. Specialized neurons in the organum vasculosum lamina terminalis (OVLT) function as osmoreceptors, sensing extracellular osmolality, responding with increased neuronal activity when extracellular osmolality increases, leading to systemic osmoregulation via anti-diuretic hormone and behavioural adaptation (Vivas *et al.* 1990; Nielsen *et al.* 1995; Ciura & Bourque, 2006; Bourque, 2008). Additionally, cells of the OVLT also send processes to thalamic areas where signals are further processed and relayed to brain areas where the sensation of thirst is generated and behavioural responses are coordinated (Bourque, 2008). Amongst the above-mentioned central osmosensors, peripheral sensors exist, for example, in the oropharyngeal cavity, the splanchnic nerves, the hepatic portal vein and the liver, and it has also been discussed that such receptors are present in the GIT, e.g. in the mesenteric-portal area or in the stomach (Baertschi & Vallet, 1981; Adachi, 1984; Choi-Kwon & Baertschi, 1991; Baertschi & Pence, 1995; Carlson *et al.* 1997; Kuramochi & Kobayashi, 2000). Nevertheless, all of these osmosensory mechanisms require the CNS, where the effector neurons, the magnocellular neurosecretory cells, are located. This route of action, which also involves the humoral pathway, leads to a delay between detected osmotic shifts and homeostatic response. Thus, the existence of local regulatory mechanisms in distinct organs would be reasonable for speeding up local responses to

osmotic shifts recovering physiological osmotic conditions as fast as possible. Such an intrinsic regulatory circuit is conceivable in the GIT because the gut is the primary site where osmotic perturbations related to nutrient and water ingestion and absorption take place. After food ingestion and connected changes in luminal osmolality, return to isotonicity is reached by bidirectional movement of water and electrolytes across the intestinal wall also passing neurons of the ENS, and hence challenging them with osmotic stimuli (Leiper, 2015). Although under physiological conditions osmolality is tightly monitored by sophisticated homeostatic mechanisms, changes in plasma osmolality occur within a certain range (Gill *et al.* 1985). For example, drinking 789–1044 ml of water lowers plasma osmolality in dehydrated humans by 5–6 mOsm kg^{-1} within 30 min (Geelen *et al.* 1996). Bearing in mind that this value was measured on a systemic level, osmotic perturbations at a local level can be expected to be much higher. This assumption is supported by the fact that osmolality in portal vein blood was reduced to a significantly greater amount than plasma osmolality (292.7 $\pm$ 4.7 mOsm kg^{-1} and 304.4 $\pm$ 6.9 mOsm kg^{-1}, respectively) after intraduodenal water infusion in mice (McHugh *et al.* 2010). Here also the osmolality was measured at the portal vein level, thus, we can hypothesize that this osmotic shift in the small blood vessels of the gut will be much higher. In an older study, calculations of tissue osmolality during glucose absorption indicated an increase in villus osmolality of 150–200 mOsm kg^{-1} and 79–90 mOsm kg^{-1} in the submucosa (Bohlen, 1982). Additionally, it has been shown that changes in intraluminal osmolality after food intake affects gastrointestinal motility, independent of the caloric load of the meal (Keinke *et al.* 1984). Furthermore, increased osmolality of a non-caloric mannitol meal altered the jejunal motor activity in dogs (Schemann & Ehrlein, 1986). These findings, together with the knowledge about the sensory and integrative capabilities of the ENS, strongly suggest that it harbours local osmosensitive mechanisms.

On a cellular basis, central osmoreceptors react to changes in cell volume induced by either hypo- or hypertonic stimuli with changes in membrane cation conductance, indicating that mechanosensitivity plays a key role in osmosensory transduction (Oliet & Bourque, 1996; Ciura & Bourque, 2006; Sharif Naeini *et al.* 2006). Non-selective cation channels belonging to the family of transient receptor potential vanilloid (TRPV) channels are involved in the transduction of this stimulus (Oliet & Bourque, 1996; Ciura & Bourque, 2006; Sharif Naeini *et al.* 2006; Zhang *et al.* 2007) with TRPV2 and 4 playing a role in central osmoreception (Liedtke *et al.* 2000; Strotmann *et al.* 2000; Liedtke & Friedman, 2003; Liedtke, 2006; McHugh *et al.* 2010). The involvement of TRPV channels in central osmoreception suggests that they might also play a role in osmotransduction in the GIT. Given the combination of its osmosensitive properties mentioned above and its functional expression in enteric neurons, the most promising candidate is TRPV4 (Fichna *et al.* 2015). So far, only rare data exist on osmosensitivity in the ENS with a focus on myenteric neurons (Dong *et al.* 2015). Data on osmosensitive features of submucosal neurons are even sparser. We hypothesize that neurons of the SMP, which are strategically located between the intestinal lumen and the small enteric blood vessels, could play an important role in osmosensitivity.

Therefore, the present study was performed to test the hypothesis that submucosal neurons of the guinea pig colon are sensitive to hypoosmolar stimuli and to characterize these neurons on a functional and molecular level. We tested three different hypoosmolar stimuli to understand the number of neurons involved and their type of responses. We studied their functional responses using voltage- and Ca^{2+}-sensitive dye techniques coupled with fast neuroimaging recordings. For pharmacological experiments, we used preferentially a strong hypoosmolar stimulus as a tool to challenge all osmosensitive enteric submucosal neurons present in the ganglia of choice. In addition, the presence of TRPV4 in submucosal neurons was investigated by qRT-PCR and the functional involvement of this channel was investigated by applying a specific activator (GSK1016790A) and antagonist (HC-067047) of TRPV4.

This study describes osmosensitivity in neurons of the SMP and provides valuable mechanistic insight into the process of osmotransduction in the ENS by pharmacological experiments. The data provide a better understanding of local osmosensitive regulatory circuits in the ENS which can be critical not only in physiological but also in pathological (e.g. secretory diarrhoea) conditions.

Material and methods

Ethical approval

Protocols for killing the guinea pigs were approved by the Animal Welfare Commissioner of TUM School of Life Sciences, are in line with methods stated in Annex IV of Directive 2010/63/EU and German animal welfare law and conform to the principles and regulations as described in the Editorial by Grundy (Grundy, 2015).

Protocols for killing the guinea pigs which were used for additional experiments with an isoosmolar solution with reduced NaCl content and those used for experiments with an intermediate hypoosmolar stimulus, were approved by the Animal Welfare Commissioner of the University of Veterinary Medicine Hannover (Hannover, Germany) and were also in line with methods stated in Annex IV of Directive 2010/63/EU and German animal welfare law. The investigators understand the ethical principles under

which the journal operates and confirm that this work complies with the animal ethics checklist.

Animals and tissue preparation

Male and female Dunkin Hartley guinea pigs (Envigo RMS GmbH, Horst, Netherlands; animals for additional experiments: Institute for Physiology and Cell Biology, University of Veterinary Medicine Hannover) weighing 399 ± 137 g were group-housed (two to five animals per group) in an approved animal housing facility under standardized conditions (20–24°C room temperature, 60 % humidity and a day:night cycle of 12:12 h) at the Chair of Human Biology for at least 1 week after arrival. The animals for additional experiments were bred and kept under the same conditions as stated above in the approved animal breeding and housing facility of the Institute for Physiology and Cell Biology, University of Veterinary Medicine Hannover. Animals received a pelleted standard diet (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany and ssniff Spezialdiäten GmbH, Soest, Germany) and drinking water *ad libitum*. They were killed by percussive blow to the head or by a spring-loaded captive bolt device leading to concussion followed by exsanguination.

