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1 **D-Serine agonism of GluN1-GluN3 NMDA receptors regulates the activity of**
2 **enteric neurons and coordinates gut motility**

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25 **Running title: D-Ser signaling in the enteric nervous system**

26 **Abstract**

27 **The enteric nervous system (ENS) is a complex network of diverse molecularly defined classes of**
28 **neurons embedded in the gastrointestinal wall and responsible for controlling the major functions**
29 **of the gut. As in the central nervous system, the vast array of ENS neurons is interconnected by**
30 **chemical synapses. Despite several studies reporting the expression of ionotropic glutamate**
31 **receptors in the ENS, their roles in the gut remain elusive. Here, by using an array of**
32 **immunohistochemistry, molecular profiling and functional assays, we uncover a new role for D-**
33 **serine (D-Ser) and non-conventional GluN1-GluN3 N-methyl D-aspartate receptors (NMDARs) in**
34 **regulating ENS functions. We demonstrate that D-Ser is produced by serine racemase (SR) expressed**
35 **in enteric neurons. By using both *in situ* patch clamp recording and calcium imaging, we show that**
36 **D-Ser alone acts as an excitatory neurotransmitter in the ENS independently of the conventional**
37 **GluN1-GluN2 NMDARs. Instead, D-Ser directly gates the non-conventional GluN1-GluN3 NMDARs in**
38 **enteric neurons from both mouse and guinea-pig. Pharmacological inhibition or potentiation of**
39 **GluN1-GluN3 NMDARs had opposite effects on mouse colonic motor activities, while genetically**
40 **driven loss of SR impairs gut transit and fluid content of pellet output. Our results demonstrate the**
41 **existence of native GluN1-GluN3 NMDARs in enteric neurons and open new perspectives on the**
42 **exploration of excitatory D-Ser receptors in gut function and diseases.**

43

44 Keywords: Intestine; serine racemase; glutamate; enteric neurons; NMDA receptors;

45

46

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66 **Conflicts of interest**

67 The authors disclose no conflicts.

68

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72 Cindy J. Lee, Céline Rouget, Marina Fortea: conducted experiments. Jumpei Sasabe, Jonathan V.
73 Sweedler, Pieter Vanden Berghe: designed experiments, analyzed and interpreted data. Patrick
74 Delmas, Jean-Pierre Mothet: designed and supervised the study, analyzed, and interpreted data and
75 wrote the paper. All authors have critically revised the manuscript and approve the final version before
76 submission.

77

78 Introduction

79 The gastrointestinal (GI) tract and its auxiliary organs is an inherently complex ecosystem that is
80 essential for life as it is the primary site for digestion of ingested food¹. Therefore, GI functions require
81 finely tuned control of muscular activity, fluid and electrolyte secretion and blood flow to efficiently
82 propel the alimentary bolus, break down macroscopic food particles, and efficiently extract nutrients.
83 Such control is accomplished by the enteric nervous system (ENS), which is intrinsically distributed
84 throughout the GI wall^{1,2}. Although the ENS is regulated by the autonomic nervous system, it can
85 uniquely function independently of the central nervous system (CNS)³. The importance of the ENS is
86 emphasized by the life-threatening consequences of certain ENS congenital neuropathies, including
87 chronic intestinal pseudo-obstruction or Hirschsprung's disease^{4,5} but also by its increasingly evident
88 implication in inflammatory bowel diseases⁶ and brain disorders⁷.

89 The ENS is composed of the outer myenteric plexus and the inner submucosal plexus^{1,8}. The
90 myenteric plexus is mainly involved in the control of GI peristalsis, whereas the submucosal plexus
91 controls mucosal functions such as electrolyte secretion, paracellular permeability, and intestinal
92 epithelial cell proliferation^{1,8,9}. These ganglia are composed of diverse classes of neurons and glial cells
93 that share many phenotypical features with their CNS counterparts, forming interlaced and expansive
94 networks along the GI tract with defined reciprocal signaling¹⁰⁻¹². Enteric neurons and glia cells use
95 plethora of cell-cell signaling molecules to support GI functions^{1,12}. Virtually every class of
96 neurotransmitters, and their receptors/transporters found in the CNS, has also been detected in the
97 ENS^{1,9}. Accordingly, pathogenic mechanisms that give rise to CNS disorders might also lead to ENS
98 dysfunction⁷.

99 Several studies have reported that glutamate can serve as a neurotransmitter in the ENS¹³⁻¹⁷.
100 The idea that glutamate and its receptors are key signaling components in the GI arises from the
101 observation that glutamate evokes fast and slow depolarizing responses of enteric neurons¹³.
102 Subsequently, morphologic studies demonstrated the presence of some glutamatergic receptors in
103 enteric neurons^{14,16,18} and functional studies have described the importance of glutamate in the

104 modulation of acetylcholine release in the guinea-pig ileum and colon^{15,19}, as well as in motor and
105 secretory functions in the gut^{18,20}. Therefore, alterations of glutamatergic transmission have been
106 proposed to contribute to the pathogenesis of GI disorders^{21,22}.

107 Despite the consensus in the literature about glutamate as being a signaling molecule in the
108 ENS^{16,21}, the identity of glutamatergic receptors involved in enteric neuronal network communication
109 remains poorly defined²³. Although the expression of functional α -amino-3-hydroxy-5-methyl-4-
110 isoxazolepropionic acid receptors (AMPA) and metabotropic glutamate receptors (mGluRs) in the
111 ENS^{14,18,24-26} is well established, evidence for a functional role of the NMDA receptors (NMDARs) in the
112 GI tract come from pharmacological studies in pathological conditions, where expression of these
113 receptors is upregulated upon inflammation²⁷⁻²⁹. Whether and how NMDARs could contribute to the
114 physiology of ENS in the small and large intestine remains to be established²⁶.

115 NMDARs are heterotetrameric ligand-gated ion channels that associate two obligatory GluN1
116 subunits with two identical or different GluN2 or GluN3 or a mixture of GluN2 and GluN3 subunits^{30,31}.
117 The activation of GluN1-GluN2 containing NMDARs requires the binding of glutamate plus a co-agonist
118 glycine or D-Ser³⁰⁻³² whereas diheteromeric GluN1-GluN3 receptors are not gated by glutamate^{30,31}.
119 Mounting evidence gathered at central synapses indicate that D-Ser which is synthesized from L-Ser by
120 serine racemase (SR)³³ would be preferred over glycine thus controlling many NMDAR-dependent
121 functions including synaptic plasticity and cognitive performances³⁴⁻³⁷.

122 While significant progress has been made in the understanding of the functions of D-Ser in the
123 CNS, the role of D-Ser in the ENS is still unknown. However, indirect observations suggest that D-Ser
124 may be an important signaling molecule in the gut. Indeed, acute kidney injury (AKI) induces gut
125 dysbiosis by altering the metabolism of D-Ser while microbiota-derived D-Ser offers protection against
126 AKI³⁸. Furthermore, D-Ser produced by yet unidentified enteric cells regulates sleep in flies³⁹. Whether
127 D-Ser can be produced in the mammalian gut by host cells and can regulate the activity of the ENS and
128 GI functions has remained unexplored.

129 Here, using a combination of pharmacological and genetic approaches, along with *in situ* patch
130 clamp recording and calcium imaging, we have investigated the physiological role of glutamatergic
131 signaling in the ENS. We report evidence that enteric neuron excitability and GI motility are not
132 regulated by conventional GluN1-GluN2 containing NMDARs. Strikingly, we demonstrate that D-Ser is
133 synthesized by SR in enteric neurons and gates the non-canonical GluN1-GluN3 receptors to shape ENS
134 network activity for normal GI motility and transit.

135

136 Results

137 D-Serine is present in the gut and is metabolized by enteric neurons

138 We first assessed whether the amino acids (AAs) D-Ser and L-Ser are present within the mouse
139 intestine. AAs were quantified by CE-LIF in the lumen and the wall (lacking the mucosa and submucosa)
140 of the medial parts of mouse ileum and colon. **Figure 1a,b** reveal the presence of a substantial amount
141 of D- and L-Ser in both the lumen and the wall fractions. The D-Ser peak identity was validated by spiking
142 with D-Ser standards (not shown) and confirmed *via* enzymatic degradation by PkDAAO, as the enzyme
143 shows high catalytic activity towards D-Ser (insets in **Fig. 1a**). Given the abundance of microbiota-
144 derived D-Ser in the gut^{38,40}, we then compared the levels of D-Ser in WT and in serine racemase (SR)
145 null-mutant (SR^{-/-}) mice. A statistically significant decrease of ~50% was observed in D-Ser content of
146 both ileum and colon from SR^{-/-} mice compared with WT mice (**Figure 1b**). Although free D-AAAs in the
147 murine intestinal tract may be microbial products⁴¹, our observations suggest that an important
148 proportion of free D-Ser in the gut originate from its local biosynthesis by the host intestinal cells.

149 We therefore explored whether SR is present in the intestine to synthesize D-Ser. RT-PCR
150 performed from mouse colon samples showed the presence of SR mRNA in the distal and proximal
151 sections of the colon of WT mice, but not in tissues extracted from SR^{-/-} mice (**Figure 1c**). Interestingly,
152 mRNA for SR was also detected in LMMP (Longitudinal Muscle and Myenteric Plexus) preparations of
153 mouse colon, which lacks the circular muscle layer and the mucosa-submucosa but still comprises the
154 myenteric neuronal network (**Figure 1c**). Western blot analysis confirmed that SR is present in the

155 colon of WT mice as well as in guinea pigs and humans (**Figure 1d**). Although mouse brain SR was
156 detected as a monomeric form at 37kDa, in all colon samples and guinea pig brain multiple bands of
157 higher sizes were observed, which most likely corresponds to previously reported variants of SR⁴² or
158 to unreducible multimers^{43–47}. The absence of any signal from SR^{-/-} mice further demonstrated that our
159 antibody was specific for SR and that the higher molecular weight bands were indeed caused by SR
160 forms resistant to both denaturation and reductive treatment. The lower molecular weight bands in
161 human samples may indicate the presence of proteolytic fragments of SR⁴⁸.

