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14. ABSTRACT Up to 80% of ASD patients exhibit gastrointestinal (GI) problems, but the underlying mechanisms are unknown. Many ASD associated mutations modify synaptic proteins and hence alter synaptic function in the brain. We propose that some of these mutations also alter the enteric nervous system (ENS) to produce bowel disorders. NL3 ^{R451C} mice express a neuroligin-3 mutation identified in ASD patients and are more responsive to the GABA neurotransmission in the brain. We investigated motility patterns in isolated jejunum and colon of the mouse model and found significant differences in motility between NL3 ^{R451C} and wildtype jejunum in control solutions, in part due to altered pacemaker activity. Patterns in the colon are identical in control solutions, but NL3 ^{R451C} colon is more sensitive to blockade of GABA _A receptors. Immunohistochemical localization of neuroligin 3 in both regions confirmed that it is found within the myenteric plexus and appears downregulated in the NL3 ^{R451C} mouse. Surprisingly, immunoreactivity was prominent in presynaptic varicosities (antiserum specificity confirmed by Western blot) suggesting that neuroligin 3 may act presynaptically. The data provide strong evidence that gastrointestinal dysfunction in autism is related to mechanisms within the enteric nervous system and the intrinsic regulation of gut muscle		

15. SUBJECT TERMS

Small intestinal motility functions differ between NL3R451C mice and their WT under control conditions in vitro
Increased sensitivity of NL3R451C colon to tropisetron is unlikely to be due to altered sensitivity of 5HT receptors alone
Localization of Neuroligin-3 protein at the presynapse

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BODY:

Research accomplishments associated with each task outlined in the Statement of Work.

Milestones, major goals/objectives of the proposed research:

Aim 1 To compare the motor patterns in isolated duodenum, jejunum and colon of NL3, Balb/cJ and wild type mice and the effects of GABA_A antagonists. (Task 1 – 9 months)

Aim 2 To localize the neuroligin 3 protein

Aim 3 To characterise motility in neonatal NL3 mice

• List of Tasks and subtasks (including number of animals required).

Task 1. Comparison of motor patterns (video recording): PI Bornstein (9 months: months 3-11, 96 Balb/cJ mice, 192 NL3 (WT/KI) mice = 288 mice)

1a. Duodenum: GABAA antagonists, serotonin antagonists, GABAA and serotonin interactions (3 months: 3-5). 32 Balb/cJ & 64 NL3 (WT and KI) mice required.

1b. Jejunum: GABAA antagonists, serotonin antagonists, GABAA and serotonin interactions (3 months: 6-8). 32 Balb/cJ & 64 NL3 (WT and KI) mice required.

1c. Colon: GABAA antagonists, serotonin antagonists, GABAA and serotonin interactions (3 months: 9-11). 32 Balb/cJ & 64 NL3 (WT and KI) mice required.

Task 2. Localize the neuroligin 3 protein: PIs Bornstein (cell classes and mutated NL3 expression, 10 months: months 15-24) **and Hill** (synapses, 10 months: months 3-12) (immunohistochemistry and confocal microscopy); 8 adult mice.

2a. Cell classes expressing NL3: Tissue from 4 WT mice labelled for NL3, GABA, Calretinin, Calbindin, nNOS, 5HT, VIP, ChAT, (5 months: months 15-19).

2b. Expression of mutated NL3: Tissue from 4 NL3 mice labelled for NL3, GABA, Calretinin, Calbindin, nNOS, 5HT. (5 months: months 20-24)

2c. Synapses expressing NL3: Tissue from 4 WT mice labelled for NL3, GABA, 5HT, synaptophysin (and non-neuronal cells if present: S-100B, kit). (10 months: months 3-12)

Task 4. Time course of expression of neuroligin 3, altered motility in neonatal NL3 mice. PI Young (RT-PCR, FACS, immunohistochemistry, confocal microscopy; 22 months: months 3-24) **and PI Bornstein** (neonatal colonic motility; 12 months)

4a. qRT-PCR experiments in developmental tissue. 30 NL3 mice (5 months: months 3-7).

4b. FACS experiments developmental tissue. 15 Ret-GFP mice (5 months: months 8-12)

4c. Immunohistochemistry in developmental tissue (12 months: months 13-24).

4d. Motility measurements in P6 and P10 NL3 mouse colon (12 months, in parallel with 4c)

Introduction

Disorders in bowel function are common in autism. Up to 80% of ASD patients exhibit gastrointestinal (GI) problems, notably chronic constipation, but the underlying mechanisms are unknown. Many mutations identified in ASD patients modify synaptic proteins and hence alter synaptic function in the brain. We propose that some of these mutations also alter the enteric nervous system (ENS) of the GI tract to produce bowel disorders. We have shown that adhesion molecules important to synaptic function in brain including neuroligin-3 and neurexins 1 and 2 are expressed in mouse myenteric plexus. NL3 mice, which express a human neuroligin-3 mutation identified in ASD patients, are more responsive to the inhibitory neurotransmitter GABA in the brain. This project aims to further investigate changes in gastrointestinal function in NL3 mice compared with WT littermates by examining motility in different regions of the GI tract in *in vitro* preparations from adult and developing animals. We will also study the spatiotemporal distribution patterns of NL3 and related proteins and mRNA in gut tissue from these mice. This project aims to determine biological mechanisms contributing to gastrointestinal dysfunction in patients with ASD.

Body

Comparing motor patterns in isolated mouse small intestine and colon

As outlined in the original application, data from the current laboratory showed for the first time that jejunal motility in WT C57Bl6 mice is more sensitive to the serotonin antagonists granisetron and SB207266 compared with in Balb/cJ mice (Neal et al., 2009). We have also shown that blockers of GABA_A receptors, bicuculline and gabazine, depress motor activity in the colon of another mouse model of autism, the NL3 mouse, but to a lesser extent or do not in wild type colon. To further understand the impact of the NL3 mutation, this component of the project is focused on investigating whether mouse models of autism, in particular the NL3 R451C model show altered small intestinal motility as well as further characterising changes in colonic motility. Based on the scientific review of the original grant application, we delayed experiments on BalBc mice while seeking funds to set up a colony of SHANK3 mice. Unfortunately, we have just been advised that our application to the National Health and Medical Research Council of Australia, which included a request for these funds, was unsuccessful. We will therefore proceed with the BalbC experiments in the next year.

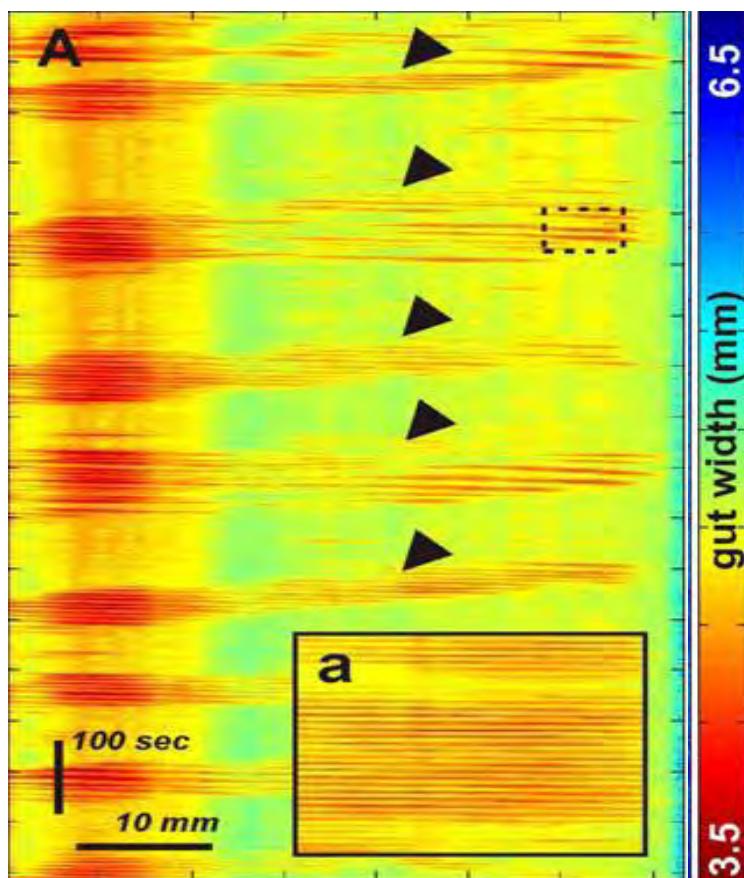
Small intestinal motility patterns

The small intestine produces a variety of motility patterns to ensure appropriate mixing and propulsion of contents during absorption, digestion, and excretion of food. In the guinea pig, these include peristaltic contractions (Hennig et al., 1999), segmental contractions (Gwynne et al., 2004), and pendular movements, all of which can be studied *in vitro*. There is also a fasted state activity known as the interdigestive motor pattern or migrating motor complex (Galligan et al) that has only been seen *in vivo*. In contrast, only one consistent contractile pattern has been identified in mouse duodenum or jejunum, the propagating contractile complex (PCC), defined as a complex of contractions that propagate in a consistent direction for 50% or more of the length of a small intestinal segment (Neal et al., 2009). While superficially similar to the colonic migrating motor complexes (CMMCs) that provided the preliminary data for this project, PCCs differ from the spontaneous motor activity in the colon in several key ways. First, in both duodenum and jejunum, PCCs often propagate from the anal end of an isolated segment to the oral end, while over 90% of CMMCs propagate

anally. Second, PCCs consist of a burst of contractions that result from the summation of activity in excitatory motor neurons and an ongoing rhythmic pattern generator intrinsic to the smooth muscle layer. The latter is seen in the muscle as regular oscillations in membrane potential or “slow waves” and these are produced by modified smooth muscle cells, interstitial cells of Cajal (ICC). The frequency of these oscillations is normally thought to be independent of neural activity. Although ICC are found in mouse colon (Ward et al 1999), slow waves do not appear to contribute to the CMMCs recorded with video imaging (Roberts et al 2007). A further difference between PCCs and CMMCs is that PCCs are enhanced by distension, while CMMCs occur spontaneously in undistended tissues.

Jejunal motor activity in NL3R451C and WT mice (Task 1b)

Our initial studies of PCCs in jejunum rapidly confirmed the differences between these motor patterns and CMMCs. As a result, we have decided to delay analysis of duodenal PCCs until we have refined the analytical tools needed to analyse this motor activity. As the normal



luminal environment of the jejunum contains various nutrients after a meal, we compared jejunal motor activity with saline in the lumen to activity in the presence of decanoic acid, which induces segmentation in guinea-pig jejunum (Gwynne et al 2004, 2007; Chambers et al 2011; Ellis et al 2013).

Figure 1: Spatiotemporal map illustrating Propagating Contractile Complexes (PCCs, arrow heads) in jejunum of adult mouse. Inset shows the higher frequency contractions

corresponding to slow waves that form part of the contractile complex. Colour scale on right shows intestinal diameter with red being maximally constricted.

In order to characterise PCCs in WT and NL3 tissue, the number of complexes, PCC duration, inter PCC interval and the peak slow wave frequency are calculated for each spatiotemporal map, and averaged between animals (Fig 2).

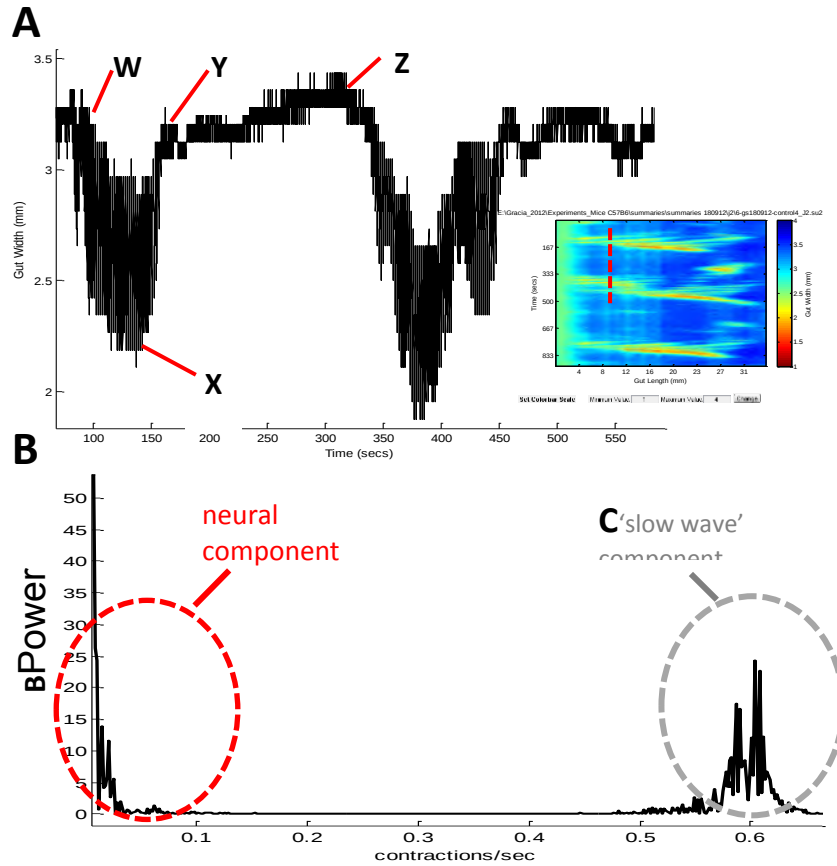


Figure 2: Overview of analysis procedure for quantifying PCC properties. **A:** Inset: a vertical slice (dashed line) identifies a specific location along the gut (jejunum) length in a spatiotemporal map recorded from an NL3 mouse. This leads to a plot of the diameter at that point as a function of time so that inter PCC interval (Z-Y) and PCC duration (W-Y) can be calculated from this 2 dimensional plot. **B:** Power spectrum generated from the vertical section indicated in the spatiotemporal map in A. Contractions cluster within two frequency ranges indicating two types of contractions (low frequency activity ie 0-0.1Hz indicates the neural component (red circle); high frequency activity indicates the slow-wave component (grey circle)).