The abdominal cavity was opened along the linea alba and 2 cm of the distal colon (approximately 2 cm distal from the colonic flexure) were quickly removed and further dissected in cold, carbogen-aerated (95 % CO_2, 5 % O_2, pH 7.40) Krebs solution containing (in mM): 117 NaCl, 11 glucose, 4.7 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$. The mucosa was gently removed to avoid damage to the plexus lying beneath. The submucosal plexus was carefully pulled off the subjacent circular muscle layer and pinned over the rectangular opening (2 × 1 cm) of a silicone ring with the mucosal side facing upwards. This ring was placed in a recording chamber continuously perfused with 37°C aerated Hepes solution containing (in mM) 136 NaCl, 10 glucose, 5 KCl, 10 Hepes, 1.2 $MgCl_2$, 2.5 $CaCl_2$ (pH 7.40) at a rate of 11 ml min^{-1}. Hypoosmolality of the Hepes solution was established by reduction of the NaCl content to 33 mM (94 mOsm kg^{-1} H_2O), 58 mM (144 mOsm kg^{-1} H_2O) or 83 mM (194 mOsm kg^{-1} H_2O). For a set of control experiments a solution with a reduced NaCl content of 33 mM which osmolality was restored to approximately 300 mOsm kg^{-1} H_2O by addition of 200 mM mannitol was used.

Measurement of the plasma osmolality of the guinea pig

One millilitre of arterial blood was collected from the carotids during exsanguination using Li-Heparin-containing vacutainers (Sarstedt AG & Co, Nürmbrecht, Germany). After centrifugation the supernatant was checked for turbidity. Only plasma samples free from turbidity were used for osmolality measurement, with a freezing point osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany).

Neuroimaging

For the detection of action potentials from enteric neurons an ultrafast neuroimaging technique was used. This technique, allowing the recording of neuronal activity with a high spatial and temporal resolution, was previously described in detail (Michel *et al.* 2011; Mazzuoli-Weber & Schemann, 2015b; Filzmayer *et al.* 2020). Individual ganglia of the SMP were stained with 20 μM di-8-ANEPPS (1-(3-sulfanatopropyl)-4-[beta[2-(di-n-octylamino)-6-naphthyl]vinyl] pyridinium betaine) by local intraganglionic application through a microinjection glass pipette. Di-8-ANEPPS was dissolved in DMSO (Acros Organics, Geel, Belgium) and Pluronic F-127-containing Krebs solution. Staining does not affect the electrophysiological properties of the neurons (Neunlist *et al.* 1999). The perfusion chamber containing the preparation was mounted on an Olympus IX 71 epifluorescence microscope (Olympus, Hamburg, Germany). Illumination of the preparation was achieved using a green high power LED (LE T A2A true green (521 nm) 700 mA; OSRAM GmbH, Munich, Germany) powered by a software-operated, self-manufactured LED driver and a filter-set containing a 545/30 nm bandpass excitation filter (AHF Analysetechnik AG; Tübingen, Germany), a dichroic mirror with a separation wavelength of 565 nm and a 560/15 nm bandpass emission filter (AHF). Due to the high light intensity needed for a feasible signal-to-noise ratio, oil immersion objectives with a high numerical aperture were used (Olympus UApo 40× OI3/340 Oil NA 1.35–0.5 and Olympus UPlanApo 100x Oil NA 1.35–0.5 Olympus Corporation, Tokyo, Japan). Signals were detected using an ultrafast charge coupled device (CCD) camera (NeuroCCD-SMQ imaging system, RedShirtImaging LLC, Decatur, GA, USA) which, due to its framerate of 1 kHz, reliably resolved action potentials. Together with the magnification of the microscope, recordings with the 40× or 100× objectives resulted in a spatial resolution of 30 or 4 μm^2 per pixel, respectively. Fluorescence signals were detected and analysed using the NeuroPlex 8.3 software (RedShirt Imaging). For the initial investigation of osmosensitivity and the response kinetics, an interval recording paradigm with four distinct recording periods (one 2 s recording under baseline conditions, and three following 2 s recording periods under hypotonic conditions; each recording period was separated by a 8 s non-recording interval, see Fig. 1*A*) was used. This was done in order to cover a long period under

stimulated conditions while reducing phototoxicity. A more detailed examination of the latency of the response was performed using a single recording paradigm, which lasted 12 s (2 s under baseline conditions and 10 s under hypotonic conditions, see Fig. 1*B*). The effect of the TRPV4 activator was tested using a 4 s continuous recording paradigm. Before and after osmotic shifts or a pharmacological stimulus, the overall viability of the ganglion of interest was tested using electrical stimulation with a monopolar point electrode positioned on an interganglionic fibre evoking fast excitatory postsynaptic potentials (EPSPs) in postsynaptic neurons by delivering rectangular constant current pulses of 20–90 μA with a duration of 600 μs. For the analysis, only ganglia that displayed similar fast EPSPs in response to the second electrical stimulation, following an osmotic or pharmacological stimulus, were considered. To evaluate the effect of HC-067047, a potent and specific inhibitor of TRPV4 (Everaerts *et al.* 2010), on the overall excitability of enteric ganglia, they were stimulated electrically before and after perfusion of the blocker and the amplitude of the resulting fast EPSPs were compared. Neuronal responses to hypoosmolar stimuli were evaluated under the influence of HC-067047. To account for a possible desensitization, these experiments were conducted in an unpaired design: neuronal responses to hypoosmolar stimulation after perfusion with the antagonist were compared with responses of another set of neurons stimulated by hypoosmolar solution without prior addition of HC-067047. The same method was used to verify complete synaptic blockade through ω-conotoxin GVIA, a blocker of N-type Ca^{2+} channels (Olivera *et al.* 1985). GSK1016790A, a specific activator of TRPV4 (Thorneloe *et al.* 2008), was applied locally onto the ganglia via a glass micropipette using a pressure-controlled picospritzer (PDES-2L, NPI electronic GmbH, Tamm, Germany). The hypoosmolar solution was applied locally to single ganglia using a programmable syringe pump (Ultra Micro Pump III, WPI, Berlin, Germany), which was connected to a 0.2 mm cannula. This technique allowed local osmotic shifts on a ganglionic level. The total volume of each application was 20 μl with a 4 μl s^{-1} application rate, resulting in a total application time of 5 s. The total volume of the experimental chamber was approximately 8 ml. To gain knowledge about the actual osmotic shift delivered to the ganglion of interest, preliminary experiments had to be carried out. In these experiments, the fluorescence intensity (resting light intensity, RLI) of a known concentration of Fast Green (Sigma-Aldrich) was compared with the RLI of the same concentration of Fast Green applied into the recording chamber with the self-manufactured device. The first step of this process was to establish a calibration curve. Fast Green concentrations of 0.050, 0.100, 0.125, 0.250 and 0.500 mM were introduced into the recording chamber. The resulting RLI measured with the CCD camera was then plotted against the Fast Green concentration. For every concentration of Fast Green, five measurements were performed and the mean RLI was calculated. Due to unspecific background fluorescence, the RLI of the chamber filled just with pure H_2O had to be subtracted from the RLI measured for the different Fast Green concentrations. In a second step the same concentrations of Fast Green were administered into the water-filled chamber using the application device described above. The position of the tip, applied volumes and application speed were set to exactly the same values as in the following experiments. Here again RLI was measured and plotted against the used Fast Green concentrations. The mean accordance between the RLI resulting from