162 To identify which cells in the ENS may synthesize D-Ser, we next performed immunostainings in
163 the guinea pig ENS, the best molecularly characterized ENS model⁸. LMMP immunostainings for D-Ser
164 revealed a high density of cell body labelling in the myenteric plexus of the ileum (**Figure 1e-j and**
165 **Extended data Fig.1**). Double immunostainings with the pan neuronal marker HuC/D revealed the
166 presence of D-Ser in neuronal cell bodies (**Figure 1e and Extended data Fig. 1a**). On the contrary,
167 double labelling for the glial marker S100 β or the glial fibrillary acidic protein (GFAP, data not shown)
168 and D-Ser showed that D-Ser is not detected in glia cells (**Figure 1f and Extended data Fig. 1b**).

169 As the ENS comprises diverse molecularly and functionally defined classes of neurons, we next
170 investigated the identity of the neurons containing D-Ser by comparing its distribution with known
171 markers. Double immunostainings revealed that 49% of D-Ser⁺-neurons also express NeuN, an
172 exclusive marker of Dogiel type II neurons, which are considered as intrinsic primary afferent neurons
173 (IPANs) in the guinea-pig GI tract^{49,50} (**Figure 1g,i and Extended data Fig. 1c**). Consistently calbindin,
174 another preferential marker of IPANs, was also found to be expressed in a substantial proportion of D-
175 Ser⁺ myenteric neurons (**Figure 1i**). Sixteen percent of D-Ser⁺ neurons were co-labelled with the
176 neuronal nitric oxide synthase (nNOS), which is specific for inhibitory motoneurons and descending
177 interneurons⁸ (**Figure 1h,i and Extended data Fig. 1d**).

178 Because the presence of D-Ser in neuronal cell bodies does not necessarily reflect its synthesis
179 site, we stained LMMPs for SR. A very similar labelling profile was observed for SR and NeuN in the
180 myenteric plexus, thus suggesting that IPANs are one of the main cells potentially producing D-Ser in

181 the gut (**Figure 1j** and **Extended data Fig. 1e**). Finally, we asked whether the D-Amino Acid Oxidase
182 (DAAO), which is degrading D-Ser⁵¹ and is highly expressed in the hindbrain, is also present in the
183 mouse gut. We found DAAO to be present in the small intestine but not in other regions of the GI tract
184 in agreement with previous data⁴¹ (**Extended data Fig. 2 & 3**). Collectively these observations suggest
185 that enteric neurons express SR and may produce D-Ser throughout the GI tract, pointing to a putative
186 role of D-Ser in the regulation of ENS functions.

187

188 **Molecular profiling reveals the presence of multiple glutamatergic receptor subunits in the** 189 **myenteric plexus**

190 In addition to GluN1-GluN2 containing NMDAR, D-Ser can also bind to the ionotropic GluN1-GluN3
191 unconventional NMDARs^{52,53} and be a ligand for the GluD1-D2 receptors⁵⁴⁻⁵⁶. To identify the putative
192 receptor for D-Ser, we performed RT-PCR for the GluN1, GluN2A-D, GluN3A-B and GluD1-2 receptor
193 subunits on LMMPs isolated from the colon of WT mice (**Extended data Fig. 4a**). Amongst the
194 transcripts tested, only GluN1, GluN2D, GluN3A, GluA2, GluD1 and GluD2 transcripts were detected.
195 The presence of receptor subunit proteins was further analyzed by immunoblotting with previously
196 characterized antibodies (**Extended data Fig. 4b**). Consistent with RT-PCR data, immunoblots showed
197 the presence of GluN1 and GluN2C/2D in the colon, whereas GluN2A/B were not detected in mouse
198 LMMPs. As the antibody we used does not distinguish between GluN2C and GluN2D (**Extended data**
199 **Fig. 4b, middle panel**), we conclude for the presence of GluN2D based on the results of RT-PCR, which
200 show the presence of GluN2D but not GluN2C transcripts. Immunoblottings demonstrated the
201 presence of GluN3A and GluN3B subunits in mouse colon (**Extended data Fig. 4b, middle panel**). Of
202 note, the expression of the glutamatergic receptor subunits was unchanged in SR^{-/-} mice (**Extended**
203 **data Fig. 4b**).

204 Our data indicate that GluN1, GluN2D, GluN3A and GluN3B are detected in the mouse colon.
205 The presence of these receptor subunits supports two main scenarios for a modulatory role of D-Ser.
206 In the first scenario, D-Ser would bind to diheteromeric GluN1-GluN2D receptors or triheteromeric

207 GluN1-GluN2D-GluN3 receptors, which both require glutamate for activation and are inhibited by the
208 glutamate site competitive antagonist D-AP5^{30,31}. In the second scenario, D-Ser would bind to the
209 unconventional diheteromeric GluN1-GluN3 NMDARs, which are not gated by glutamate or NMDA and
210 not blocked by D-AP5^{30,31}.

211

212 **Enteric D-Ser does not act through post- or pre-synaptic GluN1-GluN2 containing NMDARs**

213 We next performed *in situ* patch clamp recordings, both in voltage and current clamp, of myenteric
214 neurons in guinea-pig LMMPs⁵⁷ to first probe potential interaction of D-Ser with GluN1-GluN2
215 containing NMDARs.

216 In voltage clamp mode, we found that local application of glutamate evoked large post-synaptic
217 inward currents in myenteric neurons (**Figure 2a,b**). However, these post-synaptic currents were
218 evoked with relatively high concentrations of glutamate (20 mM) and were not blocked by the selective
219 NMDAR antagonist D-AP5 (**Figure 2a**). In addition, the glutamate-induced current-voltage (I/V)
220 relationship determined using slow voltage ramps lacked the typical negative slope of NMDAR currents
221 due to voltage-dependent Mg²⁺ block (**Figure 2c**). The lack of functional GluN1-GluN2 NMDARs was
222 further substantiated by the absence of responses to NMDA (100 μM-1 mM) in myenteric neurons that
223 either responded to glutamate or kainate (**Figure 2d,e**). Additionally, application of NMDA in the
224 continued presence of Tricine, which alleviates any blockade by endogenous Zn²⁺ of GluN2A-containing
225 NMDARs³⁶, did not evoke any currents at very positive potentials (**Figure 2e**).

226 Current clamp recordings were used to test whether NMDA would alter the resting membrane
227 potential in identified myenteric neuron populations. This recording configuration indeed allows for
228 distinguishing IPANs from interneurons and motor neurons, also called S neurons⁵⁰. IPANs are AH
229 neurons, in which the action potential shows a shoulder, sustained by Ca²⁺ and Nav1.9 channel
230 activation, and a slow afterhyperpolarizing (AH) potential, whereas S neurons have monophasic action
231 potential repolarization and no slow afterhyperpolarization^{58,59}. Neither AH neurons, nor S neurons

232 from the colon and the duodenum were found to be sensitive to NMDA exposure under a variety of
233 conditions (D-Ser, Tricine, salicylate, free $[Mg^{2+}]_o$) that would promote NMDAR function (**Figure 2f,g**).

234 To examine whether GluN1-GluN2 NMDARs may be active at the presynaptic terminal, we
235 coupled patch clamp recordings of postsynaptic myenteric neurons in LMMPs with electric stimulation
236 of presynaptic nerve bundles. We applied single electric stimuli at low frequency (1 ms every 15 s),
237 which evoked fast excitatory post-synaptic currents (fEPSCs) mediated by nicotinic receptors. Local
238 application of NMDA failed to modulate the presynaptically-evoked fEPSCs (**Figure 2h,i**). Therefore,
239 our electrophysiological recordings failed to detect functional GluN1-GluN2 containing NMDARs at
240 both post- and pre-synaptic neurons, thus raising questions about the identity of the putative receptor
241 for D-Ser.

242

243 **Acutely applied D-Ser raises intracellular calcium concentrations in colonic myenteric** 244 **neurons**

245 We next performed calcium imaging as readout for investigating the effects of D-Ser on a large
246 population of myenteric neurons in LMMPs prepared from mouse colon. Using a local perfusion
247 pipette, we exposed Fluo-4 loaded myenteric ganglia to 100 μ M D-Ser for 20s (**Figure 3a1-a3**). This
248 local D-Ser exposure evoked calcium responses in 30 % of myenteric neurons (range from 28 to 92%)
249 with a mean increase of fluorescence change ($\Delta F/F_0$) of 20%. By comparison the fluorescence change
250 induced by depolarization with high $[K^+]_o$ was about 10 fold higher (191%, not shown). The D-Ser-
251 induced Ca^{2+} signals had a relatively slow onset, peaked after 10-20s and declined within 20s to resting
252 values (**Figure 3a4**). The slow kinetics of the responses suggests that most D-Ser-induced Ca^{2+} signals
253 were not associated with abrupt changes in membrane potential^{12,60}.

254 To test the involvement of unconventional GluN1-GluN3 NMDARs we took advantage of the
255 properties of the pharmacological compounds CGP-78608 and dichlorokynurenic acid (DCKA). While
256 binding of D-Ser to the high-affinity GluN3 subunit triggers channel opening, D-Ser binding to the
257 associated low-affinity GluN1 subunit has an opposite effect, causing auto-inhibition of the

258 receptor^{61,62}. Thus, the CGP-78608 compound by preventing D-Ser binding on GluN1 can act as a
259 potentiator of GluN1-GluN3 receptor⁶². Conversely, DCKA prevents D-Ser binding to both subunits and
260 consequently blocks GluN1-GluN3 receptor activity. To avoid experimental bias caused by variability
261 among preparations, new control experiments were included for every set of experiments to enable
262 drug comparisons with control experiments performed at the same time and in the same animals. In
263 this dataset local perfusion of D-Ser (100 μ M) evoked transient increases in $[Ca^{2+}]_i$ in $30.9 \pm 29.4\%$ of
264 Fluo-4 loaded neurons in each ganglion (**Figure 3b,d**). As in all datasets, this was significantly higher
265 than the spontaneous and reverberating activity that occurred in the myenteric neuronal network (8.0
266 $\pm 6.1\%$ neurons, $p = 0.001$) (**Figure 3d**). D-Ser-induced Ca^{2+} transients in myenteric neurons ($Fi/F0$: 1.09
267 ± 0.04) were significantly higher compared to baseline variations in amplitude ($Fi/F0$: 1.06 ± 0.03 ; $p =$
268 0.009) (**Figure 3b,d**). In the presence of CGP78608 (500 nM, preincubation for 15 min), the proportion
269 of neurons responding to D-Ser increased to $45.5 \pm 31.9\%$, which was significantly higher compared to
270 the spontaneously active proportion ($13.5 \pm 10.7\%$, $p = 0.0001$) (**Figure 3c,d**). Also the $[Ca^{2+}]_i$ response
271 amplitude was enhanced compared to baseline ($p = 0.0019$) (**Figure 3c,e**). Importantly, DCKA (10 μ M)
272 strongly reduced the proportion of D-Ser responding neurons in the continued presence of the
273 CGP78608 from 45.5% to $16.6 \pm 16.5\%$ ($p = 0.02$) (**Figure 3c,d**). Equally, the increase of D-Ser-evoked
274 Ca^{2+} transients in the presence of CGP78608 was prevented by the preincubation of DCKA (**Figure 3e**).
275 Overall, these data support the notion that the GluN1-GluN3 receptors can be activated by D-Ser alone,
276 generating Ca^{2+} rises independently of GluN2-containing receptors.