We originally found that the frequency of CMMCs, measured as the number of events per 1 h recording period, is the most sensitive measure of the differences between NL3^{R451C} and WT colon. Using this measure (and others), we found no differences in CMMCs recorded in control conditions between NL3^{R451C} and WT. In jejunum, however, while no difference in PCC frequency between NL3^{R451C} and WT littermates was detected in control solutions, we observed unexpected differences in other parameters of the PCCs under these conditions.

The most notable of these were in the durations (defined as the time from onset of a PCC at a given point along the segment to the end of that complex of contractions) of the PCCs, which were significantly shorter in the NL3^{R451C} jejunum (Fig 3B; n = 4 NL3^{R451C} and n = 6 WT, adult male mice) and, very surprisingly, in the slow wave frequency, which was significantly lower in the NL3^{R451C} than in WT (Fig 3D).

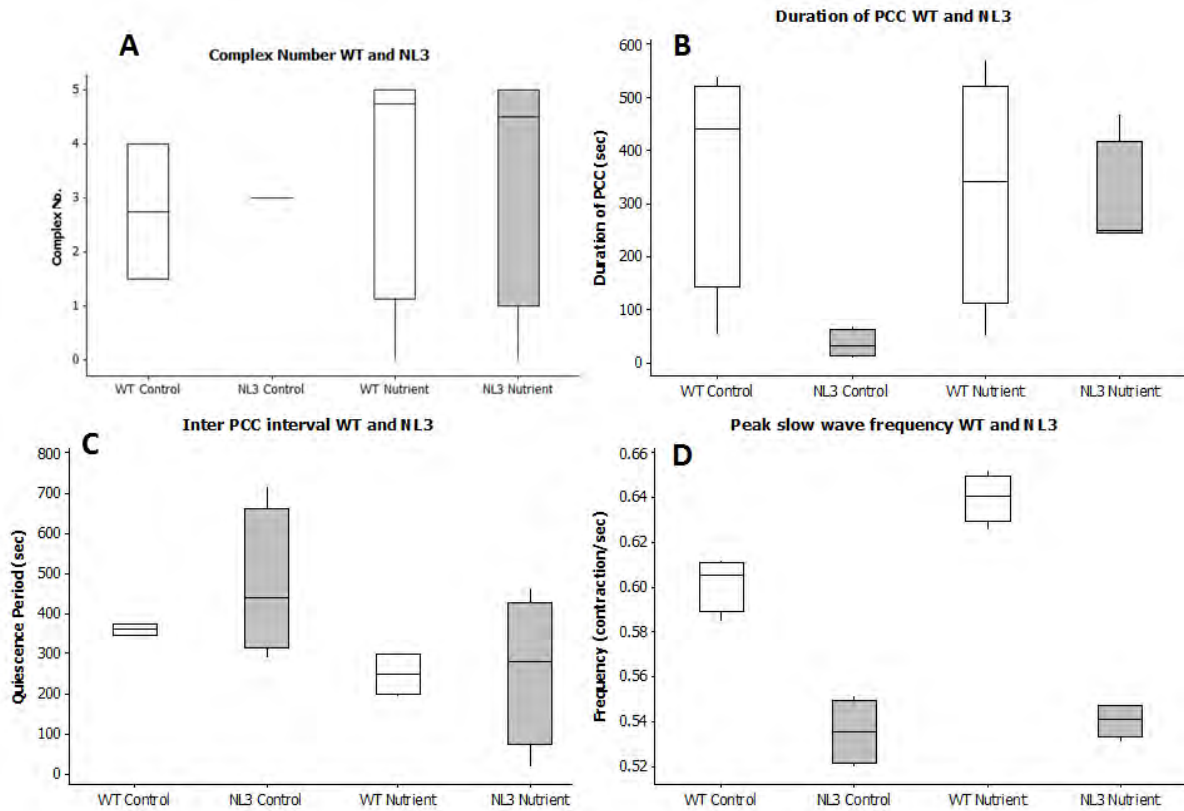


Figure 3 Propagating Contractile Complexes (PCCs) in the small intestine (jejunum) of adult WT and NL3 mouse at baseline and in the presence of 1mM decanoic acid (nutrient condition). A: Number of PCC complexes (15 min⁻¹). B: Duration of PPCs C: PCC inter event interval D: Group data illustrating peak slow wave frequency. Number of animals: 4 NL3, 6 WT. Frequency data expressed as median +/- interquartile range, graphs for other parameters expressed as mean +/- interquartile range. Statistical analyses were conducted using Minitab software.

We also saw differences between NL3^{R451C} and WT jejunum in the response to luminal decanoic acid, which did not affect the frequency of PCCs in either, but increased the durations of PCCs in the NL3^{R451C} jejunum without having a significant effect on this parameter in the WT. In contrast, decanoic acid increased the frequency of slow waves in WT, but had no effect on slow wave frequency in the NL3^{R451C} jejunum.

The finding that slow wave frequency is increased by the presence of decanoic acid in the jejunal lumen in WT mice represents entirely new physiology and raises the question of how this is achieved. As the nutrient had no effect on slow waves in the NL3^{R451C}, the issue of why this was the case also arises. Slow wave frequency is also significantly lower in the NL3^{R451C} jejunum in control, which suggests that the mutation interferes with the ability of the ICC pacemaker mechanism to respond to an external signal that is normally active and is enhanced by luminal decanoic acid. The immunohistochemical analysis discussed elsewhere in this report did not identify neuroligin 3 in the pacemaker ICC that lie at the level of the myenteric plexus, so it seems likely that any effect of the NL3^{R451C} mutation on pacemaker generation is indirect, perhaps within the ENS itself. We are now designing experiments to test this idea and will submit a modified Statement of Work when this process is complete.

The observation that PCC duration differs between WT and NL3^{R451C} littermates raises the question of whether other parameters will show strain-specific differences. We have yet to analyse the data to measure parameters like propagation speed and direction for both the neurally mediated component of the PCCs and the underlying slow waves (we have strong data that these are independent of each other) or the magnitude of the constrictions or the instantaneous frequencies of the PCCs (an index of the variability within the pacemaker mechanisms). These analyses are very time consuming and have yet to be automated; we are pursuing an update in our analysis software using funds from Australian grants to facilitate this. Once this is complete, we will undertake the pharmacology studies that we originally proposed.

Colonic motility (Task 1c)

We have already shown that CMMCs in NL3^{R451C} colon do not differ significantly in frequency, propagation speed, the distance over which they propagate or their duration from those in WT while in control solutions. Moreover, as shown in our application, NL3^{R451C} CMMCs are much more sensitive to blockade of GABA_A receptors than WT. NL3^{R451C} CMMCs are also more sensitive to tropisetron than WT, when this compound is administered at a concentration that inhibits both 5-HT₃ and 5-HT₄ receptors. In this component of the study, we first confirmed our observations on the effects of bicuculline on the frequency of CMMCs and then examined whether the effects of tropisetron are due to its action on 5-HT₃ or 5-HT₄ receptors.

We initially examined the roles of 5-HT₃ receptors using a specific antagonist, granisetron (1 μ M), which is effective in blocking these receptors in guinea-pig and rat studies. However, while this compound had effects in female mouse colon, it had no effect on CMMCs in either male WT or male NL3^{R451C} colon. Accordingly, we tested an alternative antagonist, ondansetron, which is widely reported to alter CMMCs in various mouse strains.

Ondansetron

Initial data showed that CMMCs were completely abolished by 3 μ M ondansetron in both types of mice (4 WT and 5 NL3^{R451C}; Fig 4). We then tested whether NL3^{R451C} CMMCs are more sensitive to ondansetron than WT by constructing cumulative concentration-effect curves. To date, we have assessed the effects of three concentrations of ondansetron (30, 100 and 300 nM) on colonic activity in tissue from 6 NL3^{R451C} and 6 WT adult mice. Each exposure lasted 30 min, before the ondansetron concentration was increased (ie a total of 90 minutes during drug application) prior to a 60 min washout. There were no differences in the responsiveness of the two types of mice to this antagonist at any concentration tested. Representative spatiotemporal maps illustrating colonic motility (CMMCs) in these mice are shown in Figure 5 with quantification in Fig 6.

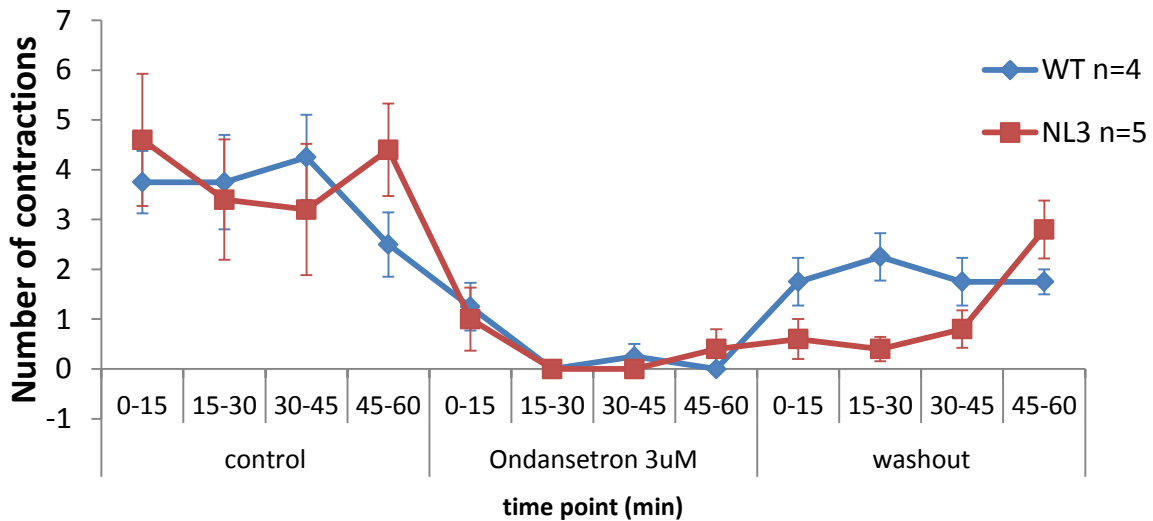


Fig 4: Effect of 3 μ M Ondansetron on CMMCs. CMMCs were completely abolished by ondansetron (3 μ M) in both NL3 and WT colon preparations; graphs show contractions per 15 min recording period in control, ondansetron and after washout.

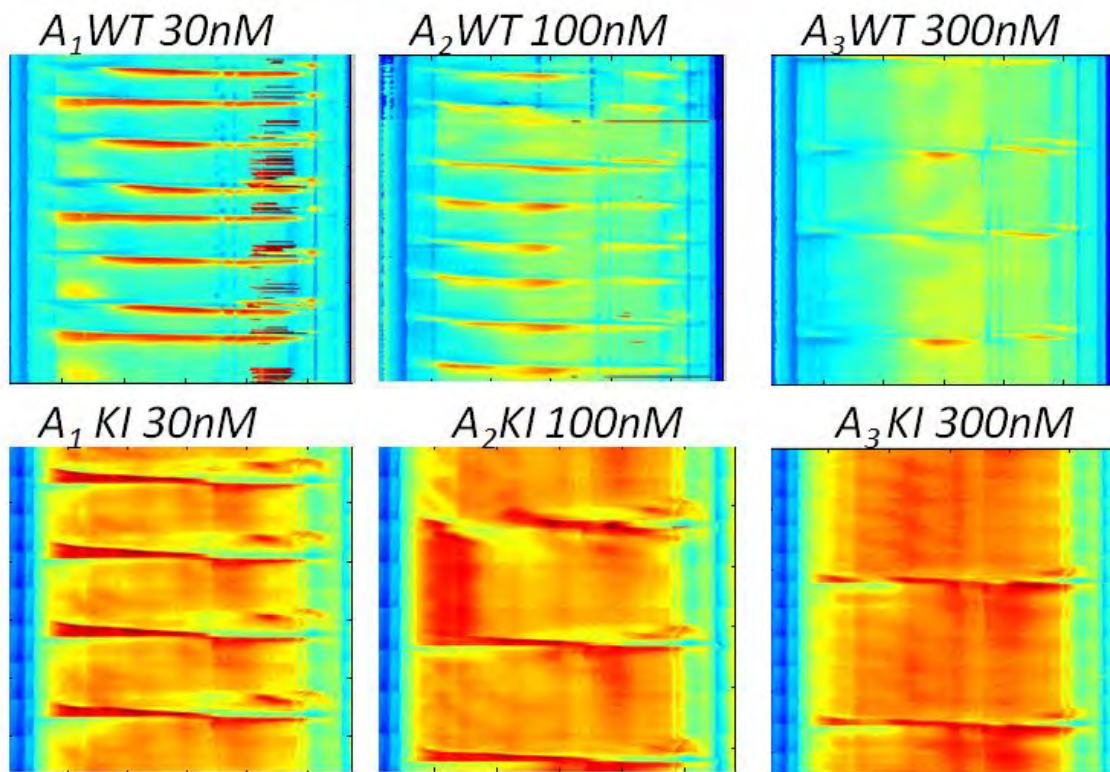


Fig 5: Effect of varying concentrations of Ondansetron on CMMCs. Representative spatiotemporal maps illustrating colonic migrating motor complexes during Ondansetron administration (30, 100 and 300 nM) in WT and NL3 mouse colon.