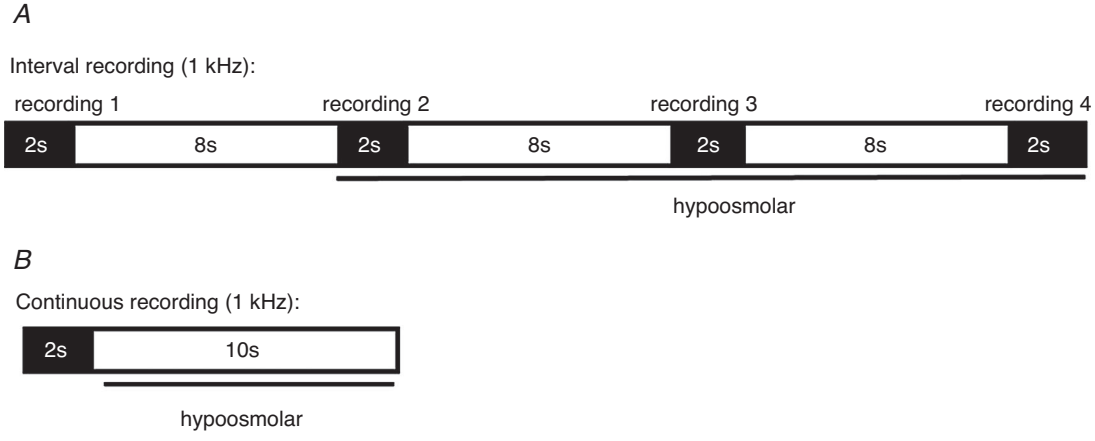

Figure 1. Schematic display of the two different recording paradigms
A, interval recording with a first recording period of 2 s under isoosmolar conditions and three following recordings (2 s each) under hypoosmolar conditions with 8 s between the recordings. *B*, continuous recording lasting for 12 s with 2 s under isoosmolar conditions following 10 s under hypoosmolar conditions.

the calibration measurements and the applications of the dye was 91.03 %. The high accordance between the two curves suggests that the concentration of the hypoosmolar solution and therefore the osmotic pressure in the area surrounding the target ganglia is nearly equivalent to the osmotic value inside the syringe lumen.

To exclude the possibility of neuronal activation due to other causes than osmotic shifts, all experiments investigating osmosensitivity compared the activity level in the same tissue after the application of isoosmolar Hepes solution (in a number of ganglia) to the activity level after application of hypoosmolar Hepes solution (in a number of different ganglia).

For Ca^{2+}-imaging experiments the Ca^{2+} reporter Fluo-4 AM (Invitrogen, Carlsbad, CA, USA) was used. SMP preparations were stained by 45 min incubation in a petri dish (diameter 2 cm) filled with 2 ml carbogen-aerated (95 % CO_2, 5 % O_2, pH 7.40) Krebs solution containing 10 μM Fluo-4 AM, 500 μM probenecid (Invitrogen) and 1 % DMSO at room temperature. Probenecid was used to inhibit organic–anion transporters located in the cell membrane to prevent quick efflux of Fluo-4 from the cells. After incubation the tissue was washed with Krebs solution for 20 min. Recordings were performed using the same recording equipment as described above but with a different light source (3 W blue LED, LE B A2A 460 nm, OSRAM) and a different filter-set consisting of a 600/60 nm bandpass excitation filter, a dichroic mirror with a separation wavelength of 660 nm and a 700/70 nm emission bandpass filter and with a frame rate of 2 Hz. High resolution images of the ganglia were taken using an Axio Cam ICm1 (Carl Zeiss Microscopy GmbH, Göttingen, Germany). For the investigation of volume changes, high resolution images of di-8-ANEPPS stained cells were used.

Quantitative real-time PCR

Samples of guinea pig colonic SMP, whole wall bladder sections and samples of the kidney as well as of the femoral extensor muscle were collected and immediately snap-frozen in liquid nitrogen. In the case of the SMP, very thorough preparation was applied to minimize contamination of the sample with smooth muscle and endothelial cells. Frozen tissue samples were homogenized in 1 ml TRIsure (Bioline, London, UK) according to the manufacturer's instructions. Precipitated RNA was transferred on spin columns (SV Total RNA Isolation System, Promega, Madison, WI, USA) and further processed according to the manufacturer's protocol. RNA was eluted in 50 μl nuclease-free water and RNA concentration was determined spectrophotometrically (Infinite 200 PRO NanoQuant, Tecan, Männedorf, Switzerland). RNA integrity was validated using the Bioanalyzer system (RNA 6000 Nano Kit, Agilent Technologies, Santa Clara, CA, USA). Synthesis of cDNA was conducted using 500 ng template RNA in a final volume of 10 μl (SensiFAST cDNA Synthesis Kit, Bioline GmbH, Luckenwalde, Germany). qRT-PCR was conducted on 384 well plates with reaction volumes of 12.5 μl per well. Each well contained 6.25 μl 2× SensiMix SYBR No-ROX (Bioline), 250 nM forward and reverse primers and 1 μl template cDNA. Samples were run in triplicates on the same plate. Primers were designed to create products spanning at least one intron to exclude quantification of PCR products derived from genomic DNA and produced by Eurofins Genomics (Ebersberg, Germany). Primer sequences were as follows:

TRPV4 5' – AGGGTGGATGAGGTGAACTG – 3' and 5'-GGCTGGAGTTCTTGTTCAGC – 3'
β-actin 5' – GATCTGGCACCACACCTTTT – 3' and 5'-GGGGTGTTGAAAGTCTCGAA – 3' (Lieu *et al.* 2011).

Following initial denaturation (95°C, 7 min), SYBR-green based detection of PCR products was conducted during 45 PCR cycles (97°C 10 s, 53°C 15 s, 72°C 20 s) using a LightCycler 480 Instrument II (Roche, Basel, Switzerland). Specificity of the primers as well as the length of the PCR products was validated using *in silico* PCR (https://genome.ucsc.edu/). Expression levels of target genes were quantified by a standard curve, consisting of pooled cDNA and were normalized to expression levels of β-actin as a constantly expressed housekeeping gene.

Statistics

Primary data from neuroimaging experiments was analysed using the Neuroplex (RedShirtImaging) software. Statistical analysis was performed using GraphPad Prism 8.01 (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as medians and $[Q_{0.25}/Q_{0.75}]$ if not stated otherwise because most data showed a non-gaussian distribution. P values <0.05 were considered as statistically significant. Asterisks indicate significance. Statistical tests were applied as indicated in figure legends. N numbers indicated in figure legends are given as numbers of animals/ganglia/neurons.

Results

Plasma osmolality of the guinea pig

The mean plasma osmolality of four guinea pigs was 293 ± 2.1 mOsm kg^{-1}.

Responses to hypoosmolar stimulus

After application of a 94 mOsm kg^{-1} Hepes-solution onto 294 neurons from 21 ganglia originating from seven guinea pigs, action potential discharge was recorded from

1.0 [1.0/2.5] cells/ganglion (corresponding to 11 [7.0/17.0] % of cells/ganglion).

Response kinetics

For further analysis of the response kinetics, two criteria were used by which responding neurons could be clearly distinguished from non-responding neurons. These were (1) the increased action potential firing frequency after the application of a hypotonic stimulus compared with isoosmotic control solution, and (2) a higher variance of action potential frequency across the four recording periods during the osmotic shift compared with the four recording periods during isoosmolar conditions.

Thirtythree out of a total number of 294 neurons fulfilled the criteria and their response kinetics were further evaluated. The action potential frequency averaged over the three recording periods in those cells was 0.0 [0.0/0.5] Hz after application of an isotonic solution and 1.0 [0.0/5.5] Hz after hypoosmolar stimulation (Fig. 2). Highest action potential frequency was recorded 10–12 s after application of the hypotonic solution. The action potential frequency in this recording period was 6.5 [2.75/9.25] Hz and therefore differed significantly from basal activity ($p < 0.001$, Fig. 3). The delay of the response after application of the 94 mOsm kg^{-1} Hepes solution was found to be 6.1 [4.7/7.8] s using the 12 s recording paradigm (see *Material and methods* section).