277

278 **D-Ser activates GluN1-GluN3 receptor-mediated slow excitatory currents in myenteric neurons**

279 We used *in situ* patch-clamp recordings to unravel the exact mechanisms of GluN1-GluN3 receptor
280 activation by D-Ser in mouse and guinea-pig myenteric neurons. The action of D-Ser was studied using
281 the whole-cell voltage-clamp mode coupled to a local perfusion system positioned over the recorded
282 neuron. In most mouse myenteric neurons tested (79%, 11/14 neurons), application of D-Ser alone
283 (100 μ M) caused fluctuations in the holding current that were difficult to distinguish from the intrinsic

284 electrical fluctuations of the enteric neuronal network (**Figure 4a**). In a minority of cases (21%, 3/14),
285 D-Ser elicited a small (-0.37 ± 0.03 pA/pF) slowly developing inward current, reminiscent of the typical
286 glycine-activated GluN1-GluN3 NMDAR current in brain neurons⁶². Pretreatment with CGP78608 (500
287 nM) significantly increased the percentage of neurons that responded to D-Ser to 55% (11/20 neurons).
288 In these cells, D-Ser-induced slow inward current reached a mean amplitude of -1.27 ± 0.18 pA/pF.
289 Overall, the D-Ser inward current in CGP78608-treated neurons was potentiated 4 fold compared with
290 non CGP78608-treated neurons (**Figure 4a,e**). D-Ser currents caused a mean depolarization of 6 mV in
291 the receiving neuron's membrane potential after CGP78608 treatment (**Figure 4d**). Pre-application of
292 CGP78608 also increased the proportion of myenteric neurons exhibiting D-Ser-induced currents (from
293 13 to 42%) as well as the amplitude of these currents (from -0.15 ± 0.06 to -0.55 ± 0.07 pA/pF; $p < 0.01$)
294 in guinea-pig LMMPs (**Figure 4b,e**). Consistent with the involvement of GluN1-GluN3 receptors in D-
295 Ser responses, DCKA fully prevented the occurrence of D-Ser-mediated inward currents in the
296 continued presence of CGP78608 (**Figure 4c,e**).

297 In few cases, bursts of fast excitatory postsynaptic currents (fEPSCs) occurred in post-synaptic
298 mouse and guinea-pig myenteric neurons during D-Ser application (**Figure 4f,g**). These events, which
299 reflect D-Ser-mediated release of neurotransmitters from presynaptic neurons, were increased in
300 myenteric ganglia treated with CGP78608 both in mice (from 6 to 29%) and guinea pigs (from 0 to
301 21%). DCKA reversed the effects of CGP78608 on D-Ser-induced fEPSPs in mouse myenteric neurons
302 (**Figure 4f,h**).

303

304 **Pharmacological manipulation of enteric GluN1-GluN3 receptors regulates colon contractility**

305 To specify the physiological roles of neuronal GluN1-GluN3 receptor activation on mouse gut motor
306 behavior, we investigated contractility parameters using *in vitro* colonic strips. Longitudinal colonic
307 muscle strips developed spontaneous activity characterized by the occurrence of rhythmic phasic
308 contractions having a mean amplitude of 0.19 ± 0.02 g, a mean frequency of 4.20 ± 0.23 contractions
309 per minute and a mean basal tone of 0.90 ± 0.02 g (**Figure 5**). In line with our patch-clamp recordings,

310 application of D-AP5 (100 μ M) had no effects on spontaneous activity (**Figure 5a-c**), confirming the
311 absence of tonically active GluN1-GluN2 NMDARs in colonic contractions under physiological
312 conditions. By contrast, exposure of colonic strips to DCKA (50 μ M), which prevents D-Ser binding to
313 both GluN1 and GluN3 subunits and consequently blocks GluN1-GluN3 receptor activity, reduced the
314 area under the curve (AUC) and frequency of ongoing phasic contractions, without affecting the resting
315 basal tone (**Figure 5d-f**). On average, DCKA-induced inhibition of spontaneous colonic contractions
316 reached 15 % when measured over a 5 min post-drug period and could reach 80% at its maximum
317 effect. On the other hand, CGP78608, which acts as a potentiator of GluN1-GluN3 receptor caused a
318 tonic colonic contraction, paralleled by a marked elevation of the basal tonus (**Figure 5g-l, inset in**
319 **Figure 5i**). The phasic contractions were usually ablated during the occurrence of the tonic contraction
320 but resurfaced once the tone began to decrease from the maximum (**Figure 5g,h**). Collectively, these
321 results suggest that ambient D-Ser tunes neuronal GluN1-GluN3 receptor activity and regulates the
322 colonic smooth muscle contractility.

323

324 **Genetically-driven loss of D-Ser levels alters gut transit**

325 Given that colonic contractility is impaired upon pharmacologically acting on the GluN1-GluN3
326 receptor D-Ser binding site, we hypothesized that deletion of SR in mice would impact GI activity. We
327 first performed histopathological analysis on hematoxylin-eosin-stained portion of the whole GI tract
328 of WT and SR^{-/-} mice. Surface area, thickness of the muscularis and submucosa layers, number, and
329 size of the villusities as well as lumen area for the ileum, proximal, medial, and distal parts of the colon
330 were analyzed. We found no obvious clinicopathological differences between WT and SR^{-/-} mice,
331 except for a modest change in the thickness of the muscularis and villi structure in the different
332 segments of SR^{-/-} colon (**Extended data Fig. 5**).

333 We further compared GI motility of WT and SR^{-/-} mice by measuring fecal pellet output and the
334 time in carmine red expulsion. The stool frequency (119 and 116 pellets/hr for WT and SR^{-/-} mice,
335 respectively) was not significantly different in SR^{-/-} mice compared with WT mice. However, the whole

336 gut transit time was significantly ($p = 0.0016$) reduced in SR^{-/-} mice to 93.63 ± 5.21 min ($n = 8$) compared
337 with WT mice (132.14 ± 7.65 min, $n = 7$) (**Figure 6a**). Consistently, the fecal pellets of SR^{-/-} mice
338 contained significantly more fluid content (**Figure 6b**). Such alterations in GI activity were not resulting
339 from differences in the total length of the GI tract as illustrated in **Figures 6c and 6d**.

340

341 Discussion

342 Our findings revealed a novel function for D-Ser in the enteric nervous system. We discovered that
343 non-conventional GluN1-GluN3A NMDARs, but not GluN2 containing NMDARs, are physiologically
344 operational in myenteric neurons of mice and guinea-pigs. We provide evidence that ambient D-Ser
345 serves as an agonist at these receptors and thus controls smooth muscle contraction and gut transit.
346 These results add a new active component to the already complex list of neuromodulators and
347 transmitters of the ENS and extend D-serine's breadth of physiological actions by uncovering its
348 involvement in GI activity.

349 The exploration of the roles of D-Ser in brain has gained extensive attention during recent years
350 and this atypical neurosignalling molecule is increasingly recognized as a major modulator of synaptic
351 plasticity and cognitive processes through its actions on the NMDARs and GluD glutamate receptors.
352 Both glial and neuronal distributions of D-Ser and SR have been reported in mammalian brains^{63,64}.
353 Although Dai and colleagues discovered that D-Ser produced by SR in the intestine regulates sleep in
354 flies³⁹ and several studies have reported the presence of substantial amounts of D-Ser in the rodent
355 gut microbiota^{40,41}, its function in GI tract has remained enigmatic. We report that D-Ser is present at
356 typical concentrations in different segments of the mouse small intestine and the colon as well. D-Ser
357 immunoreactivity was found to be localized in the cell bodies of enteric neurons, but not in glial cells,
358 at least in physiological conditions. Within enteric neurons, D-Ser and SR were commonly encountered
359 in neurons with known markers of IPANs indicating a potential for D-Ser production and secretion from
360 these neurons. Though under-represented, our data also suggests that some nNOS-positive neurons,
361 which are putative inhibitory motoneurons or descending interneurons, also accumulate D-Ser.

362 Metabolic D-Ser degradation is typically achieved by DAAO, a flavin-dependent oxidase widespread to
363 many cells and organs⁵¹. We found DAAO to be absent in the colon and present only in the proximal
364 part of the mouse small intestine, confirming previous studies^{41,65}, and supporting the absence of any
365 functional effects of CBIO, a known inhibitor^{66,67}, on colonic neurons (data not shown). Intriguingly, we
366 observed a reduction of about 50% in D-Ser amounts in the gut of SR null mutant mice, contrasting
367 with the 80-90% decrease consistently reported in brain D-Ser levels^{68,69}. Our findings thus support
368 that, additional sources, including diet and intestinal gut microbiota bacteria, are likely supplying the
369 gut with D-Ser. Collectively, these data nevertheless indicate that the molecular components for the
370 production and degradation of D-Ser, although sparsely expressed, are present in the ENS and GI tract.

371 The presence of D-Ser along with GluN1 and GluN2D subunits spurred investigations into a
372 possible functional relationship between them. To be activated, the conventional NMDARs requires
373 simultaneous binding of glutamate to the GluN2 subunit and the co-agonist glycine or D-Ser to GluN1³⁰⁻
374 ³², with the latter being more potent both at binding to the co-agonist site and stimulating the receptor.
375 Despite extensive investigation in mouse and guinea-pig myenteric neurons, we failed to detect
376 functional GluN1-GluN2 NMDARs both at post- and pre-synaptic levels, ruling out a contribution of
377 these receptors in D-Ser action. Consistently, D-AP5 failed to alter the spontaneous activity of mouse
378 colonic strips. This observation validates the lack of any physiological contribution of GluN1-GluN2
379 NMDARs in the activity of the colon, which however does not preclude a possible role for these
380 receptors in pathological conditions. Previous studies have produced conflicting data regarding the
381 presence of these conventional NMDARs in enteric neurons^{13,17,26}. While Liu et al.¹³ reported that D-
382 AP5 inhibits glutamate-induced depolarization in enteric neurons, others observed no effects of the
383 NMDAR antagonists, MK-801 or D-AP5, on glutamate-induced excitation or calcium transients^{17,26}. Our
384 *in situ* patch clamp recordings indicate that mouse and guinea-pig myenteric neurons do not respond
385 to NMDA exposure under physiological conditions, yet they exhibit fast developing inward currents in
386 response to L-glutamate. Therefore, glutamatergic responses are likely to relate to the presence of
387 other glutamate receptors such as Kainate or AMPA receptors⁷⁰.