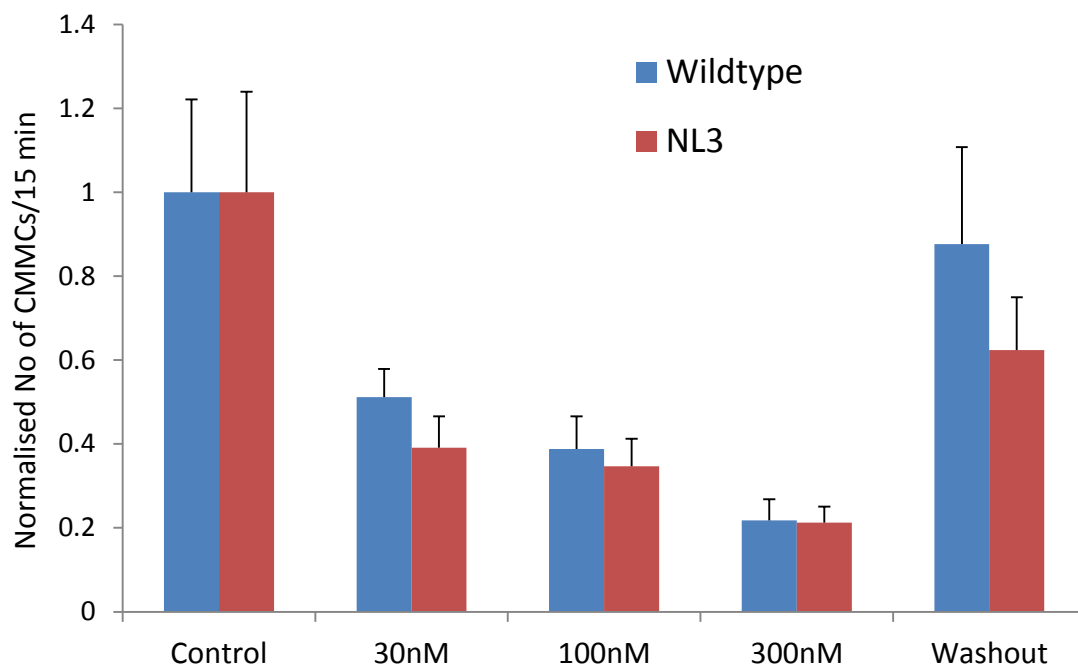


Fig 6: Concentration effect curve for Ondansetron. The normalised numbers of CMMCs per 15 min for adult mouse WT and NLW3 colon during each exposure are shown.

SB 207266, a 5-HT₄ antagonist

As sensitivity to 5-HT₃ receptor blockade appears identical for CMMCs in NL3^{R451C} and WT, we tested whether the former would be more sensitive than the latter to blocking 5-HT₄ receptors. We used the 5-HT₄ receptor antagonist SB207266, which is highly effective in blocking this class of serotonin receptor. However, although SB207266 slightly depressed CMMC activity, the effect was very small and identical in both NL3^{R451C} and WT.

These results can be explained by two alternative hypotheses. First, tropisetron is not acting on either 5-HT₃ or 5-HT₄ receptors at the concentration that we used, but rather acts on another site which is differentially sensitive between the two mouse strains. As 5-HT₃ receptors are from the Cys-loop family that includes both GABA_A receptors and nicotinic acetylcholine receptors, which are central to transmission in the ENS, it is possible that the tropisetron effect involves one of these two broad receptor classes. However, the concentration used should have been well above that required to mimic the ondansetron effect. The alternative is that there are two different types of 5-HT₃ receptor in the mouse ENS with one being sensitive to ondansetron and essential to generation of CMMCs, while the other is sensitive to granisetron and tropisetron, but is only involved in generation of CMMCs in combination with 5-HT₄ receptors. We postulate that it is this latter type of 5-HT₃ receptor whose activity is altered in NL3^{R451C} mice and will test this in the second year of the grant. We will also test whether there is differential sensitivity to nicotinic receptor blockade using the broad-spectrum nicotinic antagonist hexamethonium. We have already identified appropriate concentrations for this experiment in another strain of mouse, the C57Bl/10.

To test the possibility that block of both 5-HT₃ and 5-HT₄ receptors is required to identify differential sensitivity between the knock-in and WT mice, we will construct concentration-effect curves for tropisetron (as for ondansetron). We will then test whether granisetron and SB207266 in combination are more effective in NL3^{R451C} than in wild type mice. We will amend the statement of work to include these experiments, which are needed because of the unexpected differences between well established and clinically used 5-HT₃ antagonists.

Localizing the neuroligin 3 protein in specific classes of enteric neurons (Tasks 2a, b)

The localisation of neuroligin 3 and protein binding partners is not well characterised in the central and peripheral nervous systems. However, two studies have used immunohistochemistry to localize these proteins in the CNS (Gilbert et al., 2001, Scheiffele and Budreck, 2007).

From the CNS data, we predicted that neuroligin 3 would be a component of the post-synaptic densities at enteric synapses. To test this, we used double labelling immunohistochemistry to colocalise neuroligin 3 with a marker that labels both the somata and dendrites of myenteric neurons. Approximately 40% of myenteric neurons in the mouse colon are immunoreactive for neuronal nitric oxide synthase (nNOS) and the immunoreactivity clearly fills the entire cell body and its dendrites. These neurons have been identified as interneurons and inhibitory motor neurons and are believed to play a major role in regulation of CMMCs. Thus, we used antisera against both neuroligin 3 and nNOS in this phase of the study (nNOS; neuronal Nitric Oxide Synthase; Jackson ImmunoResearch, West Grove, USA, Neuroligin 3; Cat#129113, Synaptic Systems, Germany).

Labelling: Neuroligin 3 and nNOS

We performed double-labelling fluorescence immunohistochemistry for neuroligin-3 and nNOS in mouse myenteric plexus (Figure 2a). Unexpectedly, our data showed that neuroligin-3 is expressed in what appear to be synaptic varicosities, ie presynaptic terminals (see arrows, Fig 7). Labelling in cell bodies of nNOS neurons was often absent or indefinite and there did not appear to be synaptic accumulations that might reflect post-synaptic specializations. Similar observations were made in preparations of jejunum (Fig 8), where immunoreactivity for Neuroligin 3 in neuron cell bodies appeared to be substantially reduced in neuron cell bodies, although immunoreactivity could still be seen in apparent varicose terminals.

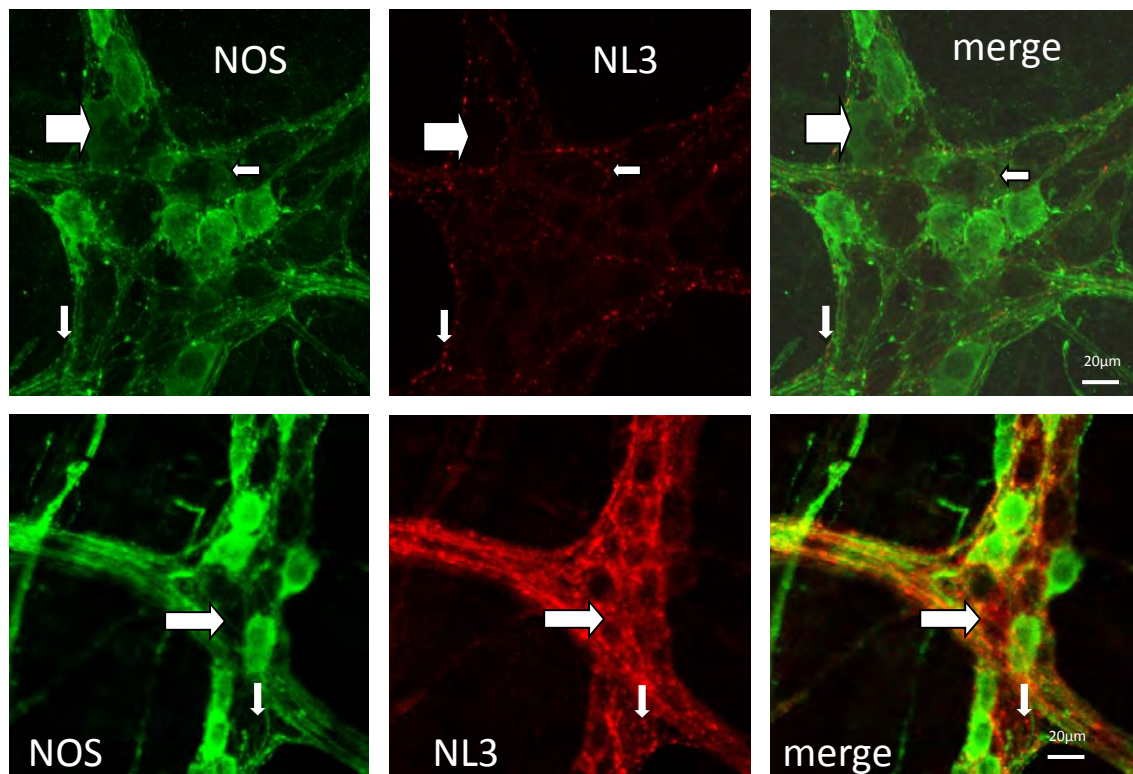


Fig 7: Localisation of nNOS and NL3 in the myenteric plexus of WT mouse colon. Representative images of immunohistochemical staining for nNOS and NL3 in WT adult mouse myenteric plexus. **Top;** thick horizontal arrow identifies a NOS positive neuron which is immunonegative for NL3; thin arrows identify beaded processes (putative axonal synaptic specializations) immunoreactive for both NOS and NL3. **Bottom;** horizontal arrow denotes a NOS negative neuron which is clearly labelled for NL3. Vertical arrow: NL3 immunoreactive (NOS negative) presynaptic specializations. Scale bar = 20 μ m.

Although these results remain to be quantified, they differ from studies of cultured hippocampal cells that suggest neuroligin 3 is ubiquitously expressed in neurons (Budreck and Sheiffele 2007).

A further key observation relevant to Part 1 is that neuroligin 3 staining was confined to the myenteric plexus and was not seen in cells that might be the ICC at the level of the myenteric plexus that generate slow waves in the colon or the jejunum (see below).

The unexpected finding of a presynaptic location for neuroligin 3 suggested that the antiserum may have also localised a different protein. Accordingly, we tested the specificity using Western blot analysis and found that the antiserum recognised only a single protein

band with an appropriate molecular weight (Fig 8). This confirms its specificity for neuroligin 3.

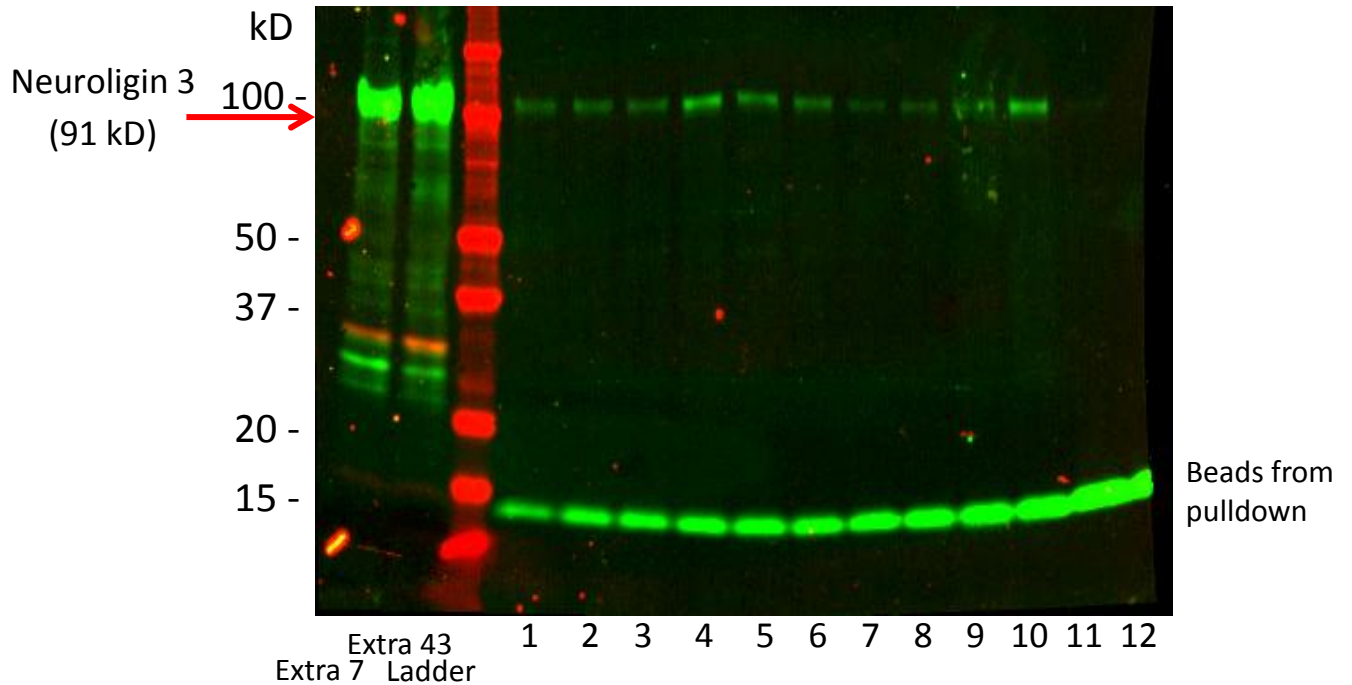


Fig 8: Western blot showing specificity of the Neuroligin-3 antibody. The Neuroligin-3 antibody (#129 113 rabbit polyclonal, Synaptic Systems) labels a 91kD protein (predicted weight of Neuroligin-3) in mouse hippocampal tissue (lanes 1-12). We have purchased antisera targeting Neurexin 1, Neurexin 2 and Neuroligin1 and have begun optimizing these antisera for use in mouse enteric tissue.