Responses to milder hypoosmolar stimuli

In another set of experiments we applied a 144 mOsm kg^{-1} Hepes solution onto 123 neurons in nine ganglia originating from four tissues and a 194 mOsm kg^{-1} Hepes solution onto 88 neurons in five ganglia from four tissues. This resulted in an increased action potential firing frequency in 18.0 [0.0/41.0] % and 7.7 [6.4/18.1] % of neurons per ganglion for the 144 mOsm kg^{-1} stimulus and the 194 mOsm kg^{-1}, respectively. Statistical analysis showed no significant differences between the numbers of osmosensitive neurons after the three stimuli.

As for the 94 mOsm kg^{-1} stimulus, the majority of cells showed the highest response activity during 10 to 12 s after application of the 194^{-} and 144 mOsm kg^{-1} stimulus with an action potential firing frequency of 1.0 [0.75/1.5] and 5.0 [2.8/8.5] Hz, respectively. For the mildest stimulus of 194 mOsm kg^{-1} the recorded response activity was significantly lower than the 144 mOsm kg^{-1} stimulus and the 94 mOsm kg^{-1} stimulus (Fig. 4).

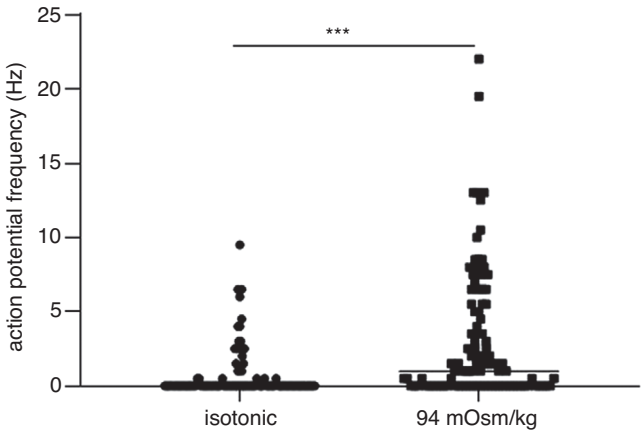

Figure 2. Averaged action potential frequency in the three recording periods after application of an isotonic or a hypotonic solution in cells defined as responders
Action potential frequency was significantly higher after application of the 94 mOsm kg^{-1} Hepes solution compared with isotonic conditions (Wilcoxon's signed rank test). n = 7/21/99. Horizontal lines within data points indicate median values.

Reproducibility of neuronal responses to hypoosmolar stimulation

In a series of paired experiments the reproducibility of neuronal responses to the hypotonic stimulus was investigated by applying the 94 mOsm kg^{-1} Hepes solution twice on the same ganglion with a stimulus interval of 20 min. This was conducted on 12 ganglia originating from four animals resulting in a total number of 218 neurons investigated. Only ganglia that displayed fast EPSPs in response to electrical stimulation after the second hypoosmolar stimulation were considered for the analysis. While 13.5 [7/20.5] % of the neurons per ganglion responded to the first 94 mOsm kg^{-1} stimulus only 2.0 [0/14] % responded to a second application 20 min later. No neuron responded solely to the second application. The averaged action potential frequency in responding neurons after the first stimulation was 3.33 [1.75/5.09] Hz and with 1.33 [0.33/3.5] Hz was significantly lower after the second stimulation ($p = 0.024$, data not shown).

Responses to isoosmolar solution with a reduced NaCl content

Application of an isoosmolar solution with a reduced NaCl content on guinea pig submucosal neurons ($n = 6/8/144$) led to neuronal activity in 21.6 [6.7/28.8] % neurons per ganglion firing action potentials with a frequency of 2.0 [0.5/3.1] Hz (data not shown). Action potential discharge was exclusively recorded within the first 2 s after application, while neurons were almost silent during the recording period 10–12 s after application.

Influence of ω-conotoxin on responses to hypoosmolar stimulus

In all tissues tested ($n = 3/4/60$), fast EPSPs were detectable prior to the perfusion with 200 nM ω-conotoxin GVIA.

After 20 min of perfusion with ω-conotoxin GVIA no fast EPSPs were detectable. In the presence of ω-conotoxin 13.9 [10.6/17.5] % of the neurons per ganglion responded to an application of 94 mOsm kg^{-1} Hepes solution. This number was not significantly different from the number of responding neurons in the absence of ω-conotoxin ($p = 0.506$, data not shown). The action potential frequency of the response was also not altered by ω-conotoxin with 3.1 [1.8/5.0] Hz in the presence of ω-conotoxin GVIA and 2.2 [0.8/3.4] Hz in the absence of ω-conotoxin GVIA, respectively ($p = 0.166$, data not shown).

Ca^{2+} responses after hypoosmolar stimulation of submucosal ganglia

Figure 5*A* shows the Ca^{2+} response of a submucosal neuron after application of the 94 mOsm kg^{-1} Hepes solution. This was tested in six ganglia from three animals. Because Fluo-4 AM does not incorporate into the cell membrane but rather stains the whole cytoplasm, it was not possible to calculate the exact number of neurons/ganglion. Only cells which showed a change in intracellular Ca^{2+} concentrations and hence a change in fluorescence intensity of the dye, could be identified on single cell level. 4.0 [3.0/7.75] neurons per ganglion showed Ca^{2+} transients in response to a treatment with 94 mOsm kg^{-1} Hepes solution. The number of neurons showing a response was significantly higher than the number of cells showing spike discharge recorded with voltage sensitive dye (VSD) imaging (1.0 [1.0/2.5]; $p < 0.001$, data not shown). The ΔRLI after application of the hypoosmolar stimulus was 100.1 [69.0/122.0] % (Fig. 5*B*). In enteric neurons, Ca^{2+} transients started with a delay of 2.7 $\pm$ 0.94 s which was significantly earlier than the onset of the responses observed in the VSD imaging experiments (5.7 $\pm$ 2.3 s) (Fig. 6). The maximum ΔRLI was reached after 14.3 $\pm$ 5.2 s. With this technique we could also record glia responses to hypoosmolar stimuli, as already shown by Mueller and colleagues (Mueller *et al.* 2011). We observed in each ganglion one or two glia responding to the stimulus. Analysing the responses of 15 glia we found that Ca^{2+} transients started with a delay of 11.5 $\pm$ 5.6 s and the maximum ΔRLI was reached after 22.0 $\pm$ 12.7 s.

Changes in visible cell surface area after hypoosmolar stimulation

Application of a 94 mOsm kg^{-1} Hepes solution caused rapid and transient swelling in the majority of neurons. Increase in visible cell surface area was 10.4 $\pm$ 5.6 % within the first 2 s under hypoosmolar conditions ($n = 3/3/5$). Within 4 to 5 s cells decreased again to their initial size. Not