388 Our findings point towards the contribution of GluN3-containing receptors. GluN3A and GluN3B
389 can associate with multiple NMDAR subunits, however, only tri-heteromeric GluN1-GluN2-GluN3 and
390 di-heteromeric GluN1-GluN3 assemblies produce surface expressed receptors in heterologous cell
391 systems. The tri-heteromeric GluN1-GluN2-GluN3 complexes respond to NMDA and glutamate and are
392 thus not at play in enteric neurons. Diheteromeric GluN1-GluN3 complexes are not activated by NMDA
393 or glutamate, are insensitive to D-AP5 and Mg²⁺ and function as excitatory glycine-gated receptors^{71,72}.

394 Our data are consistent with the involvement of GluN1-GluN3 receptors, i.e. i) activation by D-
395 Ser alone in the absence of glutamate or NMDA, ii) insensitivity to the GluN2 glutamate binding-site
396 antagonist D-AP5^{62,71,72} and iii) inhibition by the dual GluN1 and GluN3 glycine-binding site antagonist
397 DCKA. Furthermore, the GluN1 antagonist CGP78608, which unmasks GluN1-GluN3A receptors^{62,71}
398 potentiated D-Ser signals both in patch clamp recordings and calcium imaging. Our findings align with
399 single cell RNA-sequencing assays, which identified expression of GluN1 (*Grin1*) and GluN3 (*Grin3a*)
400 subunits, along with unique electrophysiological channels (*Kcnn3*, *Ano2*, *Scn11a*) in mouse IPANs^{59,73}.
401 Altogether, these results provide the first demonstration that D-Ser excitatory GluN1-GluN3 receptors
402 are functional in enteric neurons in contrast to data in the CNS where glycine is the main co-agonist of
403 these receptors⁷¹.

404 A teasing remaining question then is whether ambient D-Ser modulates GluN1-GluN3 NMDAR
405 functions *in vivo*. Our data show that D-Ser-induced currents in *ex-vivo* LMMPs are barely detectable
406 in control conditions but consistently turn into more prominent responses in the presence of
407 CGP78608. This observation suggests that the main fraction of GluN1-GluN3 NMDARs exists in a D-Ser-
408 bound inhibited/desensitized state. Accordingly, acute application of D-Ser only elicited calcium
409 transients in a fraction of neurons, but this effect was potentiated by CGP78608, as would be expected
410 from GluN1's inhibition relief of D-Ser-bound GluN1-GluN3 receptors. Because D-Ser binds to the
411 GluN3A subunit with a ~10-fold higher affinity than GluN1, we suggest that D-Ser concentration in
412 LMMPs is high enough to maintain those receptors silent, consistent with the high level of D-Ser found
413 in LMMPs and gut tissues. Hence, only a subset of GluN1-GluN3 NMDARs is available for activation in

414 LMMPs. Yet, the final concentration of D-Ser that reaches the target effector cells is still unknown *in*
415 *vivo*. Reduction of D-Ser levels by ~50% in SR^{-/-} mice was found to speed-up overall GI-transit time *in*
416 *vivo*, indicating that D-Ser through GluN3 receptors may be important in controlling small intestine
417 motility. In addition, SR^{-/-} mice have loose stools, indicating impaired absorptive function, which is
418 expected from a change in GI-transit that deprives the fecal material of adequate exposure to mucosa.
419 Collectively these phenotypic alterations are in line with the idea that GluN1-GluN3 receptors in the
420 ENS coordinate muscle movements underlying propulsion of content and absorption. However, it is
421 also possible that changes in the activity of the extrinsic innervation of the GI tract, arising from both
422 the sympathetic and parasympathetic branches of the autonomic nervous system, may contribute to
423 the observed effects *in vivo*. At the organ level, investigation of contractility parameters using *in vitro*
424 colonic strips suggests that activation of GluN1-GluN3 NMDARs acts on muscle contraction, as DCKA
425 reduces contraction amplitude while CGP78608 has an opposed effect. Thus, GluN1-GluN3 NMDARs
426 in the colon are important for normal colorectal motility. Because the absorption rate in the colon is
427 slow compared to other GI segments (due to tight mucosa), an important requirement of colonic
428 motility is to propel the luminal content slowly to prolong exposure to the mucosa. Thus, variation in
429 the amount of D-Ser may lead to colonic dysmotility leading to constipation or diarrhea.

430 Decoding the role of GluN1-GluN3 NMDAR and D-Ser in ENS remains exceptionally challenging,
431 owing to its complex organization, particularly with regards to its heterogeneous population of enteric
432 neurons that innervate different targets and interactions with the microbiome and immune system.
433 Collectively, our data suggest that D-Ser produced by enteric neurons may act at GluN1-GluN3 NMDARs
434 to regulate gut neural pathways underlying peristalsis and the balance between transit and absorption.
435 Since the ENS and gut microbiota reside in close anatomical proximity, microbiota-derived D-Ser might
436 also modulate GluN1-GluN3 NMDARs and GI functions. Indeed, gut motility is highly dependent on
437 microbiota⁷⁴ and different studies have demonstrated that several commensal bacteria produce D-
438 Ser⁴⁰. Because the gut epithelial barrier expresses transporters for AAs including D-Ser, it is tempting
439 to imagine that part of the microbiota-derived D-Ser can reach the enteric GluN1-GluN3 receptors.

440 Such crosstalk between microbiota and the ENS metabolism implying D-Ser is offering an appealing
441 framework for tailoring new strategies for treat or prevent GI diseases by targeting D-Ser pathway.

442

443 **Methods**

444 **Animals**

445 All experiments were conducted in accordance with European and French directives on animal
446 experimentation and were approved by the CNRS, Aix-Marseille University and KU Leuven Animal
447 Ethics committees (2010/63/UE). Animals were bred in-house in polycarbonate cages and maintained
448 on a 12:12-h light/dark cycle in a temperature (22°C) and humidity-controlled room. Animals were
449 given access to food and water *ad libitum* except during food fasting period preceding some
450 experiments. Age-matched (12–16-week-old) SR knock-out (SR^{-/-}) mice and wild-type (WT) mice on a
451 C57BL/6 background were used. Tricolor strain guinea pigs weighing 250-400 g from the inbred colony
452 (Marseille, France) were also used. Mice and guinea pigs were killed before experiments by cervical
453 dislocation or decapitation after isoflurane anesthesia.

454

455 **Determination of D- and L-AAs levels by capillary electrophoresis with laser induced fluorescence** 456 **(CE-LIF)**

457 Luminal contents and muscularis layers from the small intestine from WT and SR^{-/-} mice were isolated
458 following the protocols described earlier⁴¹ with slight modifications. Middle segments (2-3 cm) of the
459 ileum and colon were freshly dissected. The inner part of each piece was washed out 3 times with 2
460 mL of ice-cold sterile PBS, resulting in 6 mL of washout referred to as 'luminal content' or 'lumen'. Then
461 tissue pieces were opened longitudinally, and the mucosa and the submucosa were dissected away.
462 The remaining tissue pieces referred to as 'muscularis layers' or 'wall' were dried and placed in
463 cryogenic tubes filled with ice-cold methanol. Samples were kept at -80°C until used for analysis. Each
464 luminal content tube was supplemented with 500 µL methanol and then dried using a SpeedVac
465 system. The muscularis layers were homogenized, and the supernatants were collected and dried using

466 a SpeedVac. The pellets were sequentially rinsed with 1 mL acetonitrile, 1 ml 80:20 methanol: H₂O,
467 and 500 µL of water. Each of these solutions was combined with the original dried supernatant and
468 evaporated to dryness using a Speedvac to finally give the tissue extract samples. All samples were
469 reconstituted in water and then derivatized before determination of D- and L-AAs levels using capillary
470 electrophoresis with CE- laser induced fluorescence (LIF).

471 Naphthalene-2,3-dicarboxaldehyde (NDA) derivatization for LIF detection was accomplished by
472 mixing samples and D-Ser standards with potassium cyanide (20 mM KCN in 100 mM borate buffer)
473 and NDA (20 mM in acetonitrile) in a 1:4:4 ratio. Following derivatization, the reaction mixture was
474 diluted in water and was analyzed by CE-LIF. To confirm the presence of D-Ser, gut samples were
475 treated with D-amino acid oxidase from porcine kidney (*PkDAAO*) (Catalog# A5222, Sigma-Aldrich, St.
476 louis, MO). The sample was mixed with *PkDAAO* (15 U/mL), flavin adenine dinucleotide (5 mM),
477 catalase (68 µg/mL) from bovine liver, and PBS 1X in a 1:2:1:1:5 volume ratio. The mixture was
478 incubated in a water bath at 37 °C for 6 h. The enzyme-treated sample was dried and reconstituted in
479 1 µL water prior to derivatization and analysis by CE-LIF.

480 Separations were performed using a PA 800 plus Pharmaceutical Analysis System equipped with
481 LIF detection (AB SCIEX, Framingham, MA). The laser emitting wavelength of 440 ± 8 nm was operated
482 at 3 mW. A band-pass filter of 490 ± 15 nm was used to only permit the fluorescence emission band
483 for detection. Bare fused-silica capillary was used for all separations. The capillary had the
484 total/effective lengths of 40/30 cm with inner/outer diameters of 50/360 µm. The separation buffer
485 with final concentrations of 62 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6), 7 mM
486 potassium bromide, and 330 ppm quaternary ammonium β-cyclodextrin solution was prepared daily
487 into water. For D-Ser separation, 10 kV of reverse polarity at 20 °C was applied. The standard calibration
488 curve was generated to quantitate D-Ser in the gut samples based on the analyte peak area. Protein
489 quantification was performed using the Micro BCA Protein Assay Kit (ThermoFisher, Walham, MA) to
490 further compare amino acids levels between samples.