Similar observations were made in preparations of jejunum (Fig 9), where immunoreactivity for neuroligin 3 was seen in neuron cell bodies, but not in all NOS neurons, but appeared to be substantially reduced in neuron cell bodies of NL3^{R451C} jejunum, although immunoreactivity could still be seen in varicose terminals.

To test if the altered sensitivity to GABA seen in NL3^{R451C} colon is due to changes in the number of GABA neurons, we also undertook triple labelling immunohistochemical analysis of the proportions of GABA and nNOS (some of these neurons are known to colocalise with GABA) neurons in both knock-in and their wildtype littermates. All neurons were identified

using an antiserum recognising the pan-neuronal marker Hu: total numbers of neurons, the numbers of GABA neurons, of nNOS neurons and of neurons staining for both were counted in proximal, mid and distal colon. There were no significant differences in the total numbers of neurons or in the proportions of individual neurochemical subtypes between NL3^{R451C} and WT in each colonic region (n = 3 animals in each case).

These results indicated that we cannot account for the increased sensitivity of NL3^{R451C} colon to GABA_A antagonists by a change in the number of GABA neurons. They also indicate that the NL3^{R451C} mutation does not alter general enteric neuronal differentiation or differentiation of at least some key neurochemical subtypes of colonic neurons.

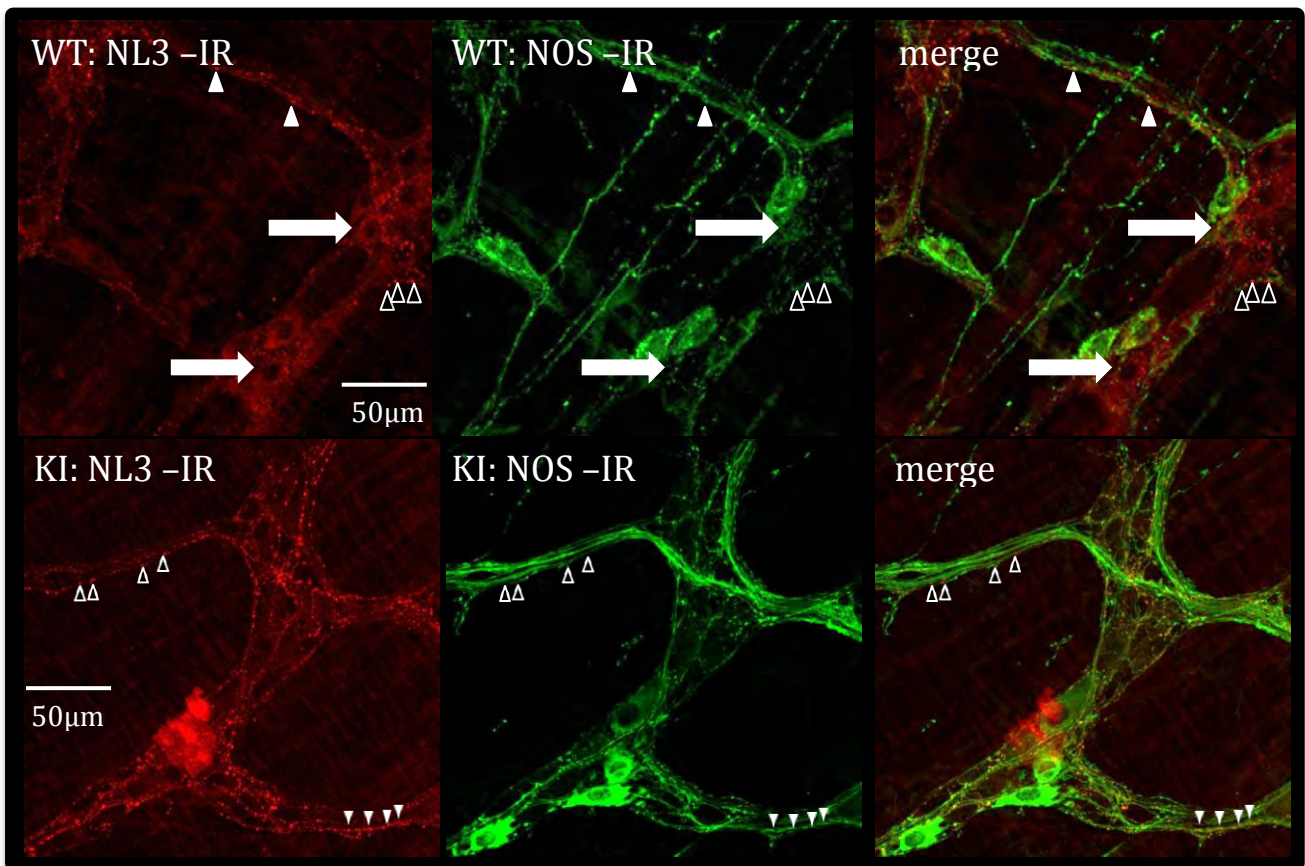


Figure 3: Localisation of NL3 and nNOS in the myenteric plexus of mouse jejunum.
Top: Adult WT mouse jejunum myenteric plexus labelled for NL3 (red, 1:250) nNOS (green, 1:1000). **Horizontal arrows** show cell bodies labelled for NL3 but negative for nNOS.
Bottom: Adult KI (NL3 Knock-in) mouse jejunum myenteric plexus labelled for NL3 (red, 1:500) and nNOS (green, 1:1000). **Open arrow heads** show presynaptic varicosities clearly labelled for NL3 but negative for nNOS. **Filled arrow heads** show presynaptic varicosities colocalizing NL3 and nNOS. Fewer NL3 immunoreactive cell bodies are visible in the KI.

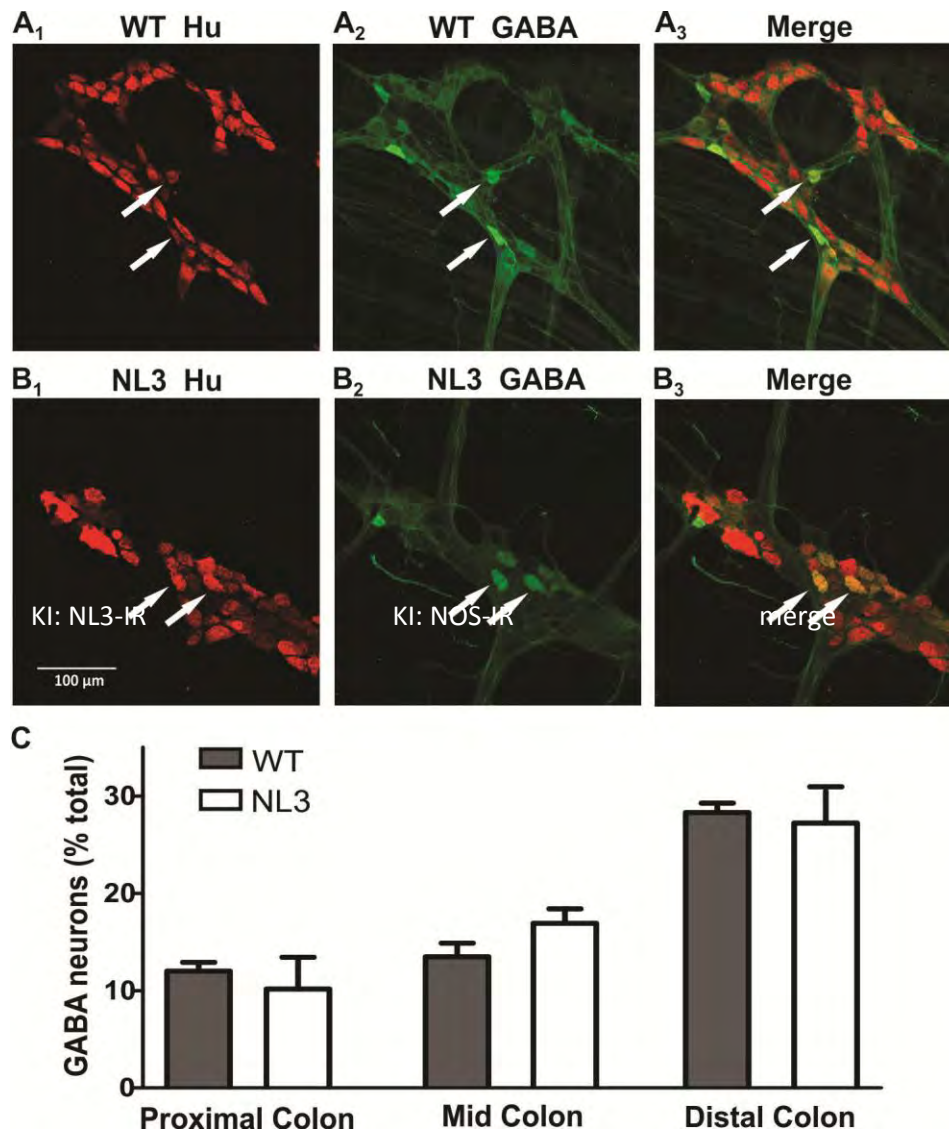


Fig 10: GABA labelling in WT and NL3 mice

We observed no change in neuronal proportions immunoreactive for GABA in NL3^{R451C} (n = 3) compared to WT (n = 3) colon. Representative images of myenteric plexus of (A) WT and (B) NL3^{R451C} distal colon illustrating neurons immunoreactive for Hu (A₁, B₁; red) and GABA (A₂, B₂; green) and merged images (A₃, B₃). Arrows indicate neurons labelled for Hu and GABA. C: Bar graph indicates the percentage of Hu-positive neurons immunoreactive for GABA in WT and NL3^{R451C} proximal, mid and distal colonic regions. There is an effect of region (p<0.0001). Data shown as mean ± s.e.m.

Training related to this project

Since the submission of the original application, Ms Sari Faustiwan (sp) has decided to pursue an alternative career. We are seeking a replacement.

Identifying functional and developmental expression of neuroligin 3

While progress has been made on this part of the study, see Report for PI Young, PI Bornstein's group will undertake their component in months 13-24 so there is no progress to report.

Key Research Accomplishments

- Demonstrated that small intestinal motility functions differ between NL3R451C mice and their WT under control conditions in vitro
- Identified an unexpected difference in slow wave activity between NL3R451C and WT jejunum indicating that smooth muscle pacemaker activity in interstitial cells of Cajal is disturbed in the mouse model of autism
- Identified an altered response of NL3R451C jejunum to the medium chain fatty acid, decanoic acid, when compared with the WT
- Showed that the increased sensitivity of NL3R451C colon to tropisetron is unlikely to be due to altered sensitivity of 5-HT₃ receptors alone
- Showed that neuroligin 3 is not expressed by all myenteric neurons with some neurons immunoreactive for nNOS lacking neuroligin 3 immunoreactivity
- Showed that neuroligin 3 is probably located in some presynaptic terminals within the mouse jejunum and colon, but not in interstitial cells of Cajal
- Showed that the NL3R451C mutation does not lead to altered differentiation of enteric neural crest cells into neurons and that it is unlikely that it affected the neurochemical differentiation of at least two major subclasses of myenteric neurons
- Demonstrated that the increased sensitivity to GABAA receptor blockade of CMMCs in NL3R451C is not due to changes in the number of GABA neurons in the myenteric plexus

Reportable Outcomes

1. Manuscripts:

1. *In preparation*: Burrows EL, Ellis M, Swaminathan M, Koyama L, Taher M, McKeown SJ, Parry LJ, Churilov L, Oezguen N, Savidge T, T O'Brien TJ, **Hill-Yardin EL**, Hannan AJ, **Bornstein JC**. Gastrointestinal dysfunction and aggression in a mouse model of autism. (attached)
2. *Invited manuscript*: Swaminathan, M, Balasuriya, G, **Hill-Yardin EL.**, **Bornstein, JC**. Video imaging of isolated colon preparations to study enteric nervous system function in mice *Journal of Visualized Experiments*

2. Abstracts:

- Gastrointestinal dysfunction mediated by GABA_A receptors in the Neuroligin-3^{R451C} mouse model of autism. **Elisa L Hill-Yardin**, Melina Ellis, Numan Oezguen, Tor Savidge, **Bornstein JC**. Accepted for poster presentation at the Society for Neuroscience meeting San Diego, Nov **2013**.
- Altered inhibitory neurotransmission in the enteric nervous system of Neuroligin-3 R451C mice Bioautism 2013, Melbourne. Swaminathan M, Foong JPP, Ellis M, **Hill-Yardin EL**, **Bornstein JC**.
- Investigating neuronal subtypes and colonic motility in the neuroligin-3 R451C mouse model of autism. Swaminathan M, Foong JPP, Ellis M, **Hill-Yardin EL** and **Bornstein JC**. Australian Neuroscience Society (ANS), Melbourne, **2013**.
- The neuroligin 3 Arg451cys (NL-3) mouse model of Autism shows altered colonic function *in vitro*. Melina Ellis, Ali M. Taher, Sonja McKeown, **Elisa L. Hill**, **Joel C. Bornstein** Digestive Diseases Week, **2012**, San Diego.

