SMN deficiency disrupts gastrointestinal and enteric nervous system function in mice

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Abstract

The 2007 Consensus Statement for Standard of Care in Spinal Muscular Atrophy (SMA) notes that patients suffer from gastroesophageal reflux, constipation and delayed gastric emptying. We used two mouse models of SMA to determine whether functional GI complications are a direct consequence of or are secondary to survival motor neuron (Smn) deficiency. Our results show that despite normal activity levels and food and water intake, Smn deficiency caused constipation, delayed gastric emptying, slow intestinal transit and reduced colonic motility without gross anatomical or histopathological abnormalities. These changes indicate alterations to the intrinsic neural control of gut functions mediated by the enteric nervous system (ENS). Indeed, Smn deficiency led to disrupted ENS signaling to the smooth muscle of the colon but did not cause enteric neuron loss. High-frequency electrical field stimulation (EFS) of distal colon segments produced up to a 10-fold greater contractile response in Smn deficient tissues. EFS responses were not corrected by the addition of a neuronal nitric oxide synthase inhibitor indicating that the increased contractility was due to hyperexcitability and not disinhibition of the circuitry. The GI symptoms observed in mice are similar to those reported in SMA patients. Together these data suggest that ENS cells are susceptible to Smn deficiency and may underlie the patient GI symptoms.

Introduction

Proximal spinal muscular atrophy (SMA) is a pediatric neuromuscular disease in which lower motor neurons degenerate causing patient paralysis and eventual respiratory failure (1). In the most common form, patients are diagnosed by 6 months of age and die within 2–3 years (2). SMA is caused by a deficiency of the survival motor neuron (SMN) protein. SMN is ubiquitously expressed and involved in the assembly of mRNA splicing machinery (3,4). In humans, SMN is encoded by two nearly identical genes called SMN1 and SMN2. SMN1 makes predominantly full-length protein, while SMN2 has a silent base change that causes the majority of its transcripts to be misspliced resulting in a partially functional, truncated protein (5). SMA occurs when all normal copies of SMN1 are lost leaving SMN2 as the only source of full-length SMN protein (1).

In mice, removal or restoration of Smn exclusively in spinal motor neurons affects motor neuron health without large changes in survival (6–8). An interpretation of these data is the involvement of other neurons in SMA pathology. The autonomic nervous system may be sensitive to SMN deficiency based on...
reports in some patients. Approximately 24% of long-lived Type 1 patients reported symptomatic bradycardia in one study, while tachycardia was reported in 7/10 cases in another study (9,10). Seven of ten patients displayed hyperhidrosis (excessive sweating) and 6/10 had abnormal finger cold-induced vasodilatation (9). Correlates for some of these symptoms exist in SMA animal models (11–13). According to the 2007 Consensus Statement for Standard of Care in SMA, gastrointestinal dysmotility occurs in SMA patients, but GI health has not been investigated in patients or animal models (14,15). GI dysmotility may be of special interest in SMA because the gastrointestinal tract is innervated by a subdivision of the autonomic nervous system called the enteric nervous system (ENS). The ENS is embedded in the muscular layers of the GI tract and is estimated to contain as many neurons as the spinal cord (16,17). These neurons can be broadly classified into motor neurons, interneurons and sensory neurons (18). Excitatory motor neurons represent 37% of the cells in the ENS, project to the longitudinal and circular muscles along the entire GI tract and are the excitatory output of the network (17,19,20). ENS dysfunction can cause constipation, reflux and delayed gastric emptying (21,22). Determining the etiology of GI disruptions in SMA is complicated by the sedentary nature of the patients and the accompanying interventions involved in SMA treatment. Inactive lifestyle is a known risk factor for constipation, while Bilevel Positive Airway Pressure (BiPAP) can lead to gastric insufflation and delayed gastric emptying (23,24).

In the current work, we utilize two mouse models of Smn deficiency to investigate the role of Smn in GI motility. In the first model, a nestin-cre transgene selectively promotes recombination in CNS and ENS neurons and glia (25). Nestin-cre-mediated recombination of mouse Smn on the SMNΔ7 background caused constipation, reduced colonic motility and slowed intestinal transit without intestinal inflammation, morphological changes to intestinal tissues or enteric neuron cell loss. However, neuronal signaling to colonic smooth muscle was disrupted. In a second mouse model of SMA, CNS gene therapy was applied to SMNΔ7 mice to extend survival to allow for GI and ENS testing. In nestin-Cre machinery, cells that underwent cre recombination expressed the fluorescent red tdTomato reporter protein (tdTomato expression was detected in neurons (HuD staining) and glial cells (S100 staining) in the myenteric plexus of the stomach, duodenum, jejunum, ileum, cecum and colon (Fig. 1A–DD). In the colon, 18.6% of myenteric neurons and 100% intraganglionic glial cells expressed tdTomato reporter protein (n = 5) (Fig. 1E). tdTomato expression was limited to choline acetyltransferase (ChAT), calretinin and calbindin positive neurons and was not observed in VIP or neuronal nitric oxide synthase (nNOS) positive cells (Fig. 2A–O), indicating a preference for excitatory, sensory and ascending interneurons. Nearly half of tdTomato positive neurons were labeled by ChAT, a third by calretinin and ~6% were labeled by calbindin (Fig. 2P). This represented ~32% of total colonic ChAT neurons, 12% of calretinin neurons and 15% of calbindin neurons (Fig. 2Q).