491

492 **Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

493 Total RNA was extracted from brain and colon LMMP preparations of WT and SR^{-/-} mice with TRIzol
494 reagent (Ambion) according to the manufacturer's guidelines. Reverse transcription reactions with
495 oligo(dT)₂₀ primers were performed with the Superscript III reverse transcriptase (Invitrogen)
496 following the manufacturer's protocol (Invitrogen) using 3 µg of total RNA. PCR on reverse-transcribed
497 cDNA was performed with specific primers to mouse transcripts. Sequences of primers and expected
498 sizes of the amplicons were described in the **Supplementary Table 1**. PCR products were separated on
499 a 2% agarose gel.

500

501 **Western Blot analysis**

502 Brains and colonic LMMP preparations isolated from mice or guinea pigs were homogenized by
503 sonication in ice-cold lysis buffer (Tris 50 mM, pH 6.8 containing SDS 0.5%, EDTA 2 mM, PMSF +
504 Complete EDTA free protease inhibitors cocktail (Roche Diagnostics) w:v = 1:10) and centrifuged 10
505 min at 1,500g to remove the crude nuclear pellet. Protein extracts (20 µg for brain and 40 µg for LMMP
506 per lane) were subjected to electrophoresis (10% (vol/vol) SDS-polyacrylamide gel) and electroblotted
507 onto nitrocellulose membranes (0.2 µm; Pall Life Science). Immunodetection was accomplished by
508 enhanced chemiluminescence (ECL) using the ECL Prime Western Blotting Detection Reagent
509 (Amersham Biosciences). Molecular sizes were estimated by separating prestained molecular weight
510 markers (10.5–175 kDa) in parallel (Nippon Genetics). Primary and secondary antibodies and their
511 dilutions are listed in **Supplementary Table 2**. Digital images of the chemiluminescent blots were
512 captured using the GBOX system (Syngene) with the GeneSys image acquisition software.

513 For DAAO immunoblots, two 6-week-old mice were anesthetized and perfused with ice-cold PBS
514 before dissection of intestinal tissue. The protocol for isolation of intestinal epithelial layer was
515 described previously⁴¹. Non-epithelial tissue was rinsed in PBS twice. Tissue was stored in -80°C until
516 use. Epithelial and non-epithelial tissue was homogenized in a lysis buffer (150 mM sodium chloride,
517 1.0% NP-40, 50 mM Tris (pH 8.0), and a protease inhibitor cocktail, Complete EDTA-free (Roche, Basel,

518 Switzerland)) and spun down at 15,000g at 4°C for 5 min. Supernatants were subjected to SDS-PAGE
519 and protein was transferred to nitrocellulose membranes. Blots were blocked in 10% skim milk in PBS
520 with 0.1% Tween-20 (PBST) with constant shaking for 1 hour. The membranes were rinsed in PBST and
521 immersed in PBST with a rabbit polyclonal antibody to mouse DAAO⁴¹ or a rabbit monoclonal antibody
522 to GAPDH at 4°C overnight. Membranes were subsequently rinsed in PBST, then incubated with an
523 appropriate secondary antibody conjugated with horseradish peroxidase for 1 hour. The membranes
524 were rinsed in PBST and bound antibodies detected with ECL (GE Healthcare Life Sciences, Chicago, IL,
525 USA).

526 LMMP preparations isolated from human colons were placed into RIPA buffer (0.5 M Tris-HCl,
527 pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA), containing a cocktail of protease
528 inhibitors (Roche Diagnostics), snap frozen on dry ice and homogenized. Protein concentration was
529 measured using a BCA protein assay kit (Bio-Rad, Hercules, CA). Aliquots containing 40 µg of proteins
530 were resolved in 8% SDS-PAGE under reducing conditions and transferred to a nitrocellulose
531 membrane. The membrane was blocked with 1% BSA in PBS + 0.05% Tween-20 for 1 hour at room
532 temperature and incubated with primary antibodies overnight at 4°C. After washing, the membrane
533 was incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibody diluted
534 in PBST milk before imaging.

535

536 **Immunohistochemistry and confocal microscopy analysis**

537 The LMMP preparation has been detailed previously^{57,59}. In brief, a 2 cm segment of each intestine
538 portion was removed from animal, placed in a Sylgard coated dish containing oxygenated standard
539 Krebs solution (in mM: 118 NaCl, 4.8 KCl, 1 NaH₂PO₄, 25 NaHCO₃, 1.2 MgCl₂, 2.5 CaCl₂, and 11 glucose,
540 equilibrated with 95% O₂-5% CO₂, pH 7.4 and supplemented with 2 µM atropine and 6 µM nicardipine).
541 The myenteric plexus was exposed by dissecting away the mucosa, the submucosal plexus, and the
542 circular muscle layers. LMMPs were then fixed by immersion for 1-2hr in PBS containing 4%
543 paraformaldehyde (complemented with 0.5-1% glutaraldehyde for D-serine immunolabelling). Whole-

544 mount preparations were subjected to immunohistochemistry as described previously⁷⁵. Antibody
545 details are supplied in **Supplementary Table 2**. Images were acquired through the 40X objective
546 (PlanFluor, 0.75 NA) of an upright Leica confocal laser scanning microscope. No staining was observed
547 in SR^{-/-} mice or when the anti-D-Ser antibody was incubated in the presence of liquid conjugate against
548 D-Ser. The confocal images were acquired using the Leica TCS software with a sequential mode to avoid
549 interference between each channel and without saturation of any pixel. Moreover, emission windows
550 were fixed for each fluorophore in conditions where no signal is detected from the other fluorophore.
551 Stack images were taken, and a Z-projection was made with ImageJ software
552 (<http://rsb.info.nih.gov/ij>) in average projection mode.

553

554 **Histochemistry for DAAO**

555 Activity-based DAAO labeling was performed using a previously reported method⁴¹. After deep
556 anesthesia, 6-week-old mice were perfused transcardially with 2% paraformaldehyde in PBS. Tissues
557 were dissected, cryoprotected and 15 µm sections were made on a cryostat (Leica, Wetzlar, Germany).
558 Sections were rinsed in PBS and immersed in DAAO-activity staining solution as defined previously⁴¹.
559 Fluorescence signals were visualized using a BZ-9000 fluorescent microscope (Keyence, Osaka, Japan).
560 Each section being compared was imaged under identical conditions.

561

562 **Histopathological analysis of SR^{-/-} mouse gut**

563 We favor the so-called gut bundling technique as it provides high quality cross sections through villi
564 and crypts and enable quantification of cell features or villus/crypt lengths. The entire GI was extracted
565 from fasted mice (6 WT and 6 SR^{-/-}). The lumen was gently flushed with ice-cold PBS to remove any
566 fecal and digesta content. The large and small intestine was cut to isolate the medial part of the ileum
567 (48.5-51.5%), the proximal (10-12.5%), medial (48.5-51.5%) and distal (77.5-80%) parts of the colon.
568 Gut segments were then placed overnight with 10X excess of tissue volume 4% paraformaldehyde
569 fixative and thereafter transferred to tissue cassette to be embedded in paraffin. Cross sections (10

570 μm) were obtained using a cryostat prior to be floated onto glass slides. Sections from WT and SR^{-/-}
571 mice were simultaneously colored with hematoxylin/eosin/safran (HES; Merck, Darmstadt, Germany).
572 Once stained, slides were scanned and proceeded for morphometric analyses. Villus size (height and
573 width), lumen and total sections areas, thickness of both smooth muscle layer and submucosa layer
574 were scored from at least 10 sections per mice. Villus height was measured from the basal layer of the
575 submucosa to the ending of the villus in the ileum and colon.

576

577 **Calcium Imaging**

578 Colon segments (approximately 5 cm long) were collected and immersed in 95% O₂/5% CO₂ -bubbled
579 Krebs solution (in mM: 120.9 NaCl; 5.9 KCl; 1.2 MgCl₂; 1.2 m NaH₂PO₄; 14.4 NaHCO₃; 11.5 Glucose; 2.5
580 CaCl₂). Tissues were opened along the mesenteric border and pinned flat with mucosa up in a silicone
581 elastomer-coated dish. After removing the mucosa and submucosa, the tissues were flipped over and
582 the longitudinal muscle layer was removed to expose the myenteric plexus. These preparations (up to
583 5 per colonic segment) were stabilized over an inox ring using a matched o-ring⁷⁶. The ring preparations
584 were loaded for 20 min in bubbled Krebs containing 0.01% (vol/vol) Cremaphor and 1 μM Fluo-4-AM.
585 After a 20 min washout period the tissues were transferred to a coverglass chamber mounted on the
586 microscope stage. 3D recordings of the Fluo-4 signals were made on an inverted spinning disk confocal
587 microscope (Nikon Ti-Andor Revolution - Yokogawa CSU-X1 Spinning Disk (Andor, Belfast, UK) with a
588 Nikon 20x lens (NA 0.8), excitation 488 nm and detection 525/50 nm. A Piezo Z Stage controller (PI)
589 was used to record fast 3D stacks at 1Hz. For other sets of experiments, 2D recordings were made on
590 an inverted Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany), 20x 0.8 NA objective lens,
591 equipped with a monochromator (Poly V) and cooled CCD camera (Imago QE), both from TILL
592 Photonics (Gräfelfing, Germany). Fluo-4 was excited at 470 nm (50 ms exposure) and images were
593 acquired at 520/50 nm at a 2 Hz framerate. The tissues were constantly supplied with 95% O₂ / 5%
594 CO₂-bubbled Krebs at room temperature via a gravity fed perfusion system (1 ml/min). The pipette tip,
595 in which 5 perfusion lines converged, was positioned just above (250 μm) the ganglion of interest. A

596 magnetic valve system allowed instantaneous switching between control and drug-containing Krebs
597 solutions. For CGP78608, a preincubation (15 min) was used before preparations were transferred to
598 the recording microscope. A 75 mM K⁺ depolarization (5 s) was used to depolarize the ganglion and
599 identify all Fluo4 labelled cells. All solutions contained 2 μM nifedipine (N7634, Sigma-Aldrich) to
600 minimize spontaneous movement.