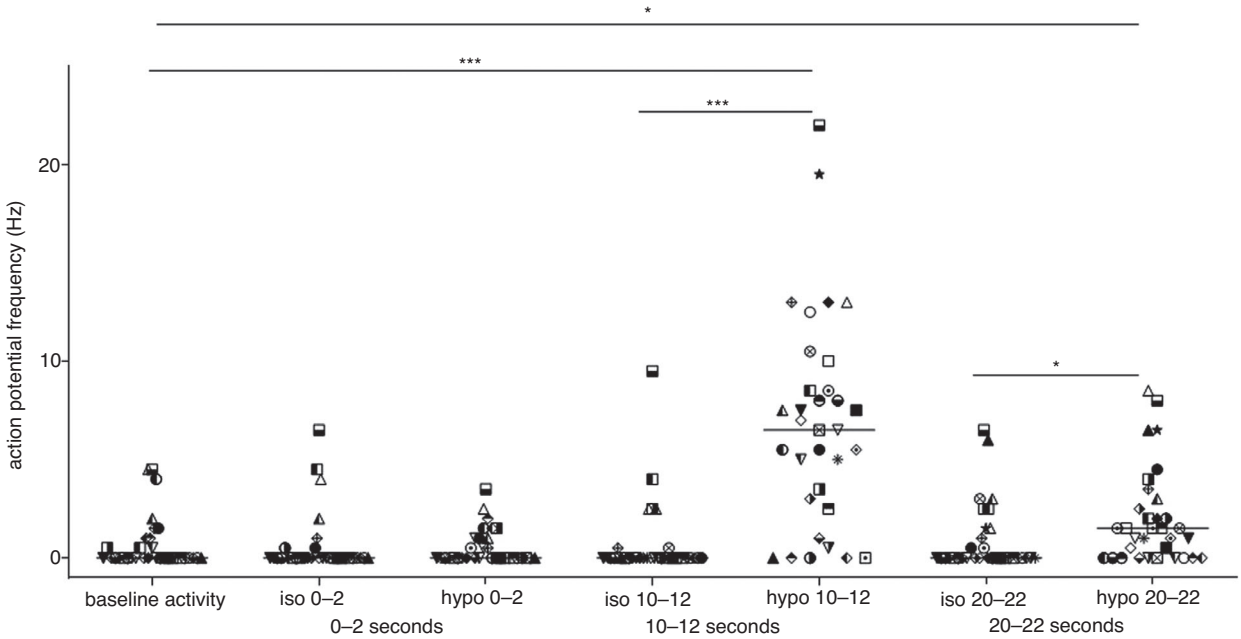

Figure 3. Temporal distribution of action potential frequencies after application of an isoosmolar or hypoosmolar solution
Each symbol represents a certain neuron. Baseline activity was compared with every other recording period under isoosmolar as well as hypoosmolar conditions (Friedman's test, $p < 0.0001$). Comparison of mean action potential frequency between iso- and hypoosmolar in one recording period was performed with Dunn's multiple comparison test and significant differences are indicated by asterisks. $n = 7/21/33$. Horizontal lines within data points indicate median values.

all cells showed an increase in cytoplasmic area after hypoosmolar stimulation and in a number of cells the visible cytoplasmic area even decreased under hypoosmolar conditions. Measurements of the cytoplasmic area of seven cells indeed showed a decrease of 8.5 ± 4.2 % after hypoosmolar stimulation. In those cells the maximum change in visible cytoplasmic area was reached within the first 2 s after the hypoosmotic stimulus. After application of an isoosmolar solution, no change in cell surface area was observed.

Expression of TRPV4 in the submucosal plexus of the guinea pig colon

All tissues investigated expressed TRPV4 mRNA with the highest expression levels in the kidney and lowest expression levels in the skeletal muscle (Fig. 7).

HC-067047 reduces the number of neurons responding to hypoosmolar stimulation

The effect of 150 nM HC-067047 on hypoosmolar responses of 156 neurons from 10 ganglia originating from three guinea pigs was tested and neuronal responses were compared with the responses of 19 neurons from eight ganglia originating from three guinea pigs stimulated without addition of HC-067047. HC-067047 had no effect on the fast EPSP in response to electrical stimulation. In the absence of HC-067047 the amplitude of the fast EPSP was 0.56 [0.44/0.78] % RLI compared with 0.47 [0.27/0.75] % RLI in the presence of HC-067047 (data not shown). In the presence of 150 nM HC-067047 the percentage of neurons responding to the hypoosmolar stimulus significantly decreased (from 18.0 [13.3/20.8] % to 0.0 [0.0/6.3] %; Fig. 8*A*). The action potential frequency

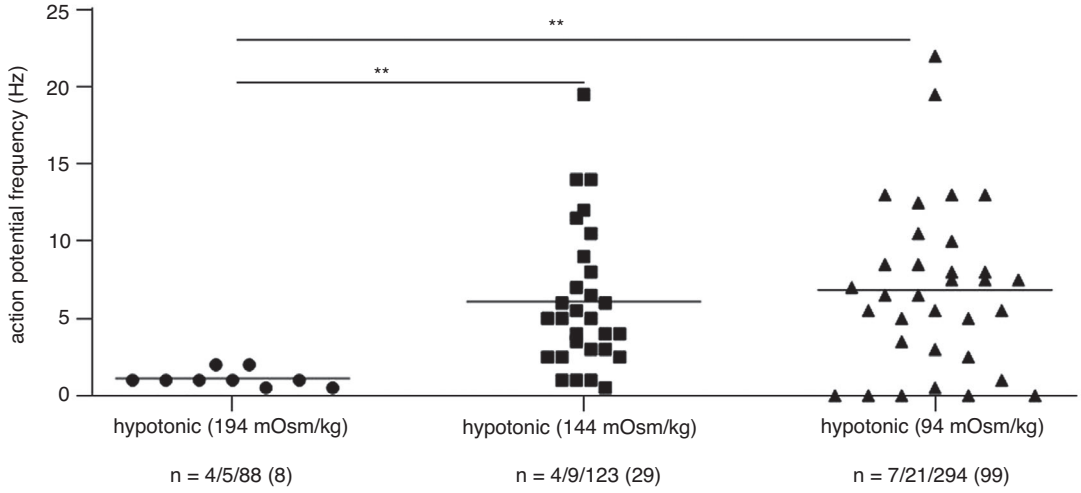

Figure 4. Comparison of the neuronal responses to three hypoosmotic stimuli
Action potential frequency of the neurons responding to the 194 mOsm kg^{-1} Hepes solution was significantly lower than that of the neurons responding to 94 and 144 mOsm kg^{-1} Hepes solution (Kruskal–Wallis test; exact *P* values were calculated with Dunn's multiple comparison test and significant differences are indicated by asterisks; $p < 0.01$). *N* numbers indicate guinea pigs/ganglia/tested neurons (osmosensitive neurons). Horizontal lines within data points indicate median values.

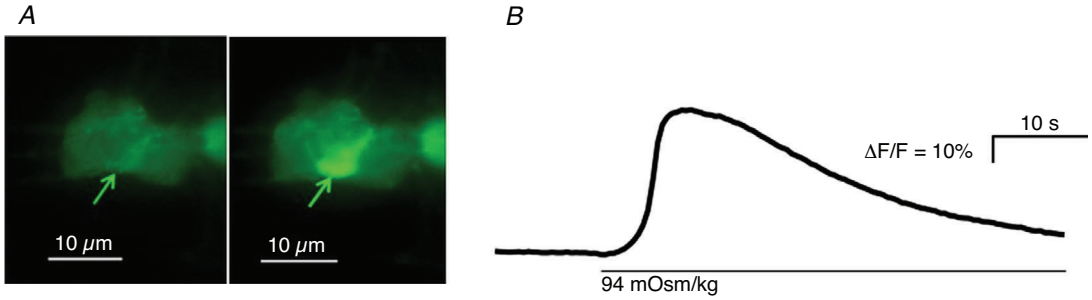

Figure 5. Ca^{2+} response after application of a hypoosmolar Hepes solution
A, fluorescence of the calcium indicator Fluo-4 before (left) and after (right) application of a 94 mOsm kg^{-1} Hepes solution onto a ganglion of the SMP (recorded with a framerate of 2 Hz). *B*, change of fluorescence of the cell responding in *A* over a time course of 1 min during recording period 3. [Colour figure can be viewed at wileyonlinelibrary.com]

in cells that responded to the osmotic stimulus despite the presence of the TRPV4 antagonist was 2.8 [1.7/5.3] Hz and not different compared with the responses without the antagonist which was 2.2 [0.7/3.8] Hz (Fig. 8*B*).