3. Presentations/Seminars:

- Examining the role of the ENS in genetically based disease, what we need to know and don't. Invited presentation at ENS II 2014, Adelaide South Australia
- The GI symptoms of autism – a role for the enteric nervous system. Invited seminar as part of the DDC GI forum at Texas Children's Hospital in Houston, TX, May 2013

Conclusion

The data obtained in this component of the project during the first year confirm that the NL3^{R451} mouse model of autism has altered gastrointestinal function resulting from changes in the behaviour of the enteric nervous system. The most notable data come from our studies of the jejunum and its response to luminal fatty acid, which show that motility patterns in the NL3^{R451C} differ from the WT both in control conditions and in the presence of the fatty acid. Changes in both the neural pacemaker within the enteric nervous system and, unexpectedly, in the intrinsic pacemakers of the intestinal smooth muscle have been identified in this work. Importantly, the differences between the NL3^{R451C} and their WT littermates could not have been secondary to alterations in central nervous system function, because they were seen in completely isolated tissues.

These data raise a number of key issues including the changes in neural circuitry that lead to altered motility in the jejunum at rest and why this type of alteration does not seem to be present in the colon in the absence of pharmacological intervention. The studies already proposed for year 2 of this project should shed light on these two questions.

However, another key question comes from the finding that there is a significant difference in the frequency of pacemaker activity in the muscle itself; activity that is generated by interstitial cells of Cajal (ICC), which are modified smooth muscle cells, not neurons. Pacemaker ICC are usually believed to operate independently of neural activity, but our current data suggest that this may not be the case with decanoic acid enhancing pacemaker frequency in WT jejunum, but not in the NL3^{R451C}. As the initial immunohistochemical localization study of neuroligin 3 in the myenteric plexus did not identify this protein in ICC in this layer of the jejunum or colon and myenteric ICC are the muscle pacemakers, it seems unlikely that the mutation directly affects ICC behaviour. Rather the most probable explanation is that the mutation is interfering with neural regulation of ICC function in some way. This is novel physiology and we will pursue this issue during the 2nd year of the project by investigating the possibility that neuroligin 3 is present in other ICC and confirming its absence from myenteric ICC. It is likely that this work will lead to a basic research proposal to the Australian Research Council in 2015, the first year that PI Bornstein is eligible to apply. Altered ICC function has been widely implicated in a variety of gastrointestinal diseases and is the subject of intensive investigation, the possibility that it may play a role in

ASDs has not previously been considered. However, our present data indicate that it may be a very real contributor in some cases.

Our work in the last year has also confirmed the presence of neuroligin 3 protein within the enteric nervous system, taking our earlier demonstration of mRNA expression further. However, the immunohistochemistry produced two surprising results. First, neuroligin 3 was not expressed ubiquitously in myenteric neurons and did not appear to be located in post-synaptic densities when it was expressed. This needs confirmation and we will be completing this aspect of the study, together with examining the expression and distribution of identified binding partners for this protein. We now have access to a super-resolution microscope, which should facilitate these studies at no extra cost to the project.

Second, neuroligin 3 appears to be located in some presynaptic terminals within both the jejunum and colon. While this remains to be confirmed by colocalization studies using presynaptic markers like synaptophysin, it suggests that current ideas about the function of neuroligin 3 at synapses in the central nervous system may need substantial modification. There is some support in the literature for a presynaptic function for neuroligin 3, notably the finding of increased spontaneous synaptic currents in NL3^{R451C} cortex, but the main focus has been on a post-synaptic role. Neuroligin 3 in synaptic varicosities might indicate that there are axo-axonic synapses in the enteric neural circuitry, but this in itself would be a highly unusual finding. In the next year, we will identify the subtypes of neurons that provide the varicose terminals immunoreactive for Neuroligin 3, identify whether these varicosities contain other Neuroligin 3 binding partners and determine the effect of the NL3^{R451C} mutation on these synapses.

Our data also indicate that the altered sensitivity to GABA_A receptor blockade of colonic motility, which formed the basis of our original application, is not due to a difference in numbers of enteric GABA neurons between the models and their littermates. This indicates that the changes in sensitivity to the antagonists are likely to be within the synapses made by these neurons and the work in the second year of the study will focus on this aspect, notably the component of the project for which PI Hill is responsible.

The observation that there was no difference in colonic sensitivity to specific 5-HT₃ or 5-HT₄ antagonists between model mice and their wildtype littermates was unexpected and has led us to identify a change in the emphasis of our pharmacological studies in both colon and jejunum. Serotonin plays a major, but still poorly characterized, role in the regulation of

intestinal and colonic motility and has long been implicated in some forms of autism. Clearly, characterizing the dysfunction seen with the mixed 5-HT₃/5-HT₄ antagonist, tropisetron, when specific antagonists do not show such a dysfunction needs to be a major goal of the next year of the study.

Overall, the work to date has major implications for both the basic science underpinning studies of autism and for the clinical picture itself. Our finding that jejunal function is modified in the NL3^{R451C} model in control conditions confirms that gastrointestinal symptoms in ASD patients may arise from enteric dysfunction, at least when there is an underlying mutation in a gene encoding a synaptic protein. This has important implications for diagnosis and treatment. Our finding of altered muscle pacemaker function in the mouse model suggests an entirely new target for intervention for the gastrointestinal symptoms of autism, the interstitial cells of Cajal. At the basic level, identification of Neuroligin 3 in apparent presynaptic terminals raises key issues for the broader field of the functions of those synaptic proteins that are implicated in ASD.

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Appendices

Summary of animals and antibodies used:

Localizing the neuroligin 3 protein in specific classes of enteric neurons

Date	Mouse ID	Weight (g)	Sex	Genotype
25.07.13	174	26.5	Male	WT/O
31.07.13	215	24.78	Male	KI/O
13.08.13	210	26.72	Male	WT/O
14.08.13	170	24.16	Male	KI/O
27.08.13	245	31.53	Male	WT/O
27.08.13	241	24.16	Male	KI/O
28.08.13	223	29.17	Male	WT/O

Antiserum	Supplier	Cat. No.	Dilution
Rabbit Anti-Neuroigin 1	Santa Cruz	sc-50393	
Goat Anti-Neuroigin 2	Santa Cruz	sc-14089	
Rabbit Anti-Neuroigin 2	Synaptic Systems	129 203	
Rabbit Anti-Neuroigin 3	Synaptic Systems	129 113	1:1000 (Mouse) 1:500 (G.Pig)
Goat Anti-Neurexin 1	Santa Cruz	sc-14334	
Goat PSD-95	Santa Cruz	sc-6926	
Neuroigin 2 Control Peptide	Synaptic Systems	129 2P	
Neuroigin 3 Control Peptide	Synaptic Systems	129 3P	1:500 (G.Pig)
Sheep Anti NOS	Jackson West Grove USA		1:1000 (Mouse & G.Pig)
Donkey anti Sheep Alexa 488 (green)	Life Technologies AustraliaInvitrogen		1:400 (Mouse & G.Pig)
Donkey anti Rabbit 594 (green)	Life Technologies AustraliaInvitrogen		1:400 (Mouse & G.Pig)

Motility experiments

Date	genotype	Experiment
18/09/2012	NL3	Time control
22/01/2013	NL3	Time control
29/01/2013	NL3	Time control
30/01/2013	NL3	Time control
13/02/2013	NL3	Time control
5/03/2013	NL3	Time control
5/03/2013	NL3	Time control
29/01/2013	WT	Time control
30/01/2013	WT	Time control
6/03/2013	WT	Time control
6/03/2013	WT	Time control
13/02/2013	WT	Time control
19/09/2012	NL3	Decanoic acid
19/09/2012	NL3	Decanoic acid
11/12/2012	NL3	Decanoic acid
11/12/2012	NL3	Decanoic acid
17/04/2013	NL3	Decanoic acid
17/04/2013	NL3	Decanoic acid
26/04/2013	NL3	Decanoic acid
1/05/2013	NL3	Decanoic acid
1/05/2013	NL3	Decanoic acid
21/05/2013	NL3	Decanoic acid
21/05/2013	NL3	Decanoic acid
22/05/2013	NL3	Decanoic acid
22/05/2013	NL3	Decanoic acid
10/09/2012	WT	Decanoic acid
24/09/2012	WT	Decanoic acid
13/11/2012	WT	Decanoic acid
13/11/2012	WT	Decanoic acid
14/11/2012	WT	Decanoic acid
14/11/2012	WT	Decanoic acid
21/11/2012	WT	Decanoic acid
21/11/2012	WT	Decanoic acid

Manuscripts

Published manuscripts:

1. Hao MM, **Bornstein JC**, Vanden Berghe P, Lomax AE, **Young HM**, Foong JP. The emergence of neural activity and its role in the development of the enteric nervous system. *Dev Biol.* 2012 Dec 19. doi:pii: S0012-1606(12)00672-0. 10.1016/j.ydbio.2012.12.006. [Epub ahead of print]
2. Thornton PDJ, Gwynne RM, McMillan DJ, **Bornstein JC** Transmission to interneurons is via slow excitatory synaptic potentials mediated by P2Y₁ receptors during descending inhibition in guinea-pig ileum. *PLoS ONE* 10.1371/journal.pone.0040840 (2013)
3. ELLIS M, CHAMBERS JD, GWYNNE RM, **BORNSTEIN JC** Serotonin (5-HT) and cholecystokinin (CCK) mediate nutrient induced segmentation in guinea pig small intestine. *AmJPhysiolGastrointestLiverPhysiol* **304**, G749-G761(2013)
4. WAFAL L, TAHER M, JOVANOVSKA V, **BORNSTEIN JC**, DASS CR, NURGALI K Effects of oxaliplatin on mouse myenteric neurons and colonic motility. *Front Neurosci* 7: 30 doi: 10.3389/fnins.2013.00030 (2013)
5. Hao MM, **Bornstein JC**, **Young HM**. Development of myenteric cholinergic neurons in ChAT-Cre;R26R-YFP mice. *J Comp Neurol.* 2013 Oct 1;521(14):3358-70. doi: 10.1002/cne.23354. PMID:23649862
6. CHAMBERS JD, THOMAS EA, **BORNSTEIN JC** Mathematical modelling of enteric neural motor patterns. *ClinExpPharmacolPhysiol* (in press)

In preparation:

3. Burrows EL, Ellis M, Swaminathan M, Koyama L, Taher M, McKeown SJ, Parry LJ, Churilov L, Oezguen N, Savidge T, T O'Brien TJ, **Hill-Yardin EL**, Hannan AJ, **Bornstein JC**. Gastrointestinal dysfunction and aggression in a mouse model of autism.

Invited manuscript:

Swaminathan, M, Balasuriya, G, **Hill-Yardin EL.**, **Bornstein, JC**. Video imaging of isolated colon preparations to study enteric nervous system function in mice *Journal of Visualized Experiments*

Conference Abstracts

Accepted as a poster presentation at the **Society for Neuroscience meeting San Diego 2013**, meeting date November 9th-13th 2013. Poster session 531, Neural mechanisms associated with Autistic behaviors in Animals. Tuesday November 12th in Halls B-H.