Nestin-F7 mice activity and food and water consumption is similar to wild type mice

The short lifespan and reduced mobility of SMA mouse models confounds studies of gastrointestinal function. Based on the lack of tdTomato expression in spinal motor neurons, we hypothesized that nestin-cre-mediated Smn reduction would not impact mouse locomotor activity, thereby creating a viable model to study GI function. To test this hypothesis, the nestin-cre transgene was added to the SMNΔ7 background then crossed to SMNΔ7 mice with a floxed murine Smn allele (see Methods). The resultant affected mice (nestin-Cre; SMNΔ7fl/+, SMNΔ7fl/−; Smn+/-) are referred to as “nestin-F7” mice. In nestin-F7 mice, cells that underwent cre recombination had a genotype equivalent to affected SMNΔ7 mice (29). Western blots of brains and spinal cords of nestin-F7 mice and wild-type littermates confirmed Smn reduction (Supplementary Material, Fig. S2). Nestin-F7 mice were significantly smaller than wild type littermates, weighing an average of 13.02 ± 1.27 g versus 19.43 ± 0.81 g (weights collected over P26–40), respectively (t(9) = 4.41, P = 0.0017, Fig. 3A) but had a median survival greater than 200 days (Supplementary Material, Fig. S2). Because mobility, caloric intake and hydration can affect GI function, measurements of each were collected. In the open field assay, wild type mice made an average of 2144 ± 160.08 and nestin-F7 mice made an average of 2094 ± 178.3 beam breaks in a 10 min period (P40, wild type n = 10, nestin-F7 n = 4). Wild-type mice (P40–50, n = 10) consumed an average of 1.81 ± 0.08 grams of food and 2.41 ± 0.12 ml of water in 24 h, while nestin-F7 mice (P40–50, n = 4) consumed 2.17 ± 0.19 g of food and 1.95 ± 0.28 ml of water per day. No significant differences in activity, food intake or water intake were found between groups (P = n.s., Fig. 3B–D) indicating nestin-F7 mice were suitable for studies of GI function.

Smn reduction in nestin-F7 mice causes GI functional defects in vivo

Smn is assumed to be expressed in every cell type, but expression in the ENS had not been previously established. We used immunofluorescence to confirm Smn expression in discrete nuclear structures of myenteric neurons of wild type mice (Supplementary Material, Fig. S3). Because constipation is a common complaint in SMA patients, we assessed defecation frequency and stool content in nestin-F7 (n = 5) and wild-type mice (n = 6, P26–40) (15,30). On average, wild type mice produced 12.27 ± 0.61 pellets per hour, while nestin-F7 mice produced 8.44 ± 0.71 pellets, significantly fewer than wild type controls (t(9) = 4.10; P = 0.0027, Fig. 4A). Likewise, total stool weight produced by nestin-F7 mice was significantly reduced compared with wild type mice (t(9) = 2.55, P = 0.0311, Fig. 4B). Nestin-F7 pellets had comparable fecal
solids compared with controls, but had significantly decreased fluid content ($t(9) = 2.81$, $P = 0.0203$, Fig. 4C and D). Dry, infrequent stool is considered a sign of constipation. Next, colonic transit was tested in the bead latency assay (30). Wild type and nestin-F7 mice took an average of $131 \pm 39.6s$ and $869 \pm 75s$, respectively, to expel a bead inserted 2 cm into the rectum (Fig. 4E). Smn

Figure 1. Nestin-cre recombination in myenteric glia and neurons. To determine the expression pattern of the nestin-cre driver in the myenteric plexus, nestin-cre mice were crossed with floxed-stop tdTomato reporter mice. Immunofluorescent staining for tdTomato (A, F, K, P, U, Z), HuD (neurons, B, G, L, Q, V, AA) and S100 (glia, C, H, M, R, W, BB) revealed recombination in both neurons (D, I, N, S, X, CC) and enteric glia (E, J, O, T, Y, DD) within the myenteric plexus through the entire GI tract. The myenteric ganglia remain attached to longitudinal smooth muscle in all of the panels. The black background in the tdTomato panels indicates a lack of recombination in smooth muscle. Boxes in CC and DD show the approximate locations of the inset images. White arrowheads in insets indicate co-expression. Scale bars A-DD are 100 µm and 20 µm in insets. Total enumeration cell counts in the distal colon show modest recombination (18.6%) in enteric neurons and complete recombination (100%) in enteric glia (EE).
reduction in the ENS therefore produced a significant delay in colonic transit, almost 6 times longer than wild type mice (t(5) = 8.81, P = 0.0003). Finally, gastric emptying and small intestine transit in nestin-F7 (P145, n = 3 gastric emptying, n = 6 transit) and wild type mice (P145, n = 3 gastric emptying, n = 7 transit) were tested when mice received an oral gavage of FITC-conjugated dextran and euthanized 1 h later (30). Measurements of fluorescent intensity were taken from the stomach and 10 segments of equal length of the small intestine to determine transit. After 60 min, nestin-F7 mice retained significantly more FITC-dextran in the stomach (2.19 × 10^10) compared with controls (6.88 × 10^9), indicative of delayed gastric emptying (P = 0.0162, Fig. 4F). Nestin-F7 mice had slower intestinal transit, indicated by significant differences in the mean geometric center (MGC) of fluorescence in the small intestine (t(11) = 3.057, P = 0.011, Fig. 4G). MGC values were 6.75 ± 0.47 and 4.50 ± 0.58 in wild type and nestin-F7 mice, respectively, indicating the FITC-dextran traveled greater than 2 segments further in unaffected animals. Average fluorescent intensity peaked in segments 4 and 6 in nestin-F7 mice (segment 1 being most oral, 10 being most aboral), reaching average intensities of 6.0 × 10^6 arbitrary units. Fluorescent intensity was highest in segments 7, 8 and 9 in wild type mice, with maximum intensities also measuring 6.0 × 10^6 units.