GSK1016790A triggers specific responses in submucosal enteric neurons

The effect of 10 μM (n = 4/13/235) and 20 μM (n = 9/22/397) of the TRPV4 agonist GSK1016790A on the neurons of the SMP was tested. Application of 10 and 20 μM GSK1016790A led to action potential discharge in 3 [1.5/5.0] cells/ganglion (corresponding to 15.0 [7.5/24.5] %) and 3.5 [1.0/6.25] cells/ganglion (corresponding to 21.0 [10.5/29.8] %), respectively. Recorded firing frequencies after application of 10 μM and 20 μM of GSK1016790A were 1.8 [1.3/2.3] Hz and 1.3 [0.5/2.3] Hz, respectively (data not shown). In addition to the VSD experiments, we also investigated the effect of the application of 20 μM GSK1016790A on 13 ganglia from four animals using Ca^{2+} imaging and compared the number of responding neurons per ganglion with results obtained in the VSD experiments. With 3.0 [2.0/3.5] cells per ganglion responding to the application of the activator detected with the Ca^{2+} imaging, this

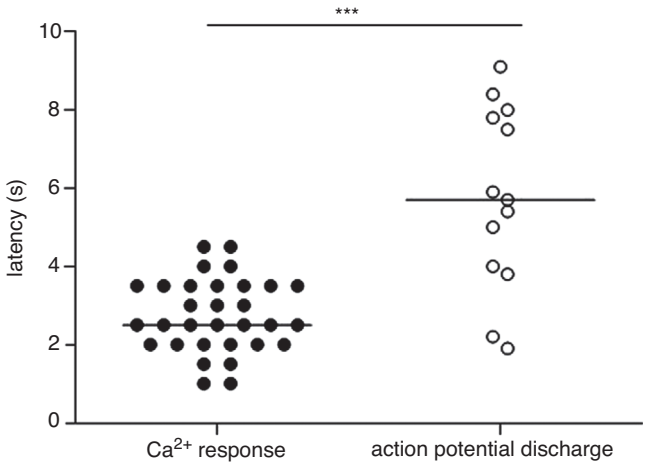

Figure 6. Latency of Ca^{2+} response and action potential discharge after hypoosmolar stimulation of enteric neurons
Increase in $[Ca^{2+}]_{in}$ started significantly earlier than the action potential discharge observed in experiments using the VSD technique. Unpaired t test. For Ca^{2+} experiments: n = 3/6/31, for VSD experiments n = 2/3/13. Horizontal lines within data points indicate median values.

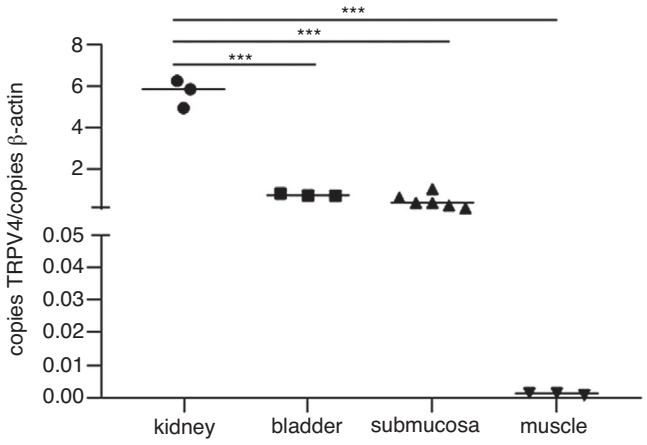

Figure 7. Relative mRNA expression of TRPV4 in kidney, bladder, submucosa and muscle
Ordinary one way ANOVA ($p < 0.001$). Comparison of individual specimen by Dunn's multiple comparison test. n = 3–6 guinea pigs. Horizontal lines within data points indicate median values.

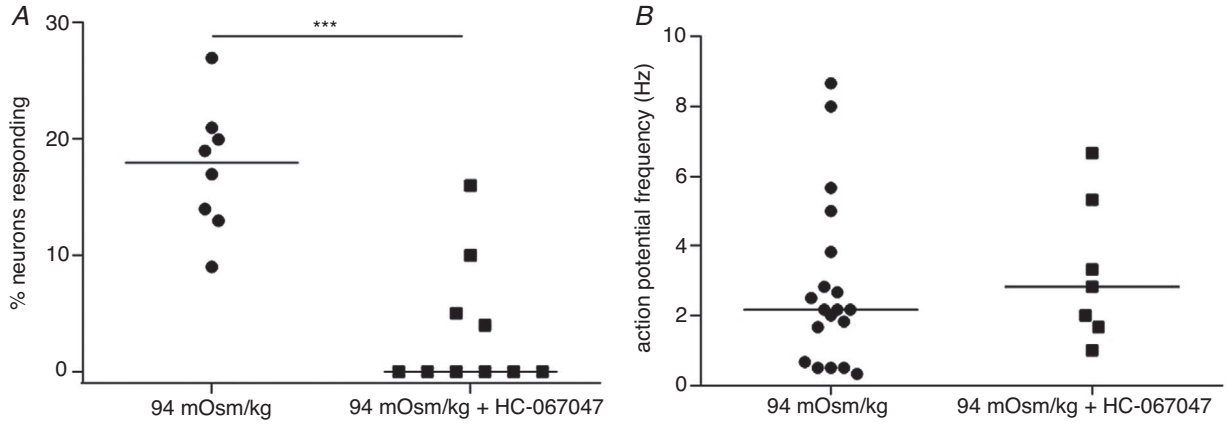

Figure 8. Effect of specific TRPV4 blocker HC-067047 on the neuronal response to hypoosmolar stimulation
A, percentage of responding cells after hypooosmolar stimulation with and without HC-067047; Mann–Whitney's test. *B*, action potential frequency in responding cells after hypoosmolar stimulation with and without HC-067047. Mann–Whitney's test. n = 3/8/19 (94 mOsm kg^{-1}), n = 3/10/7 (94 mOsm kg^{-1} + HC-0670479). Horizontal lines within data points indicate median values.

value was not significantly different from the number of cells per ganglion responding to the agonist in the VSD experiments ($p = 0.34$, data not shown). Perfusion with 6 μM HC-067047 significantly reduced the percentage of cells responding to the activator ($p = 0.032$, data not shown) as well as their action potential discharge frequency ($p < 0.001$, data not shown).

GSK1016790A sensitive neurons are also osmosensitive

Figure 9 shows an example of the activity pattern of a submucosal neuron responding consecutively to both GSK1016790A and a hypoosmolar stimulus which was investigated in a total of 253 neurons, from 13 ganglia coming from four animals. The order of application was randomized. Forty cells responded to the application of the hypoosmolar Hepes solution and 11 out of these (27.5 %) were also sensitive to GSK1016790A application (20 μM). On the other hand, 11 out of 15 cells (73.3 %) which showed action potential discharge after application of the TRPV4 agonist were also osmosensitive.

Discussion

This study describes a completely new property of submucosal enteric neurons: they are osmosensitive. It provides valuable mechanistic insight into osmotransduction in the ENS by pharmacological experiments indicating that TRPV4 may be a candidate involved in this process.

Plasma osmolality of the guinea pig

The mean plasma osmolality of the guinea pigs was 293.5 $\pm$ 2.1 mOsm kg^{-1}. This is in accordance with early investigations finding an average plasma osmolality of 293 mOsm kg^{-1} in this species (Harpur & Popkin, 1965) and comparable to osmolality in humans (288 $\pm$ 1 mOsm kg^{-1}) (Zerbe & Robertson, 1983). Therefore, the guinea pig seems to be a suitable model to study the effects of changes in extracellular osmolality on neurons. As food and water were provided *ad libitum* the measured values apply for a state of normal hydration and satiety.