Gastrointestinal dysfunction mediated by GABA_A receptors in the Neuroligin-3^{R451C} mouse model of autism

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Gastrointestinal (GI) disorders are common in patients with autism spectrum disorder (ASD) and reduce quality of life. However, despite association of many genes influencing CNS synaptic function, the etiologies of these GI symptoms are unknown. Mutations in the neuroligin family of synaptic adhesion molecules are implicated in ASD disease progression. In order to identify underlying biological mechanisms, information on the expression of ASD candidate genes and the functional impact of the NL3^{R451C} mutation in the enteric nervous system (ENS) is required. **Methods:** Human ASD-associated neuroligin mutations were structurally mapped using homology models generated for human NLG1, 2, 3 and 4Y and aligned to core structured regions of the NLGN4-X crystal structure. Protein-protein interfaces were predicted using the InterProSurf web server. Expression of neuroligins and neurexins was assessed in gut and brain cDNA using RT-PCR. Colonic migrating motor complexes (CMMCs) were analysed in isolated colon segments using video imaging techniques and spatiotemporal maps. CMMCs were assessed at baseline and in the presence of GABA_A (bicuculline 10 μ M and gabazine 10 μ M) and GABA_B (CGP 54626; 100 nM) receptor antagonists. **Results:** The structural domain harbouring R451C contained 5 of 7 identified mutation sites (Arg451 on NLGN3 and Asp429, Asp396, Val403 and Lys378 on Neuroligin 4X), closely juxtaposed to two protein-protein interface patches. We show expression of *Nlgn3*, related genes (*Nlgn1* and *Nlgn2*) and neurexin binding partners (*Nrxn 1* and *Nrxn 2*) in the mouse ENS. Further, both bicuculline (n = 16 WT, n = 16 NL3^{R451C}) and gabazine (n = 11 WT, n = 11 NL3^{R451C}) reversibly depressed CMMCs in NL3 mice compared to WT while CGP 54626 (n = 8 WT, n = 9 NL3) had no effect. Bicuculline reduced CMMCs in NL3^{R451C} compared to WT colon (median difference: 6 contractions,

95% CI: 1, 12; $p = 0.03$). Gabazine also reduced CMMC frequency in NL3^{R451C} mice compared to WT (median difference: 5 CMMCs, 95% CI: 2, 9; $p = 0.009$). In control conditions, CMMC frequency was identical in WT ($n = 70$) and NL3^{R451C} ($n = 69$) tissues; in both WT and NL3^{R451C} the median number of CMMCs was 4 (WT: 95% CI: 1, 11; NL3^{R451C}: 95% CI: 1, 15; median difference 1, 95% CI -2, 4; $p = 0.509$). **Conclusion:** Positional association of R451C with other clinically identified mutations suggest that a functionally conserved domain in the neuroligin family may be targeted in ASD. We show that the R451C knock-in mutation in the NL3^{R451C} mouse model of ASD causes colonic motility dysfunction via a GABA_A receptor mediated mechanism. These data implicate altered enteric synaptic function as a primary underlying cause of GI disorders in ASD.

Keywords: autism, gastrointestinal motility, mouse

Support:

This research was supported by a US Department of Defense Autism Research Program Idea Development Award (AR11034) to ELH-Y and JCB.

Abstract presented as poster presentation Australian Neuroscience Society, Gold Coast, QLD, Australia 2013:

INVESTIGATING NEURONAL SUBTYPES AND COLONIC MOTILITY IN THE NEUROLIGIN-3 R451C MOUSE MODEL OF AUTISM

Swaminathan M., Foong J.P.P., Ellis M., **Hill-Yardin E.L.** and Bornstein J.C.

Department of Physiology, University of Melbourne, Parkville Vic 3010.

Gastrointestinal problems are reported in up to 90% of Autism spectrum disorder (ASD) patients. Multiple gene mutations affecting synaptic function are associated with ASD. Neuroligin-3R451C mice express a missense mutation in the *nlgn3* gene coding for the neuroligin-3 postsynaptic adhesion protein and show altered GABA-mediated colonic motility. Nitric oxide released from a subset of enteric neurons (immunoreactive for nitric oxide synthase; NOS) mediates tonic inhibition between colonic migration motor complexes (CMMCs). Some NOS neurons also express GABA. **Purpose:** To determine whether NOS-mediated colonic motility is altered in NL3R451C mice and if changes in motility correspond to altered proportions of GABA and/or NOS neurons in NL3R451C mice colon. **Methods:** Colons were isolated from C57/Bl6, NL3R451C and WT (C57/Bl6-sv129-J) mice. Effects of the NOS inhibitor L-Nitro-arginine (NOLA, 100 μ m) on colonic motility were examined using video imaging techniques. Immunohistochemistry for Hu (a pan-neuronal marker), GABA and NOS was conducted on whole-mount myenteric plexus preparations from NL3R451C and WT colon. **Results:** Application of NOLA increased CMMC frequency in C57/Bl6 colons (n=8; $p < 0.05$). Similarly, NOLA increased CMMC frequency in NL3R451C colons (n=9 in each group; $p < 0.05$). In contrast, CMMC frequency was unaffected by NOLA in WT littermates (n=9). Furthermore, when compared to WT, C57/Bl6 colons showed increased CMMC frequency in response to NOLA ($p < 0.05$). The proportion of GABA or NOS immunoreactive neurons in WT (n=3) and NL3 R451C (n=3) mice was unchanged. **Conclusion:** These results suggest that the NL3R451C synaptic mutation alters nitric oxide-mediated colonic motility and that sensitivity to NOLA is strain-specific. Altered colonic motility in NL3R451C is not due to altered GABA/NOS neuronal numbers.

Abstract presented as poster presentation at Digestive Diseases Week San Diego 2013

Nitric oxide mediated colonic motility is altered in the Neuroligin-3 R451C mouse model of Autism

Mathusi Swaminathan, Jaime Pei Pei Foong, Melina Ellis, Joel C Bornstein, Elisa L Hill-Yardin

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Purpose: Gastrointestinal function is compromised in up to 90% of patients with Autism spectrum disorder (ASD) and the underlying causes are unknown. NL3^{R451C} mice express a missense mutation in the Nlgn3 gene coding for the postsynaptic adhesion protein, neuroligin-3 (NL3) found in ASD patients. We have previously shown that the excitatory component of organized colonic motility (GABA_A- and serotonergic- mediated) is perturbed in NL3^{R451C} mice compared to wild type (WT) controls. Neuronal release of nitric oxide (NO) is responsible for tonic inhibition between colonic migrating motor complexes (CMMCs). Here, we examined whether the NO-mediated inhibitory component of spontaneous CMMCs is altered in NL3^{R451C} mice.

Methods: Full length colons were removed from NL3^{R451C}, WT littermates (control) and C57Bl/6 mice (comparison strain). Colonic motility was examined using video imaging techniques. After a 1 h control recording, the NO synthase (NOS) inhibitor L-Nitro-arginine (NOLA, 100µm) was applied into the superfusing solution and activity was recorded for 1 h. The NOLA was then washed out for 1 h while a further recording was made. The proportion of all myenteric neurons (marked by the pan neuronal marker, Hu) that contained GABA and/or NOS was investigated using immunohistochemistry. Myenteric neuronal density and connectivity between GABA+ and NOS+ neurons were examined.

Results: NOLA significantly increased CMMC frequency in NL3^{R451C} (n = 9; p = 0.031), but not in WT littermates (n = 9; p = 0.831). NOLA also increased CMMC frequency in C57/Bl6 colons (n = 9; p = 0.008). No differences in neuronal density were observed between proximal (adjacent to cecum) and mid colon regions of WT and NL3^{R451C} mice (n = 3 each; p > 0.05). Furthermore, there was no difference in the mean number of neurons per ganglion in the distal colon of WT and NL3^{R451C} mice (n = 3 each; p = 0.216). In each colonic region, the proportions of neurons that were NOS+ and/or GABA+ were similar in WT and NL3^{R451C} mice (GABA+; p > 0.05; NOS+; p > 0.05; GABA+/NOS+; p > 0.05; n=3 each). No

GABA⁺ appositions onto NOS⁺ neurons were observed, and GABA⁺ neurons rarely receive NOS⁺ appositions in NL3^{R451C} mice.

Conclusions: NL3^{R451C} mice show altered NOS-mediated colonic motility compared with WT controls. However, our findings also suggest a strain specific effect of NOLA on CMMC frequency. The changes in motility in NL3^{R451C} colon are unlikely to be due to altered neuronal density or proportions of GABA or NOS neurons in NL3^{R451C} mice suggesting that altered transmission at the level of the synapse is a likely explanation. These results provide the first evidence that a synaptic mutation associated with ASD can affect inhibitory elements of gastrointestinal motility.

Curriculum Vitae PI Bornstein

Biographical Sketch

Provide the following information for each individual included in the Research & Related Senior/Key Person Profile (Expanded) Form.			
NAME JOEL C BORNSTEIN		POSITION TITLE PROFESSOR	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).			
INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Monash University, Melbourne, Australia	BSc (Hons)	1972	Physiology, physics, maths
Monash University, Melbourne, Australia	PhD	1976	Physiology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List in chronological order the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 4 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

POSITIONS AND EMPLOYMENT

1976–1978 ASSISTANT RESEARCH PHYSIOLOGIST, UNIVERSITY OF CALIFORNIA SAN FRANCISCO, CALIFORNIA

1978-1981 RESEARCH FELLOW, DEPARTMENT OF PHARMACOLOGY, JOHN CURTIN SCHOOL OF MEDICAL RESEARCH, AUSTRALIAN NATIONAL UNIVERSITY, CANBERRA ACT, AUSTRALIA

1981-1985 NHMRC AUSTRALIAN POST-DOCTORAL FELLOW, DEPARTMENT OF HUMAN PHYSIOLOGY, FLINDERS UNIVERSITY MEDICAL SCHOOL, ADELAIDE SOUTH AUSTRALIA, AUSTRALIA

1986-1987 SENIOR RESEARCH OFFICER, DEPARTMENT OF PHYSIOLOGY, FLINDERS UNIVERSITY MEDICAL SCHOOL, ADELAIDE SOUTH AUSTRALIA, AUSTRALIA

1988-1990 NHMRC RESEARCH FELLOW, DEPARTMENT OF PHYSIOLOGY, FLINDERS UNIVERSITY MEDICAL SCHOOL, ADELAIDE SOUTH AUSTRALIA, AUSTRALIA

1991-1995 SENIOR LECTURER, DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF MELBOURNE, PARKVILLE VIC, AUSTRALIA

1996-2004 ASSOCIATE PROFESSOR, DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF MELBOURNE, PARKVILLE VIC, AUSTRALIA

2005- PROFESSOR, DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF MELBOURNE, PARKVILLE VIC, AUSTRALIA

HONOURS

2009 AMERICAN GASTROENTEROLOGICAL ASSOCIATION MASTERS AWARD FOR OUTSTANDING ACHIEVEMENT IN BASIC DIGESTIVE SCIENCES BY AN ESTABLISHED RESEARCHER

SERVICE TO AUSTRALIAN GOVERNMENT

1996-1999 MEMBER NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL “REGIONAL GRANT INTERVIEW PANEL” (CHAIR 1999)

2001-2010 MEMBER AUSTRALIAN RESEARCH COUNCIL OZREADER PANEL

2003-2008 MEMBER NHMRC GRANT REVIEW PANEL (CHAIR 2007)

2011-2013 MEMBER NHMRC GRANT REVIEW PANEL

2011- ARC DETAILED ASSESSOR PANEL

EDITORIAL BOARDS

2005- SECTION EDITOR – NEUROREPORT

2009- CHIEF EDITOR – FRONTIERS IN ENTERIC NEUROSCIENCE (NOW FRONTIERS IN AUTONOMIC NEUROSCIENCE)

RESEARCH AND PROFESSIONAL EXPERIENCE (CONTINUED). PAGE LIMITATIONS APPLY. DO NOT EXCEED 4 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

JOURNAL ARTICLES (2010-)

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Gastrointestinal dysfunction and aggression in a mouse model of autism

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Abstract

Gastrointestinal (GI) motility dysfunction and aggressive behavior are common in patients with autism spectrum disorder (ASD) and reduce quality of life. However, despite association of many genes influencing CNS synaptic function, the etiologies of ASD symptoms are unknown. Mutations in the neuroligin family of synaptic adhesion molecules are implicated in ASD disease progression. Here we demonstrate that the ASD causing missense mutation (R451C) in neuroligin-3 (NL3) is located in a highly conserved structural domain preferentially targeted by clinically identified neuroligin mutations. Importantly, *Nlgn3*, related genes (*Nlgn1* and *Nlgn2*) and neurexin binding partners (*Nrxn 1* and *Nrxn 2*) were robustly expressed in the mouse enteric nervous system (ENS), and the R451C knock-in mutation in the NL3^{R451C} mouse model of ASD caused colonic motility dysfunction via GABAergic (GABA_A) receptors. The proportion of neurons immunoreactive for GABA was unchanged in NL3^{R451C} mice suggesting that altered motility is unlikely due to abnormal cellular proliferation during development. NL3^{R451C} mice displayed an aggressive phenotype that was reversed by risperidone administration, verifying the utility of this model for ASD research. These data implicate altered enteric synaptic function as a primary underlying cause of GI disorders in ASD. Identification of specific ENS receptor subtypes mediating altered GI function may assist in the development of therapeutic targets for patient treatment.

Keywords: autism, gastrointestinal motility, mouse, neuroligin-3, aggression

Introduction

GI dysfunction and aggressive behavior are serious and commonly observed traits in patients with ASD. GI dysfunction is not currently included in the diagnostic criteria of ASD because definitive evidence for such involvement being intrinsic to ASD is lacking¹. However, GI symptoms are reported in up to 90% of ASD patients, with chronic constipation being the most prominent clinical presentation^{2, 3}. Aggression is observed in 70% of ASD patients⁴ and has a major negative impact on patient quality of life. Understanding the mechanisms underlying GI dysfunction and aggression will accelerate the development of therapies for these co-morbid symptoms, for which there are currently limited treatments. In a bid to achieve this, we investigated gastrointestinal motility and aggression in the NL3^{R451C} mouse model of ASD.