Smn reduction in nestin-F7 mice does not alter morphology of the GI tract

To determine whether the observed GI functional deficits in nestin-F7 mice were due to changes in morphology, we measured absolute and relative colon lengths in nestin-F7 (P80–100, n = 10) and wild type mice (P80–100, n = 11). Neither absolute colon lengths nor relative colon lengths (normalized to tibia length) were significantly different between groups (Absolute: wild type 49.67 ± 3.04 mm versus nestin-F7 43.43 ± 2.68 mm; P = n.s.). Relative length expressed as colon:tibia wild type 2.78 versus nestin-F7 2.54; P = n.s. Supplementary Material, Fig. S4). GI inflammation can also cause ENS dysfunction and/or cell loss (31). Sections of distal small intestine (ileum) were stained with hematoxylin and eosin (H&E) and examined for inflammation, changes in smooth muscle thickness and alterations in villi and crypt height and width (Fig. 5). Evaluation of ideal sections showed no signs of inflammation or differences in villi length, width or crypt height were observed between groups (P = n.s.). However, crypt width was increased in nestin-F7 mice compared with wild types (t(7) = 1.20, P = 0.0434). Measurements of the thickness of the smooth muscularis externa were 54.68 ± 3.35 and 54.71 ± 2.57 µm in wild type and nestin-F7 mice, respectively, and revealed no sign of smooth muscle atrophy. Finally, myenteric neuronal density in the proximal colon was unchanged between the groups (wild type 1116.88 ± 95.87 versus nestin-F7 1295.00 ± 114.5 neurons per mm^2, P = n.s., Supplementary Material, Fig. S4).

Smn reduction alters ENS neuromuscular transmission in nestin-F7 mice

To determine if deficits in neuromuscular transmission caused the GI functional deficits in nestin-F7 mice, we performed ex vivo muscle tension assays on segments of distal colon (32). Neuromuscular transmission in the GI tract is dependent on cholinergic signaling. Nestin-F7 (P80–100, n = 10) and wild type
(P80–100, n = 7) tissues responded similarly in a dose-dependent fashion when a cholinomimetic (carbachol 1–10 µM) was added to the organ bath. (Fig. 6A). Tissue relaxation in the presence of the muscarinic antagonist scopolamine (100 µM) was also similar (P = n.s., Fig. 6B). Together these results show there were no intrinsic differences in the tissues at baseline. Electrical field stimulation (EFS) was used to activate the ENS but not smooth muscle. EFS evoked biphasic longitudinal muscle responses consisting of an initial muscle relaxation followed by muscle contraction. The addition of tetrodotoxin to the baths confirmed that EFS responses were dependent on neuronal activation (Supplementary Material, Fig. S5). Contractile responses differed between groups in all conditions. Wild type tissues had higher amplitude contractions at low frequency EFS, but nestin-F7 mice had greater contractions at high-frequency stimulation (Fig. 6C and D). At 30 Hz, changes in muscle tension in nestin-F7 tissue reached 0.5 g, while wild type tissue did not contract (tension ~0.1 g, Fig. 6D). Attenuated contractions at low frequency stimulation may reflect deficits in reduced probability of synaptic vesicle release as noted at neuromuscular junctions of Smn deficient tissues (33). Relaxation peaks were similar between tissue types though nestin-F7 tissue maintained higher tension relative to baseline at all EFS frequencies (Fig. 6E). Increased contractile responses could be a result of disinhibition or hyperexcitability of myenteric excitatory signaling. To determine which mechanism might contribute to increased muscle contraction in nestin-F7 tissue, the nitric oxide synthase (NOS) inhibitor L-NAME was applied to mimic disinhibition (34). In the presence of L-NAME, nestin-F7 tissue maintained increased muscle contractions compared with wild type in response to EFS suggesting that Smn deficient myenteric signaling is hyperexcitable (Fig. 6E).