601 All image analysis was performed with custom-written routines⁷⁷ in Igor Pro 8 (Wavemetrics,
602 Lake Oswego, OR, USA). 3D image sequences were imported, and maximum projections were
603 generated from the image planes that contained the myenteric plexus. These 2D images were analysed
604 in the same way as the images recorded on the 2D widefield system. First, regions of interest were
605 drawn in the images to extract the temporal information from individual cells. Peak detection and peak
606 amplitude calculation were performed using custom written procedures as previously described⁸². The
607 average Ca²⁺-signal intensity was calculated, normalized to the initial Fluo-4 values, and reported as
608 F/F₀. Responses were considered when the [Ca²⁺]_i signal increased above baseline by at least five times
609 the intrinsic noise. [Ca²⁺]_i peaks were calculated for each response, with the peak amplitude taken as
610 the maximum increase in [Ca²⁺]_i from baseline (F_i /F₀). The total number of cells responding to each
611 drug exposure was counted and expressed as a percentage of the control.

612

613 **Whole-cell patch-clamp recordings from intact myenteric ganglia**

614 Whole-cell patch-clamp recordings were conducted at room temperature on LMMP preparations
615 obtained from duodenum and colon of both guinea pigs and mice. The LMMP preparation of gut
616 segment has been detailed previously⁵⁷. In brief, a 2 cm segment of duodenum or colon was removed.
617 The tissue was opened along the mesentery line and pinned mucosal side up on the silicon elastomer
618 basis (Sylgard; Dow Corning) of a recording dish containing oxygenated standard Krebs' solution. The
619 myenteric plexus was exposed by dissecting away the mucosa, the submucosal plexus, and the circular
620 muscle layer. The recording dish was then placed on an inverted microscope stage and the LMMP
621 preparation was continuously superfused with Krebs' solution gassed with 95% O₂-5% CO₂. The upper

622 surface of a ganglion was exposed to 0.02% protease type XIV (Sigma) for 3–5 min and the surface of
623 neurons was cleaned by sweeping over the ganglion with a hair fixed at the tip of a microelectrode.
624 The standard Krebs' solution used to bath the LMMP preparation consisted of the following (in mM):
625 118 NaCl, 4.8 KCl, 1 NaH₂PO₄, 25 NaHCO₃, 1.2 MgCl₂, 2.5 CaCl₂ and 11 glucose and was equilibrated
626 with 95% O₂-5% CO₂, pH 7.4. Atropine (2 μM) and nicardipine (6 μM) were present throughout the
627 experiments to prevent spontaneous muscle movement. Drugs, diluted into the standard Krebs'
628 solution (see above), were locally applied via a gravity perfusion system directly onto the myenteric
629 ganglion under study. Patch pipettes were pulled from thick-walled borosilicate glass capillaries
630 (Harvard Apparatus) and had a resistance of 3-5 MΩ. The intracellular solution used to record in the
631 voltage-clamp mode contained (in mM): 140 CsCl, 4 NaCl, 1 CaCl₂, 2 MgCl₂, 2 EGTA, 10 HEPES. For
632 current-clamp recordings, the intrapipette solution consisted of (in mM): 140 KCl, 4 NaCl, 1 CaCl₂, 2
633 MgCl₂, 2 EGTA, 10 HEPES. pH was adjusted to 7.35 with CsOH or KOH (~300 mOsm/L).

634 To elicit EPSPs in enteric neurons, a concentric bipolar electrode (FHC, Bowdoin, USA) was placed
635 over one of the interganglionic bundles lying circumferentially to the recorded ganglion. Nerves fibers
636 were electrically stimulated using 1 ms, 0.5- to 2-mA constant current pulses delivered every 15 s from
637 an ISO-flex stimulus isolation unit driven by a Master-8 pulse stimulator (A.M.P.I., Israel).

638 All recordings were made using an Axopatch 200B amplifier (Molecular Devices), low-pass
639 filtered at 2 kHz, and digitized at 25 kHz. The series resistance measured in the whole-cell mode was
640 5-8 MΩ and was compensated by 70-80%.

641

642 **Spontaneous contractions of isolated proximal colon from mice**

643 The entire colon was removed and placed into Krebs' buffer at room temperature. The colon was
644 cleaned from connective tissues and the proximal part (2 cm from the ileocolonic junction) was cut
645 along in the longitudinal direction. Strips (8-10 mm long; 2-3 mm large) were tied with silk threads to
646 secure them between the holder and the isometric transducer (it-1, EMKA Technologies, France) and
647 mounted under 10 mN initial tension in 5 mL organ baths (EMKA Technologies, France) containing

648 Krebs-Henseleit solution (composition in mM: NaCl 114, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2,
649 NaHCO₃ 25, glucose 11.7; equilibrated with 95% O₂-5% CO₂, pH 7.4) and kept at 37°C.

650 Contractile responses were measured using isometric tension transducers connected to
651 amplifiers and to a data acquisition system (PowerLab 8/35, ADInstruments Pty Ltd., Castle Hill,
652 Australia). Colonic tissues were allowed to equilibrate for 60 minutes during which the Krebs' solution
653 was replaced every 10-15 min. In strips demonstrating spontaneous regular contractions, after a 30-
654 min recording period, D-AP5 (100 μM), CGP78608 (500 nM) or DCKA (50 μM) were added for 15 to 30-
655 min periods. Then, tissues were washed at least three times during an additional 15-min period before
656 the addition of KCl (80 mM) to test tissue viability. The basal tone was determined as mean tension
657 between the peaks of contraction before drugs addition.

658

659 **Whole-gut transit time and pellet production**

660 Whole GI transit duration was measured with carmine red simultaneously in WT and SR^{-/-} mice after
661 fasting and according to the procedure described^{78,79}. Carmine red was prepared as 1% solution with
662 5% sucrose (Sigma-Aldrich) in 2% agar. Mice were individually housed and fasted before the test. In
663 the morning, 6 carmine red agarose pieces were given to mice. T0 refers to the time when mice started
664 to eat. When mice had eaten the totality of carmine red pieces, they were placed on iron gate cage
665 and monitored every 15 min to see the appearance of the first carmine red feces. The whole-gut transit
666 time was defined as the interval between T0 and the time of first observance of carmine red feces.
667 Fecal pellets excreted from WT (n=119) and SR^{-/-} (n=136) mice were collected at zeitgeber +4 and
668 weighed (ie wet weight). Feces were next dried 24h at 37°C and weighed again, which represents
669 weight of dry feces and fluid content was then calculated as described⁸⁵.

670

671 **Drugs, enzymes and solutions**

672 Appropriate stock solutions were diluted to the final concentration with perfusing media containing
673 vehicle (<1/1000) just before application. Controls were made with vehicle to ascertain any side

674 effects. Salts were all from Sigma (St Louis, MO, USA). Tricine, L-glutamate, Kainate, Salicylate and D-
675 Ser were purchased from Sigma. N-Methyl D-aspartate (NMDA), CGP78608, 5,7-Dichlorokynurenic
676 acid (5,7DCKA), CNQX and D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) were from Tocris
677 Biosciences. Tetrodotoxin (TTX) was from Alomone Laboratories (Jerusalem, Israël). All drugs were
678 stored as aliquots at -20°C and were diluted in Krebs to achieve the desired final concentration.

679

680 **Data Analysis**

681 Quantitative data are expressed as mean ± standard error of the mean (s.e.m.), or otherwise stated.
682 Graphics and statistical analyses were performed using GraphPad Prism (GraphPad Software, San
683 Diego, CA, USA) excepted for CE-LIF for which Origin 2018 software was used. Data were tested for
684 normality using the Shapiro-Wilks test or D'Agostino and Pearson omnibus test. Outliers were
685 identified by Grubb's test. Normally distributed data were compared by Student's t-test for paired or
686 unpaired values. Data not normally distributed were compared by Mann-Whitney test or Wilcoxon's
687 test. Difference was considered significant if $p < 0.05$.

688

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- 895

896 **Figure legends**

897 **Figure 1. D-Serine and serine racemase are present in enteric neurons.** a) Representative
898 electropherogram of a NDA-derivatized gut sample showing the presence of two peaks corresponding
899 to L-serine (L-Ser) and D-serine (D-Ser). The D-Ser peak identity was confirmed by enzymatic
900 degradation by DAAO (lower panels). **b)** Histograms showing the content of D-Ser (mean \pm s.e.m) found
901 in the lumen and the wall of the medial parts of ileum (left panel) and colon (right panel) of WT (n = 4)
902 and SR^{-/-} (n = 4) mice. *p<0.05 using one-way ANOVA followed by Tukey's multiple comparison test. **c)**
903 RT-PCR analysis showing the expression of SR transcripts in colonic LMMP (top) and proximal (PrCol)
904 and distal (DiCol) colon sections of WT but not SR^{-/-} mice. (-) indicates no RT. **d)** Western blot analysis
905 of the expression of SR in brain and colonic LMMP from WT and SR^{-/-} mice, brain and colon tissues from
906 guinea pigs and the medial part of human colon with (mColon) and without (MyColon) the mucosa.
907 Protein loading was normalized to actin (42 kDa). **e)** Immunostaining for D-Ser (magenta) and the
908 neuronal pan marker HuC/D (green). N = 5 guinea-pigs, 5 preparations. Scale: 50 μ m. **f)**
909 Immunostaining for D-Ser (magenta) and the glial marker S100B (green). N = 4 guinea-pigs, 4
910 preparations. Scale: 50 μ m left, 10 μ m right. **g)** Immunostaining for D-Ser (magenta) and the marker of
911 IPANs, NeuN (green). N = 7 guinea pigs, 7 preparations. Scale: 50 μ m. **h)** Immunostaining for D-Ser
912 (magenta) and the neuronal nitric oxide synthase (nNOS, green), a marker of inhibitory motoneurons.
913 N=4 guinea-pigs, 5 preparations. Scale: 50 μ m. **i)** Bar graph showing the mean percentage (+/-s.e.m)
914 of D-Ser-positive myenteric neurons co-labelled with NeuN (7 guinea-pigs, 7 preparations), Calbindin
915 (Calb, 8 guinea-pigs, 8 preparations) and nNOS (4 guinea-pigs, 5 preparations). **j)** Immunostaining for
916 SR (magenta) and IPAN marker NeuN (green) (N = 5 guinea pigs, 5 preparations) Scale: 50 μ m.