Many mutations in synaptic adhesion proteins including multiple members of the neuroligin family and their binding partners, the neurexins, are implicated in ASD⁵⁻⁹. NL3 is expressed at both excitatory and inhibitory synapses in the CNS¹⁰. Although the precise role of NL3 is unclear, neuroligins and neurexins function within a large network of proteins, including PSD95 and the SHANK family of cytoplasmic scaffolding proteins that influence a broad range of synaptic functions, such as vesicular and receptor recycling, neurotransmitter release and synaptic scaffolding^{9, 11}. The NL3 R451C mutation was identified in two siblings with ASD⁶ and results in a 90% reduction in the level of neuroligin-3 protein at the postsynaptic membrane in central neurons¹². Two mouse lines expressing the R451C mutation show altered excitatory versus inhibitory neurotransmission signalling, whereby GABAergic inhibitory transmission is increased in somatosensory cortical slices¹³, alongside increased glutamatergic transmission in the CA1 region of the hippocampus¹⁴. Furthermore, GABA signalling is enhanced during development in hippocampal CA3 neurons¹⁵. These data

confirm that synaptic function is altered in these mice and that the R451C mutation contributes to a 'gain of function' of CNS neurotransmission.

The enteric nervous system (ENS) is largely autonomous and many of its functions are seen in completely isolated tissues. The ENS contains intrinsic sensory neurons, interneurons, secretomotor, excitatory and inhibitory motor neurons, which generate complex motor activity¹⁶. Many pre and postsynaptic proteins required for typical CNS function are also present in the ENS¹⁷⁻¹⁹, but ENS function has not been assessed in genetic mouse models of ASD²⁰. The mouse colon exhibits highly stereotyped repetitive motor patterns (colonic migrating motor complexes; CMMCs) that depend on neural activity, when isolated from the CNS and all humoral influences *in vitro*²¹, thus making this an ideal assay system for studying disturbances of ENS function resulting from synaptic dysfunction independently of CNS activity. GABA is found in a subset of interneurons and some motor neurons in the mouse colonic ENS²² and has been implicated in synaptic transmission within the enteric neural circuitry²³. In order to investigate GABAergic neurotransmission we measured CMMCs in the NL3^{R451C} mice.

Impairment in social interaction, an endophenotype relevant to social impairments observed in ASD patients is a key criterion for animal models of autism. The precise nature of the changes in social behavior in NL3^{R451C} mice are unclear as previous studies have yielded ambiguous results^{13, 14, 24-26}. In order to further characterize the NL3^{R451C} model of autism, we investigated aggressive behavior in these mice and the effects of the atypical antipsychotic risperidone, which is used to treat aggression in ASD patients. We show for the first time that a gene mutation associated with autism alters GI motility in mice and demonstrate that this NL3 mutation is associated with increased aggression that can be reversed by risperidone.

Materials and Methods

Animals. B6;129-Nlgn3tm1Sud/J mice were obtained from Jackson Laboratories (Bar Harbor, Maine USA) and maintained to generation F9 on a hybrid Sv129/C57Bl6 background. NL3^{R451C} and WT animals were derived by mating heterozygous females with NL3^{R451C} males, which produced 50:50 WT and NL3^{R451C} male offspring (Y/+ and Y/R451C) and genotyped as described¹³. Experimental animals were weaned at 4 weeks of age and housed in groups of four per cage with food and water available *ad libitum*. The holding room was maintained on a 12:12 hr light/dark cycle with lights on at 7 a.m. and at an ambient temperature of 20 ± 1°C. All procedures were approved by the Florey Neuroscience Institutes and The University of Melbourne Animal Ethics Committees.

Structural mapping of human ASD-associated neuroligin mutations. Homology models for human NLG1, 2, 3 and 4Y were retrieved from the Swiss-Model Repository as previously described²⁷⁻²⁹. These models were reliable as indicated by very high sequence to template identity (>81%). Core structured regions of the NLGN4-X crystal structure and models were aligned using Molmol³⁰. Surface exposed protein-protein interface patches for NLGN3 were predicted using the InterProSurf web server³¹.

RT-PCR. Total RNA was isolated from gut and brain tissues, and cDNA was synthesized from 1 µg total RNA in a 20 µl reaction mix with random hexamers (Supplementary Methods). Expression of neuroligins and neurexins was assessed using RT-PCR with intron-spanning specific primer pairs designed from full-length sequences of mouse neuroligin and neurexin genes (Supplementary Table S1).

GI motility experiments. Adult male NL3^{R451C} and WT mice (25-35g) were euthanized by cervical dislocation. The entire colon (~5 cm) was dissected, flushed and cannulated in an organ bath superfused with physiological saline (at 37°C, 95%O₂/5% CO₂, flow: 5 ml min⁻¹).

After a 30 min equilibration, video recordings were acquired using a Logitech Quickcam pro 9000 camera (30 fps, 640 x 840 resolution) in order to measure contractile motor activity for a duration of 3 hrs. Motor activity was recorded at baseline (1 hr), with bath-applied antagonist (1 hr) and during washout (1 hr). Pseudocolored spatiotemporal maps of motor patterns were created and motor pattern frequencies were quantified using in-house software³². Bicuculline (Tocris), gabazine (Sigma) and CGP 54626 (Tocris) were diluted in saline.

Immunohistochemistry. Wholmount colonic myenteric plexus and longitudinal muscle (LMMP) preparations from WT and NL3 mice were processed for immunohistochemistry using antisera raised against Hu (a pan neuronal marker, a gift from Dr. V. Lennon, Mayo Clinic, USA) and GABA (Sigma-Aldrich, Castle Hill, Australia). See Supplementary Methods.

Resident-intruder test. Male resident mice were isolated for 1 week, during which their home cages were not changed. Aggressive behaviors in 3 month-old mice were monitored during four 5 min test exposures to 8 week old C57BL/6 male intruder mice conducted over 4 days. Latency to first attack, attack incidence and non-aggressive social interactions were recorded from videotapes of each test session. Non-aggressive social interactions were defined as sniffing, climbing on and grooming the intruder. Trials were aborted if the experimenter observed tufts of hair being removed from either animal. NL3^{R451C} mice were injected intraperitoneally with a non-sedative dose of risperidone (0.05 mg/kg; Sigma) or saline 15 minutes prior to testing (Supplementary Figure S1). Testing was conducted in the light cycle between the hours of 9am-5pm, blind to genotype, and drug treatment and treatment group assignment was randomized.

Statistical analysis: See Supplementary Methods

Results

More than one hundred rare gene mutations are associated with ASD, and many of these affect synaptic function^{5, 9}. Several clinically identified mutations target the neuroligin family, which is structurally conserved in 3D topology (Figure 1A, B and Supplementary Video S1). We structurally mapped known human neuroligin missense mutations to establish whether positional correlates exist with the R451C mutation in *Nlgn3*, because this is associated with ASD symptoms in humans and mice. The structural domain harbouring R451C also contained 5 of 7 other identified mutation sites (Arg451 on NLGN3 and Asp429, Asp396, Val403 and Lys378 on Neuroligin 4X), closely juxtaposed to two protein-protein interface patches (Figure 1C and Supplementary Video S1). All of these mutation sites were highly conserved in all neuroligin isoforms, and across species in human, mouse and rat structural variants. Positional association of R451C with other clinically identified mutations suggest that a functionally conserved domain in the neuroligin family may be targeted in ASD.

The idea that a synaptic mutation could impact on the periphery by directly affecting gut function in ASD is untested³³. We first showed that relevant neuroligins and neurexins are expressed in the GI tract of mice (Figure 2). Transcripts for expression of *Nlgn1*, *Nlgn2*, *Nlgn3*, *Nrxn1* and *Nrxn2* were identified in adult mouse duodenum (Figure 2A, Supplementary Table S1) and predominantly expressed in the myenteric plexus/longitudinal muscle of both duodenum and colon (Figure 2B, Supplementary Table S1). Thus, mutations in these genes associated with ASD will be present in the ENS. Hence, we investigated if the R451C mutation in *Nlgn3* identified in ASD patients alters GI behavior in the NL3^{R451C} mouse.

NL3^{R451C} mice show altered GABA_A receptor mediated colonic motility

As NL3^{R451C} mice show enhanced GABAergic neurotransmission in the CNS^{13, 15}, we tested if enteric neural circuits in NL3^{R451C} colon show altered involvement of GABA. In control conditions, CMMC frequency was identical in WT and NL3^{R451C} tissues (Figure 3D); in WT mice the median number of CMMCs per 15 minute recording period was 4 (95% CI: 1, 11) and in NL3^{R451C} 4 (95% CI: 1, 15) (median difference 1, 95% CI -2,4; $p = 0.509$). When the neural network regulating CMMCs was challenged by exposure to bicuculline (GABA_A antagonist 10 μ M, Figure 3B, C, E, H), NL3^{R451C} mouse colon exhibited significantly fewer CMMCs than WT colon (median difference over a 1 hr duration: 6 contractions, 95% CI: 1, 12; $p = 0.03$; Supplementary Video S2). In an independent sample treated with another GABA_A antagonist, gabazine (10 μ M, Figure 3B, C, F, I), the number of CMMCs in NL3^{R451C} mice was also reduced compared to WT (median difference: 5 CMMCs, 95% CI: 2, 9; $p = 0.009$). In contrast, CMMC numbers were unaffected by the GABA_B antagonist CGP 54626 (100 nM, Figure 3B, C, G, J; median difference: -1 CMMC, 95% CI: -9, 6; $p = 0.809$), suggesting that a GABA_A receptor specific mechanism is involved. Together, these data demonstrate altered GABA_A receptor-mediated signalling in NL3^{R451C} colon and provide the first evidence that a gene mutation associated with ASD produces GI dysfunction via an effect in the ENS.

The percentage of neurons immunoreactive for GABA is unchanged in NL3^{R451C} colon

Perturbations in enteric neural serotonin during enteric development lead to changes in both number of neurons and proportion of GABA neurons, along with significant disturbances in colonic motility³⁴. In order to examine neuronal density, neuronal cell bodies (immunoreactive for the pan neuronal marker Hu) were counted in proximal, mid and distal colon preparations (Supplementary Methods). We then assessed whether the NL3^{R451C} mutation alters the percentage of neurons immunoreactive for GABA in the proximal, mid and distal colon. No difference in the mean density of neurons was detected in proximal and

mid colonic regions taken from WT and NL3^{R415C} mice (neurons per mm² in proximal colon: 317 and 315; mid colon: 314 and 306; WT and NL3, respectively). In the proximal colon, the mean difference in neuronal numbers was -2 (95% CI: -128, 124) and in the mid colon; -8 (95% CI: -135, 118; $p = 0.910$). Since neuronal populations are comparatively sparsely distributed in the distal colon, a total of 20 ganglia were counted from each preparation to compare the mean number of neurons per ganglion between WT and NL3^{R451C} mice. We found that the mean number of neurons per ganglion in the distal colon were similar between WT and NL3^{R451C} mice (24.1 21.4 respectively; mean difference: -2.58; 95% CI: -7.5, 2.3; $p = 0.216$).

Although the total neuronal numbers were unchanged in WT compared to NL3^{R451C} colon, the proportions of neuronal subsets could contribute to the functional changes observed in NL3^{R451C} colon in response to GABA_A receptor antagonists. Therefore we assessed for regional and genotype differences between the proportions of GABA immunoreactive neurons (GABA neurons) in WT and NL3^{R451C} colon as a percentage of Hu immunoreactive neurons. There were no significant differences between genotypes in the percentage of GABA neurons in the proximal colon (WT: 12, NL3^{R451C}: 10; mean difference -1.8; 95% CI: -10, 6.5,) or in the mid colon (WT: 13, NL3^{R451C}: 17; 95% CI: -5, 12). Similarly, there were no genotype differences in the percentage of distal colonic GABA neurons (WT: 28, NL3^{R451C}: 27; 95% CI: -9, 7) in WT and NL3^{R451C} mice. When data from all regions of the colon were compared, the percentage of GABA neurons was higher in the distal colon compared to the proximal and mid colonic regions ($p < 0.0001$) but no differences were observed between WT and NL3^{R451C} mice ($p = 0.953$). Thus, the increased susceptibility of CMMCs to GABA_A receptor blockade in NL3^{R451C} mice is unlikely due to alterations in numbers of GABA neurons. In light of these data we suggest that the functional changes observed in NL3^{R451C} mice may be occurring at the level of enteric GABA synapses.

NL3^{R451C} mice display heightened aggression

To clarify the nature of the social behavioral phenotype of mice expressing the R451C mutation, we tested for endophenotypes relevant to social impairments observed in ASD, particularly aggression. Using the resident-intruder assay we found heightened levels of aggression in NL3^{R451C} mice towards a novel intruder mouse compared to WT littermates. Two NL3^{R451C} animals were excluded from testing on the first day due to extreme aggression towards the intruder. NL3^{R451C} mice were 8 times more likely to initiate the first attack when confronted by an intruder mouse (95% CI: 1.52, 41.94; $p = 0.014$) at any point over the 300 s observation period (Figure 5A). Enhanced aggression in NL3^{R451C} mice was also reflected by an increase in the incidence of attacks (ratio of expected number of attacks = 5.63; $p = 0.029$; 95% CI: 1.19, 26.61; Figure 5B). To explore non-aggressive interactions, mice were scored for the duration of time that they engaged in anogenital sniffing, climbing and grooming of the intruder. NL3^{R451C} mice, on average, engaged in less non-aggressive interaction with their intruder pairs compared to controls (difference in mean duration = -46 s; $p = 0.01$; 95% CI: -81,-11; Figure 5C).