Intracerebroventricular (ICV) injection of scAAV9 GFP has minimal expression in the ENS

In order to verify our findings in the novel nestin-F7 model of Smn deficiency, we sought to test GI and ENS function in a more widely used model, the SMNΔ7 mouse (29). Normally, the short lifespan and delicate tissues of the young mice would preclude these studies. Therefore, we hypothesized that careful application of SMN gene therapy could selectively restore SMN expression in CNS tissues with only minor SMN expression in the periphery (35,36). To test the hypothesis, we performed ICV injections of 4.0 x 10^10 vg of scAAV9 CB GFP into newborn (postnatal day 1) wild type mice. The gastrointestinal tract was harvested 30–60 days post injection and the myenteric plexuses were examined for GFP expression. We have previously shown that high-dose systemic injection of scAAV9 CB GFP targets 25–57% of myenteric neurons along the GI tract (38). ICV injections resulted in few GFP positive myenteric neurons (Fig. 7E–H). Based on the GFP data, we used previously established methods to ICV inject scAAV9 CB SMN (2.7 x 10^10 vg) into P1 affected mice to extend mouse survival and study SMNΔ7 GI function (36).

CNS rescued SMNΔ7 and wild type mice have similar body weight, mobility and food and water consumption

Body mass of CNS rescued SMNΔ7 mice was 15.51 ± 0.84 g and was slightly less than 18.82 ± 1.38 g in wild type mice (P32–P40, n = 5 each group) (Fig. 8A). Activity of wild type and CNS rescued SMNΔ7 mice (referred to as SMNΔ7 from now on) was measured using the open field assay (Fig. 8A, n = 5 each group). No significant differences were observed in activity between wild type and
SMNΔ7 mice (P = n.s., Fig. 8B). To control for weight variation, food and water consumption values were normalized to 10 g body weight. Water intake was similar between groups (P32–40, n = 5 per group), but food consumption was greater by CNS rescued SMNΔ7 compared with controls (P = 0.0249). Wild type mice ate 2.26 ± 0.08 g and drank 2.16 ± 0.15 ml, while SMNΔ7 mice ate 2.60 ± 0.10 g and drank 2.40 ± 0.03 ml in a 24 h period on average (P32–40, Fig. 8C and D).

CNS rescued SMNΔ7 mice have impaired colon function

Fecal pellets were collected from treated SMNΔ7 mice and analyzed for stool content. Pellet frequency was equivalent between SMNΔ7 mice (10.58 ± 0.91, n = 3, P30–45) and wild type littermates (10.67 ± 1.76, n = 4, P30–45). Total stool mass was significantly decreased in SMNΔ7 mice (0.0014 ± 0.0019 g) compared with wild type mice (0.0031 ± 0.0028 g, P = 0.0051, Fig. 9B), indicating that pellets produced by SMNΔ7 mice were smaller overall. Fecal solids and moisture were significantly reduced in SMNΔ7 mice compared with wild type littermates (Fig. 9C and D). Dry stool content in SMNΔ7 mice averaged 0.0062 ± 0.0012 g and water content weighed 0.0026 ± 0.0005 g. Dry stool content in wild type mice averaged 0.0093 ± 0.0001 g and water weight was 0.0050 ± 0.0004 g (dry stool content (P = 0.0079) and water content (P = 0.0020)). Total stool, dry stool and water content values were normalized to body weight. In conjunction with results from nestin-F7 mice, these data suggest that Smn reduction alters fecal content. In the bead latency assay, SMNΔ7 mice (P45, n = 3) took an average of 535.70 ± 222.90 s to expel the rectally inserted bead, and wild type mice (P45, n = 3) took an average of 233.3 ± 40.58 s (Fig. 9E). Differences in latency to expel the bead were not significantly different likely due to high variability in SMNΔ7 latencies. Gastric emptying and intestinal transit were not tested in CNS rescued SMNΔ7 mice.

Intestine and colon morphology is not altered by Smn reduction in CNS rescued SMNΔ7 mice

Average colon length did not significantly differ between wild type (49.14 ± 1.67 mm, P56–80, n = 8) and SMNΔ7 (45.25 ± 1.97 mm, P56–80, n = 7) mice. The colon:tibia ratio was 2.80 ± 0.24 in wild type mice and 3.13 ± 0.21 in SMNΔ7 mice and did not significantly differ between groups. H&E stains of ileal tissues were analyzed as in nestin-F7 mice (Supplementary Material, Figure 4. Nestin-F7 mice have GI dysfunction. Nestin-F7 mice produce significantly fewer fecal pellets than wild type littermates (A). Total stool mass was significantly decreased in nestin-F7 mice (B). Total fecal solids (C) were similar between groups, but moisture content (D) was significantly reduced in nestin-F7 stool. (E) The bead latency test showed that nestin-F7 mice took nearly 6 times longer than wild type littermates to expel a rectally inserted bead. (F) Nestin-F7 mice retained significantly more FITC-dextran in their stomachs 1 hour after oral gavage than wild type littermates indicating gastroparesis. In addition, transit of FITC-dextran was slower in nestin-F7 intestines than wild type littermates (G). *P < 0.05.
Muscularis externa thicknesses were 76.46 ± 2.13 µm and 68.09 ± 1.36 µm in wild type and SMNΔ7 mice, respectively, and were significantly different (t(4) = 3.31, P = 0.0296). Villi length and width did not differ between groups and were 231.7 ± 8.44 and 91.99 ± 5.78 µm in wild type mice and 210.60 ± 8.16 µm (length) and 83.72 ± 6.05 µm (width) in SMNΔ7 mice. Crypt lengths and widths were also similar (109.90 ± 11.62 µm and 36.38 ± 2.95 µm in wild type mice and 110.20 ± 7.23 µm and 41.20 ± 2.41 in SMNΔ7 mice). No signs of inflammation were present in any tissue. Neuronal density within the myenteric plexus did not significantly differ between wild type and SMNΔ7 mice (P = n.s.). Density reached 1262 ± 70.68 and 1234 ± 111.8 neurons per mm² in wild type and SMNΔ7 mice, respectively (data not shown).