917

918 **Figure 2 - Lack of detectable functional NMDA receptors at both pre- and post-synaptic levels in**
919 **myenteric neurons.** a) Glutamate at 20 mM, but not at 1 mM, evoked inward currents that were
920 insensitive to APV (D-AP5, 100 μ M), a NMDA receptor antagonist. Recordings of *in situ* guinea-pig
921 myenteric neurons were made in the voltage-clamp mode with a holding potential of -50 mV, in the

922 presence of 500 μ M D-Ser and 10 mM tricine in the bath medium. CsCl-based intrapipette solution. **b)**
923 Quantification of 1 and 20 mM glutamate-evoked currents ($I = 0.05 \pm 0.01$ pA/pF, $n = 2$ and $I = 2.46 \pm$
924 1.06 pA/pF, $n = 8$, respectively). **c)** Upper traces: slow voltage-ramp induced I - V relationships
925 determined before and during the application of 20 mM glutamate (Glu). Lower trace: difference
926 current depicting the voltage-dependence of the glutamate-induced current. Note the absence of the
927 N-shaped I - V relationship typical of NMDAR-induced current. Currents were evoked by a 2s-voltage
928 ramp command from -80 to +40 mV (0.06 mV/ms) in the continued presence of 500 μ M D-Ser, 10 mM
929 tricine and 1 mM Mg^{2+} in the bath. **d)** Glutamate (20 mM) and kainate (50 μ M) evoked currents at
930 either negative or positive potentials, whereas NMDA, at 100 μ M or 1 mM, failed to do so. Recordings
931 were made in the voltage-clamp mode at the indicated holding potentials, in the presence of 500 μ M
932 D-Ser and 10 mM tricine in the bath medium. CsCl-based intrapipette solution. **e)** Pooled data showing
933 Glutamate-, Kainate- and NMDA-induced currents determined at $V_h = +40$ mV. Recording conditions
934 are indicated. From left to right: $I_{NMDA} = -0.21 \pm 0.19$ pA/pF; $I_{NMDA} = -0.17 \pm 0.04$ pA/pF; $I_{NMDA} = 0.01 \pm$
935 0.04 pA/pF; $I_{Glu} = 3.4 \pm 1.7$ pA/pF; $I_{KA} = 0.78 \pm 0.23$ pA/pF. **f)** Recordings of membrane potential during
936 1 mM NMDA application in a colonic AH myenteric neuron (top trace) and a colonic S myenteric neuron
937 (bottom panel). Current clamp recordings were made in the presence of 500 μ M D-Ser and 10 mM
938 tricine using K^+ -based pipette solution. AH- and S-neuronal subtypes were ascertained from their
939 action potential waveform (left panels). Left: Evoked action potentials of the AH and S neurons to
940 current injections of -2, 0, 16 and 20 pA/pF and -1.6, 0, 9.6 and 12.8 pA/pF respectively. **g)** Membrane
941 potential measurements in S- and AH-type myenteric neurons from both guinea-pig colonic and
942 duodenal myenteric neurons before and during superfusion of NMDA (1 mM) in the presence of 500
943 μ M D-Ser and 10 mM tricine. KCl intrapipette solution. From left to right (mean \pm s.e.m, mV): $V_{m_{ctrl}} =$
944 -46.6 ± 2.7 , $V_{m_{NMDA}} = -46.8 \pm 3.3$, $n = 6$; $V_{m_{ctrl}} = -51.4 \pm 2.1$, $V_{m_{NMDA}} = -51.9 \pm 2.4$, $n = 5$; $V_{m_{ctrl}} = -43.7$
945 ± 0.7 , $V_{m_{NMDA}} = -40.9 \pm 0.1$, $n = 2$; $V_{m_{ctrl}} = -49.3 \pm 3.5$, $V_{m_{NMDA}} = -49.6 \pm 3.5$, $n = 5$. **h)** Amplitude of
946 presynaptically-evoked fast nicotinic EPSCs recorded in a GP myenteric neuron from duodenal LMMP
947 before, during and after application of NMDA (1 mM). Holding potential -50 mV. Recordings made in

948 the presence of 20 μM D-Ser and 10 mM tricine. KCl-based intrapipette solution. Single shock electrical
949 stimulation was applied on nerve bundles every 15 s. Right insets: corresponding recordings (average
950 of 4-7 sweeps) of EPSCs before (1), during (2) and after (3) application of NMDA. i) Mean amplitudes
951 of electrically evoked EPSCs before (control), during (NMDA) and after (wash) application of NMDA (1
952 mM) in the presence of 20 μM D-Ser and 10 mM tricine. $I_{\text{Ctrl}} = -2.7 \pm 0.6$ pA/pF; $I_{\text{NMDA}} = -2.3 \pm 0.4$ pA/pF;
953 $I_{\text{wash}} = -2.2 \pm 0.4$ pA/pF ($n = 3$). Data were normalized to the cell membrane capacitance.

954

955 **Figure 3. Acute application of D-Ser evokes Ca^{2+} responses in mouse myenteric neurons.** a) Fluo-4
956 loaded colonic myenteric ganglia were exposed to D-Ser (100 μM , 20 s), which elicited a rise in
957 intracellular $[\text{Ca}^{2+}]_i$ in some neurons. Scale = 20 μm . **a1)** Frame of a Fluo-4 movie in which at frame 60
958 a colonic ganglion is exposed to D-Ser for 20s. **a2-3)** Averages of 10 frames of the recording at rest (**a2**)
959 and after exposure to D-Ser (**a3**). **a4)** Fluo-4 intensity traces of five individual actively responding
960 neurons. The grey area indicates D-Ser application. **b)** Normalized fluorescence intensity of the average
961 of D-Ser-induced $[\text{Ca}^{2+}]_i$ transients in control conditions ($n = 16$ neurons). **(c)** Averaged D-Ser-induced
962 $[\text{Ca}^{2+}]_i$ transients ($n = 23$ neurons) in the continued presence of CGP-78608 (500 nM) (blue trace) and
963 when CGP-78608 (500 nM) and DCKA (10 μM) were applied concomitantly. In **b** and **c**, graphs represent
964 the mean \pm 95% CI (as indicated by the shaded areas) of the normalized intensity over time. Colour-
965 coded bars indicate the condition and the stimuli used, their beginning and duration. **d,e)** Percentage
966 of responding cells (including spontaneous responses) per ganglion (**d**) and corresponding amplitude
967 of the response per ganglion (**e**) to local perfusion of D-Ser in basal conditions (Krebs, BL) as well as
968 after CGP-78608 or CGP-78608 +DCKA incubation. Data collected from 30-35 cells per ganglion and 6
969 mice for each condition. Each open circle refers to one ganglion. The grey bar represents the control
970 baseline 95% CI. Horizontal lines indicate mean values. Unpaired t test or Mann-Whitney test were
971 performed when comparing in groups. Significant p values are indicated in the graphs (* $p \leq 0.05$, ** p
972 ≤ 0.01 , *** $p \leq 0.001$).

973

974 **Figure 4. D-Ser induces GluN1-GluN3-like inward current in mouse myenteric neurons. a, b)** Example
975 traces illustrating D-Ser-evoked currents in mouse (a) and guinea-pig (b) myenteric neurons recorded
976 *in situ* in control conditions (upper traces) and following preincubation with 500 nM CGP (lower traces).
977 Holding potential -60 mV; CsCl-based pipette solution. c) Adding DCKA (10 μ M) to the CGP-containing
978 bathing solution abolished D-Ser currents in a mouse duodenal myenteric neuron. Holding potential -
979 60 mV, CsCl-based intrapipette solution. d) Example trace illustrating D-Ser-evoked membrane
980 depolarization in a mouse myenteric neuron following preincubation with 500 nM CGP. KCl-based
981 intrapipette solution. e) Quantification of D-Ser-induced currents in duodenal mouse and guinea-pig
982 myenteric neurons in control conditions, in the presence of CGP (500 nM) and when DCKA (10 μ M)
983 was added to the CGP solution. Mouse: $I_{ctrl} = -0.08 \pm 0.04$ pA/pF, $n = 14$; $I_{CGP} = -0.74 \pm 0.18$ pA/pF, $n =$
984 19 ; $I_{DCKA} = -0.04 \pm 0.04$ pA/pF; $n = 14$. GP: $I_{ctrl} = -0.02 \pm 0.01$ pA/pF, $n = 15$; $I_{CGP} = -0.23 \pm 0.07$ pA/pF, $n =$
985 19 . ** $P < 0.01$; * $P < 0.05$; Mann-Whitney test. f) Example traces illustrating D-Ser-evoked EPSCs in a
986 mouse duodenal myenteric neuron following preincubation with 500 nM CGP or 500 nM CGP + 10 μ M
987 DCKA. Holding potential -60 mV, CsCl-based intrapipette solution. g) D-Ser-evoked EPSCs in a guinea-
988 pig duodenal myenteric neuron following preincubation with 500 nM CGP. Holding potential -60 mV;
989 CsCl-based intrapipette solution. h) Proportion of mouse and guinea-pig myenteric neurons with
990 specified responses to 100 μ M D-Ser in control conditions and in the presence of CGP or CGP + DCKA.
991 Number of recorded neurons are indicated. * $P < 0.05$; χ^2 test.

992

993 **Figure 5. Pharmacological manipulation of GluN1-GluN3 receptors alters motor patterns of isolated**
994 **strips of mouse colon.** DCKA (50 μ M, d-f) and CGP-78608 (500 nM, g-i) have opposite effects on
995 spontaneous contractions of mouse colonic strips. Note that D-AP5 (100 μ M, a-c) had no effects on
996 colonic contraction, consistent with the lack of GluN2-containing receptors. The rightmost panels
997 illustrate the mean changes in amplitude, area under curve (AUC) and frequency of phasic colonic
998 contractions for individual strips. Data normalization was determined for 5 min-recording periods after

999 (post-drug) and before (pre-drug) drug application, i.e. $\left\{ \left(\frac{\text{postdrug}}{\text{predrug}} \right) * 100 \right\} - 100$. Inset in (i) illustrates
1000 the mean percentage change of the basal tonus in the 6 strips treated with CGP-78608 (500 nM).

1001

1002 **Figure 6. Deletion of SR alters gut transit and pellet output.** **a)** Bar graph showing that the duration
1003 of the whole GI transit is reduced in $SR^{-/-}$ mice ($n = 8$) when compared to the littermate wild-type (WT)
1004 mice ($n = 7$). *** $p=0.0016$ using unpaired t test. **b)** The quality of the pellet output is altered in $SR^{-/-}$
1005 mice. Both the dry weight (left) and fluid content (right) are increased ($n = 119$ pellets for WT vs 116
1006 for $SR^{-/-}$ mice). ** $p<0.01$, *** $p<0.001$ using Mann-Whitney test. **c-d)** Comparison of the total GI length
1007 between WT ($n = 14$) and $SR^{-/-}$ ($n = 13$) mice reveals no obvious changes in the anatomy of the GI from
1008 stomach (stm) to the rectum (re). Abbreviations: ce, cecum; co, colon; si, small intestine. $P = 0.5584$
1009 using Man-Whitney test. **a-d**, errors bars show s.e.m.