Suitability of the stimulus and region of the gastrointestinal tract

Given the fact that plasma osmolality in vertebrates is tightly regulated under physiological conditions (Baylis, 1983; Gill *et al.* 1985), the stimuli used in this study appear to be rather drastic. Nevertheless, it has to be taken into account that plasma osmolality represents systemic values while fluctuations in osmolality might be much higher on a local level, e.g. in the wall of the GIT. This is supported by the fact that portal vein osmolality dropped by 8 % to 285.6 $\pm$ 3.0 mOsm kg^{-1} 30 min after oral water intake in mice (Lechner *et al.* 2011) and was also significantly reduced relative to plasma osmolality after intraduodenal water infusion (292.7 $\pm$ 4.7 mOsm kg^{-1} and 304.4 $\pm$ 6.9 mOsm kg^{-1}) (McHugh *et al.* 2010). In this respect it seems conceivable that the SMP, located between the intestinal lumen and the efferent blood vessels, is exposed to even much stronger osmotic stimuli. After food or fluid ingestion, luminal osmolality is altered and in order to return it to isotonicity, water and electrolytes are moved in a bidirectional manner via the intestinal epithelium automatically passing by the SMP (Leiper, 2015). In this regard, hypertonic solutions cause a net water flux from the body water pool into the intestinal lumen (Grim, 1962), while hypoosmolar fluid composition in the intestine leads to water reabsorption from the lumen across the mucosa (Wapnir & Lifshitz, 1985). An early study by Bohlen, examining the effect of intestinal glucose absorption on submucosal osmolality in rats, calculated a 79–90 mOsm increase in submucosal osmolality compared with control conditions (Bohlen, 1982). A contrary effect of lowering submucosal osmolality and hence also osmotic environment enteric neurons are surrounded by intestinal water absorption, is therefore conceivable. With regard to potential osmosensitive properties of submucosal enteric neurons, the colon is of special interest because at this site large amounts of water are reabsorbed even against a high osmotic gradient (Ma & Verkman, 1999), hence altering extracellular osmolality in the submucosa before reaching efferent blood vessels.

We tested the responsivity of submucosal colonic neurons to three different hypoosmotic stimuli. The final osmolality reached at ganglionic level was: 94, 144 and 194 mOsm kg^{-1}. The proportion of osmosensitive neurons was not statistically different between the three stimuli; however, the firing frequency of the responsive neurons was significantly lower with the mildest stimulus in comparison with the others. This seems to indicate that we have a population of osmosensitive neurons, which show a firing pattern with dose–response effect, suggesting that smaller osmotic changes may induce a lower responsivity in the same neurons.

Submucosal neurons are sensitive to hypoosmolar stimulation

The results of the present study show that low extracellular osmolality activates a subpopulation of submucosal neurons in the guinea pig colon. Our findings agree

with the only other study addressing osmosensitivity in enteric neurons so far, which found that oesophageal motor neurons of the rat were activated by a hypotonic solution, which had an osmolality of 170 mOsm kg^{-1} (Dong *et al.* 2015). In this study the authors concomitantly recorded neuronal activity (using Ca^{2+} imaging) and cell swelling following application of a 170 mOsm kg^{-1} hypotonic solution, indicating a functional link between cell volume changes (in this case volume increase) and neuronal activation. In a similar fashion this has been demonstrated in central neurons (Prager-Khoutorsky & Bourque, 2015).

The NaCl concentration of our hypoosmolar solution was reduced from 136 to 33 mM, this might affect reversal potentials for Na^+ and Cl^-. Enteric neurons, or at least a subpopulation of them, are known to have a high intracellular Cl^- concentration. This has been measured to be around 55 mM (Starodub & Wood, 2000; Neunlist *et al.* 2001). Lowering the extracellular Cl^- concentration may lead to neuronal activation due to Cl^- efflux if there is significant resting membrane Cl^- permeability. To avoid this possible artefact, we performed control experiments by applying an isoosmolar solution (mannitol) with a reduced NaCl (33 mM) content with the same recording paradigm used for the hypoosmolar stimulus. These control experiments showed a neuronal subpopulation which was indeed activated by lowering extracellular Cl^- concentration. The responses of these neurons, however, ceased after the first 2 s and they were almost silent during the recording period of 10–12 s, when the osmosensitive neurons showed the maximal response. Thus, we concluded that they are a distinct population from the osmosensitive neurons.

Our results, also in agreement with the study of Dong and colleagues (Dong *et al.* 2015), revealed that the majority of submucosal neurons showed swelling in response to hypoosmolar stimulus. However, we could also describe a subpopulation of neurons displaying a decrease in visible cell surface area in response to the application of the hypoosmolar solution. It is noteworthy that these changes were always displayed within the first 2 s after the stimulus application. In addition, it is important to say that with the methods used, only changes in visible cell surface area could be measured while changes in the thickness of cells (Z-axis) were not detectable. Therefore, it is still possible that those cells expanded in the Z-axis but did not expand in the X–Y-plane. Ganglia are also surrounded by a ganglionic sheet, thus are quite compact structures. When one neuron shows an increase in its area, some other neurons have to show a decrease. Thus, this could be due to the experimental artefact. Another hypothesis is that the decrease in visible cell surface area could be linked to the Cl^- efflux, likely followed by a water efflux, and not related to the hypoosmolar stimulus.

The reproducibility of the neuronal responses was relatively low. There are several possible explanations: most likely this could be caused by a desensitizing effect of the stimulus. Such an effect could either be due to desensitization of the receptors, a phenomenon reported for neurons of the SMP after repeated stimulation with serotonin (Michel *et al.* 2005) or due to changes in receptor density (Ferrandiz-Huertas *et al.* 2014). On the other hand, the detected decrease in stimulated neuronal activity could be caused by disturbances in intracellular ionic concentrations due to the regulatory volume decrease that most cell types display after hypotonic stimulation

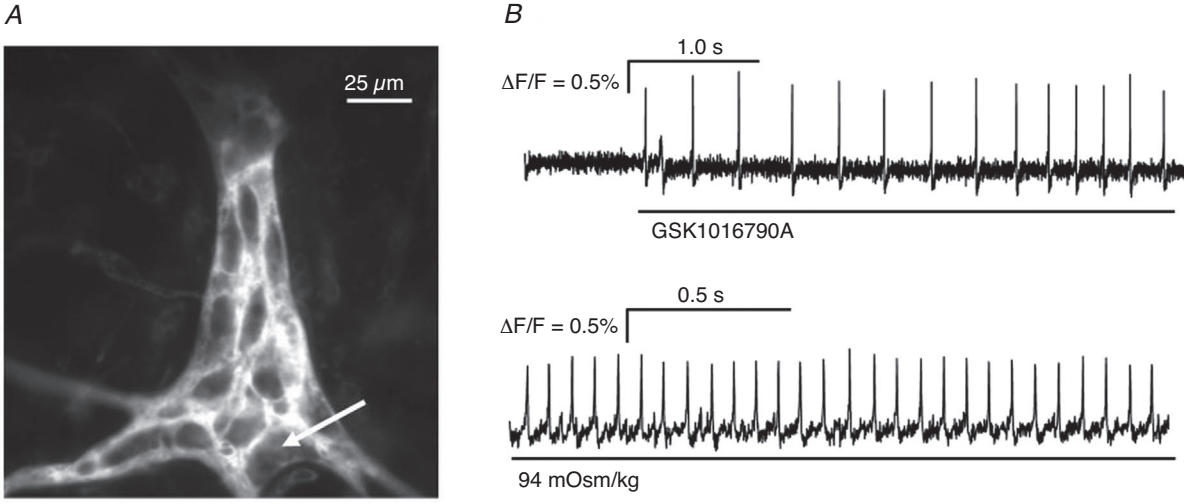

Figure 9. Neuronal activity pattern after GSK1016790A and after hypoosmolar stimulation
A, ganglion stained with Di-8-ANEPPS and exposed to 20 μM GSK1016790A and 94 mOsm kg^{-1} consecutively. White arrow indicates a cell responding to both stimuli. *B*, traces recorded from the cell displayed in *A*. The response to GSK1016790A was measured in the 4 s following the agonist application while the response to hypoosmolar stimulus during period 10–12 s of the interval recording paradigm.