Reversal of aggressive phenotype by risperidone treatment

To demonstrate predictive validity in this model, NL3^{R451C} mice were treated with a non-sedative dose (0.05 mg/kg) of risperidone (Supplementary Figure S1), an atypical antipsychotic commonly used to treat aggression in ASD patients³⁵. In mutant mice, risperidone treatment reduced the likelihood of the first attack at any point over the 300 s observation period by 6.7 times (Figure 5D, Hazard ratio = 0.15; 95% CI: 0.03, 0.69; $p = 0.015$). Risperidone treatment of NL3^{R451C} mice also reduced the frequency of attacks on intruder mice compared to saline injected NL3^{R451C} mice (ratio of expected number of attacks = 0.19; $p = 0.012$; 95% CI: 0.05, 0.69; Figure 5E). Non-aggressive social interaction was increased following risperidone administration in NL3^{R451C} mice (difference in mean duration

= 52 s; $p = 0.01$; 95% CI: 11, 92; accounting for the effect of multiple test days, Figure 5F). These data establish that a clinically effective drug reduces aggression in this monogenic mouse model of ASD, supporting predictive validity of the NL3^{R451C} mouse and utility for preclinical ASD research.

Discussion

We demonstrate that the NL3 R451C mutation is structurally associated with several other clinically identified neuroligin missense mutations, and can cause GI motility dysfunction via an enteric neural mechanism. Our data suggest that GI symptoms may be integral to ASD in patients expressing synaptic mutations and thus provide opportunities for the identification of therapeutic targets.

The expression of *Nlgn3* together with genes coding for other synaptic adhesion molecules in the myenteric plexus indicates that mutations in these genes implicated in ASD may impact ENS function. NL3^{R451C} mice demonstrate altered colonic motility involving enteric GABA_A receptors. These observations identify the GABAergic transmitter system as a potential target for therapeutic intervention in GI disorders and highlight the need for further research into neural control of GI function in ASD.

NL3^{R451C} mice exhibit heightened aggression that is reduced by risperidone; furthermore they show reduced non-aggressive interactions. These findings demonstrate both face and predictive validity of the NL3^{R451C} mouse model of autism and highlight the utility of these mice for behavioral studies. We suggest that evaluation of gastrointestinal function should be included in the battery of tests needed to characterize animal models expressing ASD associated gene mutations. We predict that GI dysfunction is not a trait unique to NL3^{R451C} mice, but will be present in other models of ASD exhibiting synaptic dysfunction. This work

highlights the need for targeted gastroenterological assessments in ASD patients as part of the routine diagnostic procedures.

Evidence that enteric synaptic dysfunction can cause GI problems in ASD patients may lead to novel therapeutic approaches with significant potential benefits for patients³³. Involvement of GABA in altered gastrointestinal motility is also relevant to the many patients with ASD who are commonly prescribed a range of medications which modulate GABAergic pathways; notably benzodiazepines including Alprazolam, Diazepam and Lorazepam (prescribed to treat anxiety, agitation, nervousness and sleep disorders)³⁶. Furthermore, animal studies have demonstrated that Diazepam potentiates GABA effects at the level of single myenteric neurons³⁷. Given that a large proportion of ASD patients also exhibit gastrointestinal issues, further investigation and consideration of the effects of GABAergic modulators on gastrointestinal function is needed. Importantly, our observations offer a novel explanation for the mechanisms underlying GI issues in ASD patients.

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Conflict of Interest Statement: The authors declare that no conflict of interest exists.

Supplementary information is available at Molecular Psychiatry's website.

Figure 1

(A) Ribbon representation of the structural alignment of human NLGN4-X linked crystal structure in light blue (2xb6.pdb) and human NLGN3 model in gray. The NLGN3 model is based on rat NLGN1 (3vkf.pdb) and was modelled by Swiss-Model^{27, 28}. Highlighted in spheres are ASD associated mutation sites. In yellow is the R451C NLGN3 site⁶ and red blue and green are the mutation sites reported for NLGN4-X^{6, 38, 39}. (B) Human NLGN3 in ribbon representation with ASD associated mutations in NLGN3 and NLGN4-X as spheres and predicted protein-protein interface as patches on the surface of NLGN3. (C) Close up of the ASD associated mutations closely juxtaposed to R451C (yellow).

Figure 2

Neuroligin (*Nlgn*) 1-3 and neurexin (*Nrxn*) 1 and 2 mRNA is expressed in brain and gut tissue. **(A)** Expression in the adult mouse whole brain (Br) and duodenum (Duo). **(B)** Expression in the myenteric plexus with associated smooth muscle (MP), but little or no expression in mucosa (M) of colon or duodenum. *Gapdh* (Glyceraldehyde 3-phosphate dehydrogenase) serves as a reference gene. RT-: no reverse transcriptase, +: brain positive control, NTC: no template water control. Gels in A and B were cropped horizontally to improve clarity and conciseness of presentation.

Figure 3

CMMC frequency is depressed by GABA_A antagonists. **(A)** Schematic diagram of a CMMC propagating from oral to anal colon (left panel) and corresponding spatiotemporal map reflecting changes in gut diameter (colored bar) across the length of the colon.

Spatiotemporal maps showing CMMC frequency in colonic preparations from WT **(B)** and NL3^{R451C} **(C)** mice under control (con) conditions **(B₁, C₁)** and in the presence of antagonists for GABA_A (bicuculline (bic), 10 μ M, **B₂, C₂**; gabazine (gab), 10 μ M, **B₃, C₃**) and GABA_B (CGP 54626 (CGP), 100 nM, **B₄, C₄**) receptors. **(D)** NL3^{R451C} and WT CMMC frequency from a large control dataset (NL3^{R451C} (n = 70) and WT mice (n = 69)) was unchanged. **(E)** Bicuculline treatment reduced the median number of contractions for NL3^{R451C} (n = 16) compared with WT (n = 16) mice. **(F)** Gabazine also reduced the median number of contractions in NL3^{R451C} (n = 11) compared to WT (n = 11) mice. **(G)** CGP had no effect in WT (n = 8) and NL3^{R451C} (n = 9) mice. In **(H-J)**, total contractions/15 min are represented as time course data (mean $\pm$ s.e.m.); **(H)** bicuculline, **(I)** gabazine, and **(J)** CGP 54626-treated. Box plots represent median interquartile range and range of the data. Vertical and horizontal scale bars in **(A)** represent 5 s, 10 mm and for **(B, C)** in **(B₁)**: 2 min, 5 mm respectively. * p $\leq$ 0.05, **p $\leq$ 0.01

Figure 4

No change in neuronal proportions immunoreactive for GABA in NL3^{R451C} (n = 3) compared to WT (n = 3) colon. Representative images of myenteric plexus of (A) WT and (B) NL3^{R451C} distal colon illustrating neurons immunoreactive for Hu (A₁, B₁; red) and GABA (A₂, B₂; green) and merged images (A₃, B₃). Arrows indicate neurons labelled for Hu and GABA. C: Bar graph indicates the percentage of Hu-positive neurons immunoreactive for GABA in WT and NL3^{R451C} proximal, mid and distal colonic regions. There is an effect of region (p<0.0001). Data shown as mean $\pm$ s.e.m.

Figure 5

Increased aggression was observed in NL3^{R451C} mice (n = 6) compared to WT mice (n = 8) in the resident-intruder test over four days of testing. (A) NL3^{R451C} mice had a significantly increased probability of initiating a first attack during the 300s observation period. (B) NL3^{R451C} mice showed an increase in attack incidence compared to WT mice and spent significantly less time in social interaction compared to WT mice (C). (D) In NL3^{R451C} mice, risperidone treatment reduced levels of aggression towards intruder mice. (E) NL3^{R451C} mice treated with 0.05mg/kg risperidone (n = 5) attacked intruder mice less frequently compared to saline injected NL3^{R451C} mice. (F) Risperidone treatment increased non-aggressive social interaction in NL3^{R451C} mice. Values are displayed as median interquartile range and range of data (attack incidence) and mean $\pm$ SD (non-aggressive social interactions). * $p \leq 0.05$, ** $p \leq 0.01$.

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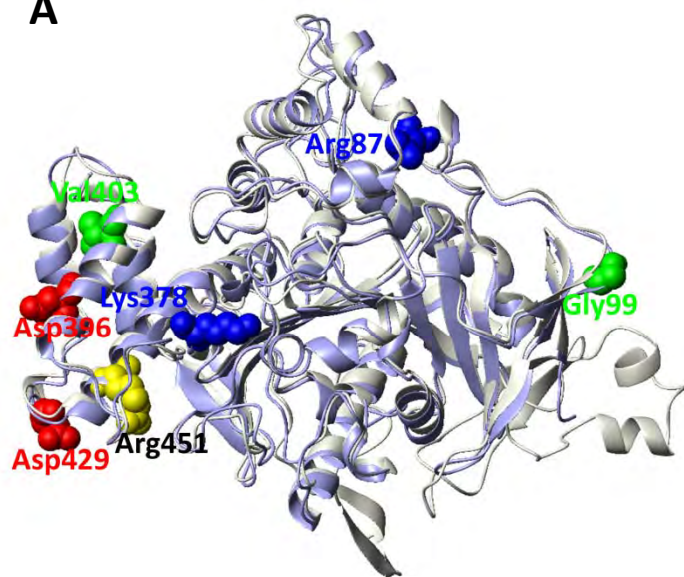
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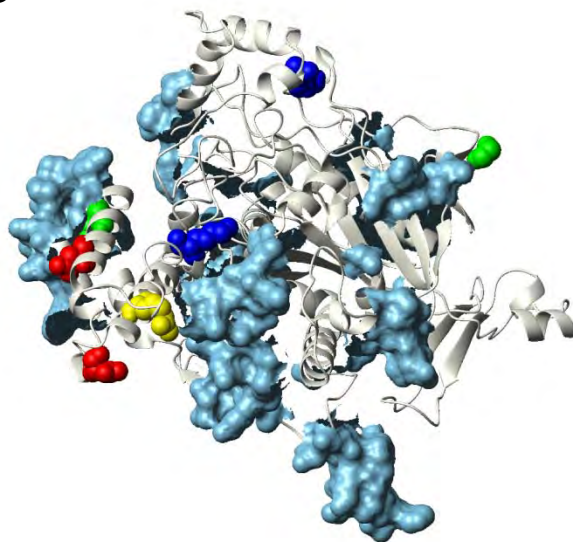
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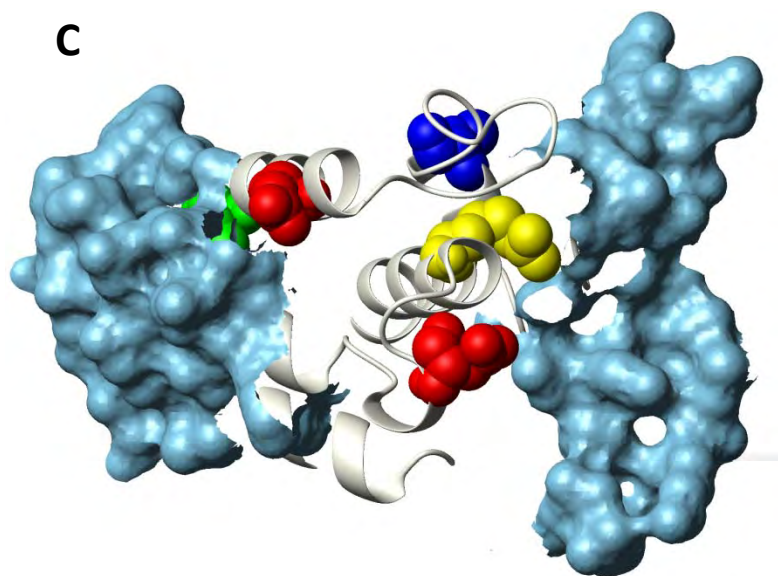
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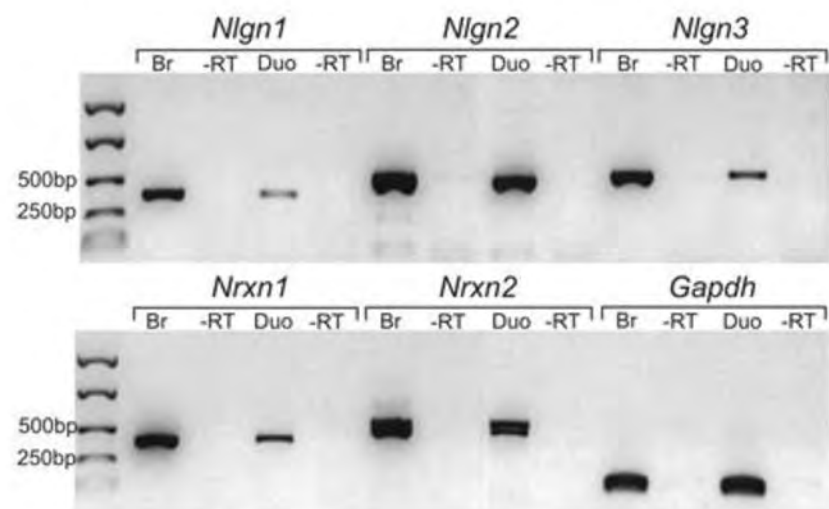


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