Figure 5. Ileal morphology is not altered in nestin-F7 mice. H&E stained sections from wild type (A) and nestin-F7 (B) mice were used for histological analysis. Villi height (C and D) and villi width (C and E) did not significantly differ between groups. Crypt length (C and F) and crypt width (C and G) were not significantly different between groups. Thickness of the muscularis externa, including circular and longitudinal smooth layers, was not significantly different between groups (C and H). GI dysfunction in nestin-F7 mice is not the result of abnormal morphology.

Figure 6. Nestin-F7 mice have abnormal ENS neuromuscular transmission. Tension assays were completed on distal colon segments from nestin-F7 and wild type littermate mice. The muscarinic agonist carbachol (added incrementally at 1, 3 and 10 µM, A) and antagonist scopolamine (10 µM, B) were added to organ bath chambers to examine innate contraction and relaxation responses of tissues from both groups. No differences were detected between wild type and nestin-F7 tissues. (C) Representative muscle tension recordings from nestin-F7 (black line) and wild type (white line) colon segments stimulated with EFS at 30 Hz frequency for 10 s total (gray box). The black arrow indicates longitudinal smooth muscle contraction in nestin-F7 tissue and the white arrow indicates opposite relaxation muscle responses from wild type tissue (C). Peak contractile (D) and relaxation (E) responses were evaluated by stimulating colon segments at multiple frequencies (2, 5, 10, 20 and 30 Hz). Nestin-F7 tissues had robust contractile responses during high-frequency EFS that were not present in wild type tissues (D). Wild type tissues consistently had greater relaxation responses than nestin-F7 tissues (E). The NOS inhibitor L-NAME (100 µM, in the presence of scopolamine) had no effect on the differences between wild type and nestin-F7 tissues, indicating that disinhibition was not responsible for the observed differences (F).
Smn reduction impairs neuromuscular transmission in CNS rescued SmnΔ7 mice

Ex vivo organ bath muscle tension assays in CNS rescued SmnΔ7 (P50–72, n = 7) and wild type mice (P50–72, n = 8) were conducted using an identical protocol to the nestin-F7 studies. The addition of carbachol to induce contraction of the muscle showed no significant differences in SmnΔ7 tissue innate contractility (Fig. 9F). Longitudinal muscle relaxation in response to scopolamine did not significantly differ between groups (Fig. 9G). Similar to nestin-F7 tissue, differences in contractile amplitude were most pronounced at the highest frequency of EFS (30 Hz, Fig. 9H–I). At 30 Hz, there was a 10-fold increase in contractile amplitude in SmnΔ7 tissue that reached tension changes from baseline

Figure 7. GFP expression in the ENS following intravascular (IV) and ICV injection of scAAV9-CB-GFP into neonatal mice. IV injection into neonatal pups results in robust green fluorescent protein (GFP) expression (A, green) in the ENS throughout the length of the GI tract (A–D). ICV injection results in little transgene expression in the ENS, and minimal GFP expression (E) was observed in neurons and processes (E–H). AAV9 delivery confines GFP expression to enteric neurons (B, F, HuD, red) and no GFP expression was observed in enteric glial (C, G, S100, blue). ICV injections of scAAV9-CB-SMN (4 × 10^{10} vg) were given to SmnΔ7 mice to increase Smn levels in the CNS and minimize Smn expression in the ENS. Scale bars = 100 µm.

Figure 8. CNS rescued SmnΔ7 mouse activity and food and water consumption. CNS rescued SmnΔ7 mice have similar body weights compared with wild type littermates (A). Activity level (B) and water intake (C) do not significantly differ between CNS rescued SmnΔ7 and wild type mice. Food consumption (D) was greater by CNS rescued SmnΔ7 compared with controls (*P = 0.0249).
of 0.08 g over wild type tissue which reached tension changes of 0.008 g. No differences were observed in relaxation responses between groups at any frequency (Fig. 9J). Finally, the NOS inhibitor L-NAME was applied to SMNΔ7 and wild type tissue to artificially produce disinhibition. Like nestin-F7 tissue, SMNΔ7 tissue maintained greater muscle tension compared with wild type tissue, indicating that the Smn deficient myenteric plexus is hyperexcitable (Fig. 9K).

**Discussion**

This work is the first to examine the direct impact of Smn deficiency on GI function. Food and water intake and inactivity are confounding factors in SMA patients, but were controlled for in our studies. Smn deficiency led to drier fecal pellets from nestin-F7 mice, and CNS rescued SMNΔ7 mice. Nestin-F7 mice also produced significantly fewer fecal pellets than controls. Dry,
infrequent stools are signs of constipation which is regarded as “nearly universal” in Type 1 patients (38). Nestin-F7 mice also had delayed gastric emptying and slow liquid transit in the small intestine compared with wild type littermates. Based on our organ bath studies, these symptoms are likely consequences of impaired neuromuscular transmission in the GI tract. Upon high-frequency stimulation, Smn deficient spinal motor neurons, and it is reasonable that excitatory enteric motor neurons respond similarly (39). Future studies are needed to determine the cell-specific effect of Smn deficiency in the ENS.

