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# Excitatory Neuronal Responses of Ca$^{2+}$ Transients in Interstitial Cells of Cajal in the Small Intestine

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1 **Excitatory neuronal responses of Ca$^{2+}$ transients in interstitial**

2 **cells of Cajal in the small intestine**

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13 **Abbreviated title:** Regulation of Ca$^{2+}$ transients in ICC by excitatory neurons

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47   **Abstract**

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49   Interstitial cells of Cajal (ICC) regulate smooth muscle excitability and motility in

50   the gastrointestinal tract. ICC in the deep muscular plexus (ICC-DMP) of the

51   small intestine are aligned closely with varicosities of enteric motor neurons and

52   thought to transduce neural responses. ICC-DMP generate $Ca^{2+}$ transients that

53   activate $Ca^{2+}$ activated $Cl^-$ channels and generate electrophysiological responses.

54   We tested the hypothesis that excitatory neurotransmitters regulate $Ca^{2+}$

55   transients in ICC-DMP as a means of regulating intestinal muscles.  High-

56   resolution confocal microscopy was used to image $Ca^{2+}$ transients in ICC-DMP

57   within murine small intestinal muscles with cell-specific expression of GCaMP3.

58   Intrinsic nerves were stimulated by electrical field stimulation (EFS).  ICC-DMP

59   exhibited ongoing $Ca^{2+}$ transients before stimuli were applied.  EFS caused initial

60   suppression of $Ca^{2+}$ transients, followed by escape during sustained stimulation,

61   and large increases in $Ca^{2+}$ transients after cessation of stimulation.  Basal $Ca^{2+}$

62   activity and the excitatory phases of $Ca^{2+}$ responses to EFS were inhibited by

63   atropine and neurokinin 1 receptor (NK1) antagonists, but not by NK2 receptor

64   antagonists. Exogenous ACh and substance P increased $Ca^{2+}$ transients,

65   atropine and NK1 antagonists decreased $Ca^{2+}$ transients. Neurokinins appear to

66   be released spontaneously (tonic excitation) in small intestinal muscles and are

67   the dominant excitatory neurotransmitters.  Subcellular regulation of $Ca^{2+}$ release

68   events in ICC-DMP may be a means by which excitatory neurotransmission

69   organizes intestinal motility patterns.

70 **Significance statement:**

71 Interstitial cells of Cajal (ICC) are innervated by enteric motor neurons and

72 thought to transduce neural responses in GI muscles.

73 $Ca^{2+}$ transients, due to $Ca^{2+}$ release from $Ca^{2+}$ intracellular stores, mediate

74 electrophysiological events in ICC by activation of $Ca^{2+}$-activated $Cl^-$ channels.

75 Neural responses in ICC in the deep muscular plexus of the small intestine (ICC-

76 DMP) were studied by confocal imaging of $Ca^{2+}$ transients in these cells.

77 Excitatory neural input was due to cholinergic and peptidergic neurotransmitters

78 (acetylcholine and neurokinins), as excitatory effects on $Ca^{2+}$ transients were

79 blocked by atropine and neurokinin receptor antagonists. Neurokinins are the

80 dominant excitatory regulators of $Ca^{2+}$ transients in ICC-DMP. ICC-DMP are

81 innervated by enteric motor neurons and mediate significant excitatory responses

82 in intestinal muscles.

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**Introduction**

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94      Muscles of the gastrointestinal (GI) tract are innervated by both excitatory

95   and inhibitory enteric motor neurons (Furness, 2012), and motility patterns of the

96   gut depend upon the outputs of the enteric nervous system.   Neural inputs are

97   overlaid upon the basal excitability of the smooth muscle cells (SMCs) that line

98   the walls of GI organs.  SMC excitability is determined by ionic conductances and

99   $Ca^{2+}$ sensitization mechanisms intrinsic to these cells but also by interstitial cells

100   that are electrically coupled to SMCs.  Together SMCs and interstitial cells (i.e.

101   interstitial cells of Cajal (ICC) and platelet derived growth factor receptor α-

102   immunopositive (PDGFRα^{+}) cells (Komuro, 2006; Sanders and Ward, 2006; Iino

103   et al., 2009; Blair et al., 2012; Baker et al., 2013) form an electrical syncytium,

104   known as the SIP syncytium (Sanders et al., 2012). It is the integrated output of

105   these cells that determines the basal excitability of GI smooth muscle tissues and

106   ultimately the responses to enteric motor neurons and other higher order

107   regulatory pathways (Sanders et al., 2014a).