(Friedrich *et al.* 2006; Lang, 2007). Nevertheless, one may expect that the 20 min period between two osmotic stimulations was enough to normalize cytoplasmic ion levels. Another reason for the low reproducibility could be due to neuronal damage induced by the stimulus itself triggering not a physiological, rather a pathological response. We recorded responses from a comparable number of enteric neurons activated by the application of three different hypoosmolar solutions suggesting an all/none response to hypoosmolar stimulation in a particular population of osmosensitive neurons. The lower firing frequency showed with the mildest stimulus indicates a dose–response effect. We can speculate that, during physiological conditions, enteric neurons do not likely undergo strong osmotic shifts, yet a population of them is able to sense and respond to moderate changes in osmolality. The increase of the firing frequency under strong osmotic shift could indicate a response to pathological conditions. Another kind of pathological response, also described by other authors, is neuronal damage in the CNS caused not by the hypoosmolar stimulus itself, but due to excessive release of neurotransmitters from the cells undergoing strong osmotic shifts (Kimelberg *et al.* 1990; Pasantes-Morales & Tuz, 2006). However, as viability control we recorded fast EPSPs from osmosensitive neurons in response to interganglionic electrical stimulation after the second hypoosmolar stimulation. This demonstrated that the neurons which responded to osmotic shifts were vital and viable, therefore suggesting that neuronal cell damage was not the reason for the low reproducibility observed.

All neurons responding are directly osmosensitive and TRPV4 is involved in the process of osmotransduction

Due to the significant delay of action potential discharge after hypoosmolar stimulation, a synaptically mediated activation of at least part of the responding enteric neurons had to be considered. Nevertheless, synaptic blockade with 200 nM ω-conotoxin GVIA had no effect on the number of activated cells or the action potential frequency of the response leading to the conclusion that the synaptic activation between neurons did not affect the proportion of responding neurons. Regarding the existence of osmoreceptors on enteric submucosal neurons responding to hypoosmolar solution, we hypothesize the involvement of members of the TRPV family. Among the TRPV members identified as osmosensitive, the most suitable candidate is TRPV4, due to its role in central osmoreception (Ciura & Bourque, 2006; Sharif-Naeini *et al.* 2008). Indeed, results of the present study show expression of TRPV4 in the colonic SMP. TRPV4 expression has also been reported in macrophages (Hamanaka *et al.* 2010) and endothelial cells (Vriens *et al.* 2005), both of which are present in the submucosal layer (Reichardt *et al.* 2013) and hence could have led to detection of the TRPV4 mRNA. Confirming this finding, we showed that application of the specific TRPV4 activator GSK1016790A led to action potential discharge as well as Ca^{2+} transients in SMP neurons. The number of recorded responses was the same in experiments using Ca^{2+} imaging and VSD imaging, indicating that GSK1016790A predominantly activated neurons. This finding contrasts with that of Poole and colleagues who described predominant activation of non-neuronal cells (indicated by an increase in intracellular Ca^{2+}) in response to an application of GSK1016790A on colonic submucosal wholemounts (Poole *et al.* 2017). This discrepancy might be explained by the different GSK1016790A concentrations used in the cited study (100 nM) and in our experiments (10 and 20 μM). Activation of myenteric neurons by application of 100 nM GSK1016790A has been described in the colon of mice, with 48 % of investigated neurons displaying an increase in $[Ca^{2+}]_{in}$ in response to an application of the TRPV4 agonist (Fichna *et al.* 2015). In our study, pretreatment with the TRPV4 antagonist HC-067047 effectively reduced the percentage of responding neurons after hypoosmolar stimulation, indicating functional relevance of TRPV4 in osmosensitivity. However, action potential frequency remained unchanged in those cells that did respond in the presence of the TRPV4 antagonist, indicating that another, TRPV4 independent, mechanism is involved in neuronal osmosensitivity. Consecutive application of GSK1016790A and a hypoosmolar stimulus to submucosal neurons revealed that most cells functionally expressing TRPV4 are osmosensitive, while only a subset of osmosensitive cells responded to the application of the TRPV4 activator, supporting the assumption of the presence of additional osmotransducers.

Hypoosmolar stimulation also activates enteric glia

In the Ca^{2+} imaging experiments we analysed a number of glia responding to the hypoosmolar stimulus. This response was, however, delayed in comparison with neuronal responses. Poole and colleagues reported activation of enteric glia in murine myenteric and submucosal wholemount preparations after application of 100 nM GSK1016790A (Poole *et al.* 2017). Enteric glia cells are known to share several morphological as well as functional features with astrocytes (Boesmans *et al.* 2013). Osmosensitivity is well described in astrocytes and is connected with the release of organic cytoplasmatic molecules such as taurine, glutamate and aspartate after hypoosmolar stimulation to regulate cell volume (Kimelberg *et al.* 1990; Pasantes-Morales *et al.* 1990).

Although those molecules are primarily responsible for the regulatory volume decrease to normalize cell volume, they also have neuroactive effects (Kimelberg *et al.* 1990). Taurine, for example, can bind to glycine receptors (Betz, 1992), which are expressed on enteric neurons (Galligan, 2002) and might modulate neuronal function via this route. Therefore, we cannot rule out that the activity of enteric neurons is additionally modulated by the release of neuroactive amino acids (for example, taurine) from enteric glia after osmotic stimulation. However, the latency in responses of neurons *versus* glia indicates that neuronal responses started before glia. Moreover, this latency of neuronal responses to hypoosmolar solution appears comparable with the pattern of responses of osmosensitive oesophageal myenteric neurons (Dong *et al.* 2015).

In the Ca^{2+} imaging experiments we observed an increased number of responding neurons; these responses might be subthreshold neuronal depolarizations mediated by Ca^{2+}- transients not sufficient for action potential firing and hence not detected using VSD imaging. We already showed in a precedent study that with Ca^{2+} imaging subthreshold signals can be recorded (Michel *et al.* 2011).

Taken together, this study was the first to describe osmosensitive properties in colonic submucosal neurons and to reveal that they are at least in part mediated by TRPV4. In future studies the physiological relevance of this finding should be evaluated, e.g. by conducting experiments in TRPV4-/- mice.

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Additional information

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

No competing interests declared.

Author contributions

All experiments have been conducted in laboratories of the Chair of Human Biology, Technical University of Munich, Freising, Germany. G.M.W. and M.S. designed and drafted the work and M.K. revised it critically. P.K. conducted the experiments, analysed the data and wrote the manuscript. K.E. conducted the experiments, analysed and interpreted the data and wrote the manuscript. S.M. conducted the experiments and analysed the data. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

The project was funded by the German Research Foundation, DFG (Graduate School 1482/3, MA-5202/1-1 and 1–2) and NIH/SPARC (1OT2OD0248999).

Acknowledgements

Open access funding enabled and organized by Projekt DEAL.

Keywords

enteric nervous system, osmosensitivity, submucosal enteric neurons, TRPV4

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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