Our studies did not find neurodegeneration within the ENS at the ages examined. This is surprising compared with the motor neuron loss in the spinal cord (29). It is possible that a longitudinal study would reveal ENS neuronal pathology, but would have to be balanced against the lifespan of SMA patients. The relevance of ENS cell loss occurring in aged SMA mice is debatable. The early cellular dysfunction rather than degeneration may be biologically significant. This could be due to developmental origin as ENS cells are neural crest derived, and motor neurons develop from the neural tube (40). GI dysmotility without neurodegeneration is found in many functional GI disorders (41).

Our studies were confined to the well-characterized SMN7 and the novel nestin-F7 mouse models of SMA. SMN7 mice were studied because of their established and robust neuromuscular phenotype. Nestin-F7 mice were used because SMN reduction occurred within ENS cells but was maintained in spinal motor neurons, therefore, promoting ambulation and an extended lifespan. A complete characterization of nestin-F7 mouse is forthcoming in a manuscript by McGovern et al. (manuscript in preparation). However, we did not observe cardiac enlargement, bladder blockage or bowel perforations. Other long-lived SMN deficient tissues had exaggeration of symptoms observed in mice have been noted in SMA patients, but their prevalence is considered rare in the general SMA population. These issues can impact the patient quality of life and represent significant concerns for the patients and caregivers. Furthermore, these non-motor symptoms may become paramount as highly promising SMA therapies that increase survival and attenuate motor deficits enter clinical trials (48-50). In summary, we have shown similar GI and ENS dysfunction in two ambulatory, hydrated mouse models of SMN deficiency. To our knowledge, these are the first in depth studies of GI function in SMA models and are highly suggestive that GI symptoms may be a primary aspect of disease in some patients.

Materials and Methods

Animals

All initial breeders for mouse lines used in these studies were generously supplied by Arthur Burghes at Ohio State University. Groups were balanced for gender. Nestin-tdTomato mice. Nestin-tdTomato mice (SMN2+/−; SMNΔ7−/−; Smn+/−; nestin-cre; tdTomato) were generated from tdTomato carrier mice (SMN2+/−; SMNΔ7−/−; Smn+/−; tdTomato+) and nestin-cre carrier mice (nestin-cre−/−; SMN2−/−; SMNΔ7−/−; Smn+/−). Mice were provided food and water ad libitum and housed in a 12:12 light:dark cycle room.

Nestin-F7 mice

Nestin-F7 mice (SMN2+/−; SMNΔ7−/−; Smn+/−; nestin-cre) were generated from nestin-crease carrier mice (SMN2+/−; SMNΔ7−/−; Smn+/−; nestin-cre) and mice with a floxed exon 7 murine Smn allele (SMN2−/−; SMNΔ7−/−; Smn+/−). No phenotypic differences were detected between unaffected littermates with and without the nestin-cre transgene, therefore, mice with both genotypes were considered wild type and used as controls in nestin-F7 experiments.

SMN7 mice

SMN7 mice (SMN2+/−; SMNΔ7−/−; Smn−/−) mice were generated from male and female carrier mice (SMN2+/−; SMNΔ7−/−; Smn−/−) (29). Breeder mice were provided with food and water ad libitum and were housed in a 12:12 light:dark cycle room. Affected male and female SMN7 pups and wild type littermates (either SMN2+/−; SMNΔ7−/−; Smn+/− or SMN2−/−; SMNΔ7−/−; Smn−/−) were used in these studies. To ensure survival past 15 days, affected SMN7 pups received ICV injections of 2 × 1010 scAAV9-CB-SMN on postnatal day 1 or 2 (referred to as CNS rescued SMN7) (29). ICV injection restores SMN expression in the CNS while minimizing SMN expression in the ENS. After weaning mice were provided with food and water ad libitum and housed in the same 12:12 light: dark cycle room.
All breeding of and subsequent experiments involving these mice were approved by the Institute of Animal Care and Use Committee at the Ohio State University.

Genotyping of animals
The genotyping of SMNΔ7 was completed as previously described (29). Genomic DNA was collected from P1 tail clips and amplified with PCR using the following primers: nestin-cre, 5′-TCTCAAGATTCTCTCAGAAAATCACC-3′ and 5′-GTGGCTGGATAGTTTTTAC7GCCAG-3′; Smn-F7, 5′-AGAAGGAAAGTGCCTACATACAAATT-3′ and 5′-TGCTTATATATGCCATCTGAG-3′.