108      In the small intestine, a network of ICC in the myenteric region (ICC-MY)

109   serves as the pacemaker cells that generate and actively propagate electrical

110   slow waves and organize contractile activity into a phasic pattern that underlies

111   segmental contractions (Langton et al., 1989; Ward et al., 1994; Ordog et al.,

112   1999; Sanders et al., 2014a).  Another class of ICC are distributed within smooth

113   muscle bundles in the deep muscular plexus (ICC-DMP; (Rumessen et al., 1992;

114   Zhou and Komuro, 1992)) throughout the smooth muscle organs of the GI tract.

115   ICC-DMP are innervated by motor neurons and transduce part of the input from

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116    enteric motor neurons (Wang et al., 2003b; Iino et al., 2004; Ward et al., 2006).

117    This conclusion is based on the fact that ICC-DMP are: i) closely apposed to

118    varicosities of enteric motor neurons, forming synaptic-like contacts (i.e. <20 nM;

119    (Rumessen et al., 1992; Zhou and Komuro, 1992); ii) express major receptors for

120    enteric motor neurotransmitters (Sternini et al., 1995; Vannucchi et al., 1997;

121    Chen et al., 2007); and iii) display evidence of receptor binding, receptor

122    internalization, and translocation of signaling molecules upon nerve stimulation

123    (Wang et al., 2003b; Iino et al., 2004). iv) electrically coupled to SMCs via gap

124    junctions (Daniel et al., 1998; Daniel and Wang, 1999; Seki and Komuro, 2001).

125    Experiments in other regions of the GI tract, where ICC-IM are lost in mutant

126    animals have shown distinct changes in motor neurotransmission in the absence

127    of ICC (Daniel and Posey-Daniel, 1984; Burns et al., 1996; Komuro et al., 1999;

128    Ward et al., 2000; Klein et al., 2013).  Nevertheless, there is controversy about

129    the importance of ICC in neurotransmission, and some investigators have argued

130    that ICC are not important elements in enteric nerve responses (Goyal and

131    Chaudhury, 2010; Goyal, 2016).

132         A fundamental mechanism involved in the activation of ICC (as

133    pacemakers and in regulating the excitability of GI muscles) is $Ca^{2+}$ release from

134    intracellular stores (van Helden and Imtiaz, 2003; Lee et al., 2007; Baker et al.,

135    2016; Drumm et al., 2017).  $Ca^{2+}$ release is important because it activates $Ca^{2+}$-

136    activated $Cl^-$ channels (CaCC), encoded by *Ano1*, that are strongly expressed in

137    ICC (Chen et al., 2007; Gomez-Pinilla et al., 2009; Zhu et al., 2015). We have

138    used mice expressing $Ca^{2+}$ sensors specifically in ICC to investigate the $Ca^{2+}$

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139    transients generated by ICC in intact intestinal muscles (Baker et al., 2016;

140    Drumm et al., 2017).

141         Excitatory transmission in the gut is mediated predominantly via

142    cholinergic and neurokinins. Tachykinin (TKs) family of peptides (substance P

143    (SP), neurokinin A (NKA) and B (NKB) are expressed throughout the

144    gastrointestinal tract (Holzer and Holzer-Petsche, 2001; Cipriani et al., 2011;

145    Mitsui, 2011; Steinhoff et al., 2014). SP, NKA and NKB are preferentially

146    mediated through the stimulation of NK1, NK2 and NK3 G-protein-coupled

147    receptors. Both NK1 and NK2 receptors mediate contractile effects in the

148    gut. small intestine, smooth muscle electrical and motor events induced by

149    electrical field stimulation (EFS) can involve either or both NK1 and NK2

150    receptors. But functional evidence support the involvement of NK1 subtype in

151    mediating NANC contractions to EFS in the mouse small intestine (lino et al.,

152    2004; De Schepper et al., 2005).

153         In the present study we investigated the hypothesis that a major

154    mechanism by which enteric motor neurotransmitters regulate ICC is through

155    modulation of $Ca^{2+}$ release events.  To test this hypothesis, we explored whether

156    excitatory neural inputs to ICC-DMP are coupled to $Ca^{2+}$ release and

157    characterized the nature of the $Ca^{2+}$ responses that constitute this transduction

158    pathway for post-junctional excitatory transmission.