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1013 **Extended data**

1014 **Extended data Figure 1. Cell-type distribution of serine racemase and D-Ser in the myenteric plexus.**

1015 Gray scale images of splitted channels corresponding to double immunostainings illustrated in Fig. 1.

1016 **a)** Immunostaining for D-Ser (left) and HuC/D (right), a neuronal pan marker. Scale: 50 μ m. **b)**

1017 Immunostaining for D-Ser (left) and S100B (right), a glial marker. Scale: 50 μ m, **c)** Immunostaining for

1018 D-Ser (left) and NeuN (right), a marker of gut sensory neurons. Scale: 50 μ m. **d)** Immunostaining for D-

1019 ser (left) and neuronal nitric oxide synthase, nNOS (right), a marker of inhibitory motoneurons. Scale:

1020 50 μ m. **e)** Immunostaining for SR (left) and NeuN (right). Scale: 50 μ m.

1021

1022 **Extended data Figure 2. The D-Ser degrading enzyme DAAO is not present in the distal small intestine**

1023 **and the colon. a)** First lane: Cross-sections showing activity based DAAO labeling. The labeling reveals

1024 the presence of DAAO in the proximal s.i, but not in the distal s.i and the colon of WT mouse. Second

1025 lane: Cross-sections revealing DAAO activity and counterstained for phalloidin and DAPI. DAAO is only

1026 present in the mucosa layer. Scale: 200 μ m. **b)** Zooms into ROI shown in **(a)** for each segment of the GI

1027 tractus. Scale: 200 μ m. **c)** Western blot showing differential expression of DAAO in mouse small

1028 intestine and the colon. Tissues extracts were subjected to electrophoresis and to immunoblotting

1029 DAAO and GAPDH. A cerebellar lysate was used as a positive control (pc).

1030

1031 **Extended data Figure 3. The D-Ser degrading enzyme DAAO is not detected in the colon by RT-PCR.**

1032 Lack of DAAO transcripts in mouse colon as shown by RT-PCR. (-) indicates no RT. Brain tissue is used

1033 as positive control.

1034

1035 **Extended data Figure 4. Molecular profiling of glutamatergic receptors expressed in the myenteric**

1036 **plexus of the colon. a)** RT-PCR performed from LMMPs of mouse colon for the different transcripts.

1037 Note that transcripts for the major glutamatergic receptor subunits are present (NMDA receptors:

1038 GluN1, GluN2D, GluN3A; AMPA receptors: GluA2 and GluD1-2). When transcripts were not positively

1039 detected (GluN2A, GluN2B, GluN2C and GluN3B) in the colonic LMMP brain extracts were shown as
1040 positive controls (not shown when amplicons from mouse colon LMMP were detected). cDNAs isolated
1041 from mouse colon LMMP and brain were amplified using primers GluN1, GluN2A-D, GluN3A-B, Grid1-
1042 2 and GluR2 (see **Extended data Table 1**). Contamination from genomic DNA was routinely tested by
1043 omitting the reverse transcriptase in the templates (-). M, 100 bp-ladder DNA size standard. **b)**
1044 Immunoblots analysis on colonic LMMPs and brain extracts collected from WT and SR^{-/-} mice. GluN1 N
1045 ter but not GluN1 C-ter is present in colon confirming previous report⁸⁵. Immunoblots show positive
1046 signals for GluN2C/D, GluN3A-B, GluA2 in the colon of WT and SR^{-/-} (-/-) mice and faint or no detection
1047 for GluN2B, GluN2A, GluD1-2 and the vesicular transport type 1 (vGlut1), which is the primary vesicular
1048 transporter for glutamate in the CNS. All sample loadings were normalized to actin.

1049

1050 **Extended data Figure 5. Histopathological analysis of GI wall in wild-type and SR^{-/-} mice.** a) Schematic
1051 representation of the GI tractus highlighting the ileum and colon segments collected for the analysis.
1052 **b)** Cross-sections from WT and SR^{-/-} mice and colored for hematoxylin/phloxine safran are illustrated
1053 for each segment. **c)** Schematic representation of a cross-section and the different parameters used in
1054 the analysis. **d-i)** Bar graphs summarizing the analysis for each segment of the GI tractus in WT and SR^{-/-}
1055 mice. In panels **d** & **e**, N=7-8 sections. In panels **f** & **g**, N=30-110 different location measurements
1056 from 7-8 cross-sections. In panels **h** & **i**, N=38-74 villi were analyzed. *, p<0.05; **, p<0.01; #, p<0.0001
1057 using multiple *t*-test.

1058

1059 **Extended data Table 1.** Sequences of primers and expected sizes of the amplicons

Gene (GenBank)	Forward Primer (5'-3') Reverse Primer (5'-3')	Expected size (bp)	Reference
Serine Racemase (NM_013761.4)	ACCCAGTGCTAACAAGCTC CTGGACTCGTCACTTGGGTC	329	This study
D-Amino Acid Oxidase (NM_010018.3)	TCGATTCACCCCGTTCAC GTGTTTAATCCAAGGGGC	523	This study
GluN1 (NM_008169.3)	GCCGATTTAAGGTGAACAGC AATTGTGCTTCTCCATGTGC	372	80
GluN2A (NM_008170.2)	TGCAAGTTACACAGCCAACC ATCGGAAAAGCGGAGAATAG	119	81
GluN2B (NM_008171.3)	CCCAGATCCTCGATTTCATT GCCAAACTGGAAGAACATGG	116	81
GluN2C (NM_010350.2)	TTCGTGGTGTGTGGAATGGT ATGGTCCACCTGACTTCTTGC	327	This study
GluN2D (NM_008172.2)	CGATGGCGTCTGGAATGG AGATGAAAAGTGTGACGGCG	260	82
GluN3A (NM_001276355.1)	TCCCAACTCTCCGTTGACCT GTTGTCAGGATGGAGAGACCG	213	This study
GluN3B (NM_130455.2)	CTTCTGCCTGCTGGTGCTAT CTATGCCGTAGCCCTCGAT	374	This study
Grid1 (NM_008166.2)	ACCTCCTGGAATGGGATGAT CCTCAGGCTTCTTGATGAGG	154	83
Grid2 (NM_008167.2)	TTCACAAAGGATCCTGGAGCTG TGTTTATGGGGGCTGTCGTC	330	This study
GluR2 (NM_001083806.1)	AACGGCGTGAATCCTTGAC GCCAAACAATCTCCTGCATT	385	This study

1060

1061

1062 **Extended data Table 2. Antibody details**

Primary Antibody	Host species	Dilution /Use	Supplier	Catalog No
Monoclonal anti-Serine Racemase	Mouse	1:400-/ WB 1:200 / IF	Santa Cruz Bio	sc-365217
Polyclonal anti-Serine Racemase	Goat	1:200 / IF	Santa Cruz Bio	sc-5752
Polyclonal anti-D-Amino Acid Oxidase	Rabbit	1:1,000/ WB	Jumpei Sasabe	Ref 41
Polyclonal anti-D-Serine	Rabbit	1:3K-1:5K/IF	GemacBio	AP041
Polyclonal anti GluN1 C-ter	Goat	1:500 / WB	Santa Cruz Bio	sc-1467
Polyclonal GluN1 N-ter	Guinea Pig	1:500 / WB	Alomone	AGP-046
Polyclonal anti GluN2A	Rabbit	1:1,000 / WB	Millipore	AB1555P
Polyclonal GluN2B	Rabbit	1:750 / WB	Millipore	AB15362
Polyclonal anti GluN2C/2D	Rabbit	1:500	Alomone	AGC-018
Polyclonal anti-GluN3A	Rabbit	1:500 / WB	Millipore	07-356
Polyclonal anti GluN3B	Rabbit	1:500 / WB	Millipore	07-351
Polyclonal anti GluD1	Rabbit	1:10,000/WB	L Tricoire	Ref 84
Polyclonal anti GluD2 C-ter	Rabbit	1:500/WB	Frontier Inst Co Ltd	Rb-Af500
Polyclonal anti GluD2 N-ter	Rabbit	1:500 / WB	Frontier Inst Co Ltd	Rb-Af1460
Polyclonal anti GluA2	Rabbit	1:400/WB	Alomone	AGC-005
Polyclonal anti VGlut1	Rabbit	1:2,000/WB	Millipore	ABN1647
Monoclonal anti β -Actin	Mouse	1:10,000/WB	Sigma-Aldrich	A1978
Monoclonal anti GAPDH	Rabbit (14C10)	1:1,000 /WB	Cell signaling Tech	2118
Polyclonal anti-GFAP	Rabbit	1:1,000/IF	DAKO	Z0334
Monoclonal anti-S100 β	Mouse	1:1,000 / IF	Sigma-Aldrich	S2532
Monoclonal anti-2H3	Mouse	1:5 / IF	DSHB	2H3*
Monoclonal anti-Calbindin	Mouse	1:100 / IF	Sigma-Aldrich	G9848
Monoclonal anti-HUC/D	Mouse	1:20 / IF	Molecular Probes	A21271
Monoclonal anti-NeuN	Mouse	1:1,000 / IF	AbCys	VMA377
Monoclonal anti-NOS	Mouse	1:500 / IF	Sigma-Aldrich	N2280
Secondary Antibody (HRP)	Host species	Dilution /Use	Supplier	Catalog No
Polyclonal purified anti-rabbit	Goat	1:4,000/WB	Abliance	BI2407
Monoclonal purified anti-goat	Mouse	1:4,000/WB	Abliance	B12403
Polyclonal purified anti-mouse	Rabbit	1:4,000/WB	Abliance	BI2413L
Secondary Antibody (Fluo)	Host species	Dilution /Use	Supplier	Catalog No
Alexa Fluor 546 anti-rabbit	Goat	1:2,000/IF	Invitrogen	A11035
Alexa Fluor 488 anti-mouse	Donkey	1:1,000/IF	Invitrogen	A21202

1063 *2H3 was deposited to the DSHB by Jessell, T.M. / Dodd, J. (DSHB Hybridoma Product 2H3).