Gastric emptying and intestinal transit
Gastric emptying and intestinal transit were tested as previously described (30). Gastric emptying and gastrointestinal transit within the stomach and small intestine were assessed by measuring the distribution of a 70 kDa FITC-conjugated dextran marker (FITC-dextran; Sigma Aldrich FD70S in water). Nestin-F7 mice (P145, n = 6) and wild type littermates (P145, n = 7) were fasted for 12 hours prior to onset of testing, but were given access to water ad libitum. Each mouse was orally gavaged with 100 µl FITC-dextran and returned to emptied home cages with no access to food or water. Sixty minutes after gavage mice were euthanized and the stomach and small intestine were removed and intestine was cut into 10 segments of equal length. Each segment was then flushed with 3 mls of phosphate buffered saline (pH 7.3) and centrifuged at 1200 rpm at 4°C for 5 min. The supernatant was collected and 1:40 and fluoroscence was measured in triplicate samples form a 96-well plate in a fluorescent plate reader (Wallac 1420 VICTOR2, Perkin Elmer, Waltham, MA). Samples were normalized to a single sample measured between plates. Results are reported as total fluorescence and the geometric center of the distribution of dextran throughout the small intestine. The MGC was calculated using this equation:

\[\text{MGC} = \sum \left(\frac{\text{fraction of amount of FITCin each segment}}{\text{segment number}}\right) \times 100\]

Colonic transit
Colonic motility was assessed using the colon bead assay, a measure previously used to assess colon motility in vivo (30). Briefly, nestin-F7 (P40, n = 5) and wild type littermates (P40, n = 6) or SMNΔ7 mice (P45, n = 3) and wild type littermates (P45, n = 3) were lightly anesthetized with isoflurane (2%), after which a lubricated plastic bead (2 mm diameter by 6 mm long) was inserted into the anus and pushed 2 cm inside the rectum by a silicone capped steel cannula. Following insertion, mice were placed in an empty plastic cage and time to bead expulsion was recorded. Timing began immediately after the mice were placed into the empty cage. Due to light anesthesia mice were awake and moving immediately upon being placed in the empty cage. A single trial was conducted and mice were returned to their home cages at the conclusion of the study.

Open field assay for activity
Adult nestin-F7 (P40, n = 4), wild type littermates (P40, n = 10), SMNΔ7 (P45, n = 5) and wild type littermates (P45, n = 5) were assayed for activity in the open field test. Briefly, all mice experienced a 30 min acclimation period prior to the onset of the test. Mice were then placed in the open field apparatus for 10 min each for a single session and beam breaks were recorded. Results are presented as average beam breaks in 10 min for each group ± SEM. The test was powered to detect a 25% change in activity since smaller changes were unlikely to impact bowel habits.

Food and water intake
Food intake was measured by weight of food consumed over a 24 h period, measured consecutively for 10 days. During that time, water intake was measured by milliliters consumed from a pre-measured water bottle and weight of food consumed was recorded in grams. Nestin-F7 (n = 10) and wild type littermate (n = 4) mice were tested between ages P40–50 and SMNΔ7 (n = 5) and wild type littermate (n = 5) mice were tested between ages P32–42. Water intake was powered to detect a 25% change in activity since smaller changes were unlikely to impact bowel habits.

Pellet production
Fecal pellets were collected from nestin-F7 (P26–40, n = 5) mice and wild type littermates (P26–40, n = 6) and SMNΔ7 (P30–45, n = 3) and wild type littermate mice (P30–45, n = 4) to determine defecation frequency and stool content. Mice were placed in individual, empty cages for 60 min sessions for a total of eight trials. Trials were conducted as previously described (30) and were conducted every other day at the same time of day. Pellets were collected into pre-weighed microcentrifuge tubes and weighed after collection to calculate total stool mass. Pellets where then dehydrated overnight at 60°C and re-weighed for dry stool weight and total moisture content was calculated.

Organ bath studies
Group sizes were based on prior studies (32). Nestin-F7 (P80–100, n = 10), wild type littermates (P80–100, n = 7), SMNΔ7 (P50–72, n = 7) and wild type littermate mice (P50–72, n = 8) were used in these experiments. Nestin-F7 and wild type littermate mice were assayed between postnatal days 95 and 110. SMNΔ7 and wild type littermate mice were assayed between postnatal days 50–60. Contraction of the smooth longitudinal muscle was recorded from 1 cm segments of the distal colon under 1.5 g passive tension with a force transducer (Radnoti LLC, Monrovia, CA, USA). Following a 45-minute equilibration period in a bath of 37°C oxygenated Kreb’s solution (final concentration 120.0 mM NaCl, 6.0 mM KCl, 2.5 mM CaCl_2, 1.2 mM MgCl_2, 1.35 mM NaH_2PO_4, 14.4 mM NaHCO_3 and 12.7 mM Ca(H_2O)_4), innate contractile and relaxation abilities of affected and wild type tissues were tested with the application of the muscarinic agonist carbachol (added cumulatively, 1, 3 and 10 µM) and the muscarinic antagonist scopolamine (10 µM). Afterward, EFS for 10 s duration (30 V, 0.5 ms, 2, 5, 10, 20 and 30 Hz) was applied to the tissue through a platinum electrode to evoke neurogenic contraction/relaxation without pharmacological intervention. Tension changes were recorded and normalized to individual baseline. Next, the NOS inhibitor L-N^ω-Nitroarginine methyl ester (L-NAME, 100 µM) was added to the bath to artificially induce disinhibition. EFS for 10s duration (30 V, 0.5 ms, 2, 5, 10, 20 and 30 Hz) was applied and tension changes were recorded. Finally, tetrodotoxin (TTX, 1 µM) was used to confirm that contractions were neurogenic in nature. At the end of the bath, tissue segments were removed, silk sutures cut away and a weight wet of tissues was obtained. Results are presented as the average maximum contractile response.
percentages of the total carbachol contractile response and are normalized to wet weight of the segment.

Immunohistochemistry

Whole mount preparations of the myenteric plexus in the stomach, duodenum, jejunum, ileum, cecum and colon were subject to dissection and immunohistochemistry, as described previously (Gombash et al., 2014). Primary antibodies utilized included anti-red fluorescent protein (RFP, 1:200; Millipore, Temecula, CA, USA), HuD (1:25; A-21271, Life Technologies, Eugene, OR, USA), SMN1 Protein Ab-2 (S100; 1:200; RB-044-A0, Neomarkers, Fremont, CA, USA), choline acetyltransferase (ChAT; 1:50; AB144P, Millipore); vasoactive intestinal peptide (VIP; 1:200; Abcam), neuronal nitric oxide synthase (nNOS; 1:200; ab1376, Abcam), calretinin (1:200; CG1, Swant, Switzerland) or calbindin (1:200; S100; 1:200; RB-044-A0, Neomarkers, Fremont, CA, USA), choline acetyltransferase (ChAT; 1:50; AB144P, Millipore); vasoactive intestinal peptide (VIP; 1:200; Abcam), neuronal nitric oxide synthase (nNOS; 1:200; ab1376, Abcam), calretinin (1:200; CG1, Swant, Switzerland) or calbindin (1:200; S100; 1:200; RB-044-A0, Neomarkers, Fremont, CA, USA). Images were captured on an Olympus FV 100 Spectral Confocal system (Melville, NY, USA) in the Ohio State University Campus Microscopy and Imaging Facility (Columbus, OH, USA).

To assess histological changes, whole ileum segments from nestin-F7 (P80–100, n = 5), SMNΔ7 (P50–72, n = 3) and wild type mice (n = 8) were flash frozen and sliced on cryostat at 20 µm thickness. Tissue sections were stained with hematoxylin and eosin. Measurements of villi and crypt length and width were taken using NeuroLucida (Microbrightfield Bioscience, Williston, VT, USA) and Zeiss Imager.M2 fluorescent microscope (Zeiss, Thornwood, NY, USA) with a digital camera (Q Imaging, Surrey, BC, Canada).

Myenteric plexus neuronal density

All analyses were carried out using a Zeiss Imager.M2 fluorescent microscope (Zeiss), digital camera 9 (Q Imaging) and NeuroLucida (Microbrightfield). To determine neuronal density within the myenteric plexus in the proximal colon of nestin-F7 (P80–100, n = 4), SMNΔ7 (P50–72, n = 3) and wild type (n = 10) mice, contours were drawn around the perimeters of myenteric ganglia and the area of each ganglia was recorded. Neurons, identified by HuD staining, within each ganglia were counted in a minimum of 500,000 µm² of outlined ganglia area for each mouse. Data are reported as density of neurons per mm².

Cell quantification

Confocal imaging (Olympus FV 100 Spectral Confocal) was used to confirm co-expression of tdTomato in enteric neurons, marked by HuD expression, and glial cells, marked by S100 expression in the GI tract in nestin-cre tdTomato reporter mice (n = 5). A total of 350 neurons (HuD) and 350 enteric glial cells (S100) were counted within the myenteric plexus. Cells co-expressing tdTomato and HuD or S100 were recorded and results are presented as the percentage of tdTomato co-expressing cells of each type. Furthermore, counts of ChAT and tdTomato positive, calretinin and tdTomato positive and calbindin and tdTomato positive neurons were recorded from colons of a second cohort of nestin-tdTomato mice (n = 3). Data are presented as percentages of tdTomato positive neurons that also express ChAT, calbindin or calretinin, or percentages of ChAT, calretinin or calbindin positive neurons that co-express tdTomato.

Statistics

All statistical tests were conducted in SigmaPlot v11 (Systat Software, Inc., San Jose, CA, USA). Mean values ± SEM are reported. In all experiments, α = 0.05 and β = 0.80. Sample sizes not based on prior experience were determined using the following calculation: n = 1 + 2C(1/2)^2 (s = standard deviation, d = difference and C = 7.85 for α = 0.05 and 1 − β = 0.80) as previously described (Gombash et al., 2014). Nestin-F7 mice were only compared with their wild type littermates and SMNΔ7 mice were only compared with their wild type littermates. Food and water consumption values were averaged together for each individual mouse over 10 trials. Final values from individual mice normalized to 10 g body weight were compared using Student’s t-tests. Likewise, fecal pellet number, total stool, dry stool and water content values were averaged for each mouse over a series of 8 trials and final values were compared using a Student’s t-test. Numbers of beam breaks in the open field activity assay were analyzed using Student’s t-tests.

All histological measurements, including neuronal density and myenteric plexus area were compared using Student’s t-tests. For neuromuscular transmission assays, all values reported were normalized to wet weight of the 1 cm colon segment being tested. Scopolamine muscle responses were compared using Student’s t-tests. Carbachol muscle responses were compared using a one-way repeated measures analysis of variance (ANOVA). Contraction and relaxation responses in response to different frequencies of EFS were compared using two-way repeated measure ANOVAs.

Supplementary Material

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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