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160 **Methods**

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162 **Animals**

163       GCaMP3-floxed mice (B6.129S-*Gt(ROSA)26Sor^{tm38(CAG-GCaMP3)Hze}*/J) and

164 their wild-type siblings (C57BL/6) were acquired  from Jackson Laboratories (Bar

165 Harbor, MN, USA) and crossed with Kit-Cre mice (c-Kit$^{+/Cre-ERT2}$), provided by Dr.

166 Dieter Saur (Technical University Munich, Munich, Germany).  Kit-Cre-GCaMP3

167 mice (both sexes) were injected with tamoxifen at 6-8 weeks of age (2 mg for

168 three consecutive days), as previously described (Baker et al., 2016) to activate

169 Cre recombinase and GCaMP3 expression. 15 days after tamoxifen injection,

170 Kit-Cre-GCaMP3 mice were anaesthetized by isoflurane inhalation (Baxter,

171 Deerfield, IL, USA) and killed by cervical dislocation.  All animals used for these

172 experiments were handled in compliance with the National Institutes of Health

173 Guide for the Care and Use of Laboratory Animals, and the protocols were

174 approved by the Institutional Animal Use and Care Committee at the University of

175 Nevada, Reno.

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177 **Tissue preparation**

178       Segments of jejunum (2 cm in length) were removed from mice and

179 bathed in Krebs-Ringer bicarbonate solution (KRB). Jejunal segments were

180 opened along the mesenteric border and luminal contents were washed away

181 with KRB. The mucosa and sub-mucosa layers were removed by sharp

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182    dissection, and the remaining *tunica muscularis* was pinned flat within a Sylgard

183    coated dish.

**Drugs and Solutions**

185        Tissues were maintained and perfused with KRB containing (mmol/L):

186    NaCl, 120.35; KCl, 5.9; NaHCO$_3$, 15.5; NaH$_2$PO$_4$, 1.2; MgCl$_2$, 1.2; CaCl$_2$, 2.5;

187    and glucose, 11.5.  The KRB was bubbled with a mixture of 97% O$_2$ – 3 %CO$_2$

188    and warmed to 37 ± 0.2 °C.

189        All drugs were purchased from Tocris Bioscience (Ellisville, Missouri,

190    USA) and dissolved in the solvents recommended by the manufacturer to obtain

191    stock solutions. Final concentrations used in experiments were obtained by

192    dilution into KRB.

193

**Fluorescence Activated Cell Sorting (FACS), RNA extraction and**

**quantitative PCR**

196        Jejunal ICC were dispersed from Kit$^{+/copGFP}$ mice as previously described

197    (Zhu et al., 2009; Zhu et al., 2011). ICC were sorted and purified by FACS

198    (FACSAria II; Becton-Dickinson) using an excitation laser (488 nm) and emission

199    filter (530/30 nm). Sorting was performed using a 130-µm nozzle and a sheath

200    pressure of 12 psi.

201        RNA was prepared from sorted ICC and dispersed jejunal cells of the

202    *tunica muscularis* before sorting using an illustra RNAspin Mini RNA Isolation Kit

203    (GE Healthcare). The PCR primers used and their GenBank accession numbers

204    are provided in Table 1. Quantitative PCR (qPCR) was performed using SYBR

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205    green chemistry on the 7500 HT Real-time PCR System (Applied Biosystems)

206    and analyzed, as previously described (Baker et al., 2016). All data sets were

207    normalized to the housekeeping gene *Gapdh*.

208

209    **Calcium imaging**

210       Jejunal muscle sheets (5.0 x 10.0 mm) were pinned to the base of a 5ml,

211    60 mm diameter Sylgard-coated dish. The muscles were perfused with warmed

212    KRB solution at 37°C for an equilibration period of 1 hour. Fluorescence imaging

213    was performed with a spinning-disk confocal microscope (CSU-W1 spinning disk;

214    Yokogawa Electric Corporation) mounted to an upright Nikon Eclipse FN1

215    microscope equipped with a 60x 1.0 NA CFI Fluor lens (Nikon instruments INC,

216    NY, USA). GCaMP3, expressed solely in ICC, was excited at 488 nm using a

217    laser coupled to a Borealis system (ANDOR Technology, Belfast, UK). Emitted

218    fluorescence (>515 nm) was captured using a high-speed EMCCD Camera

219    (Andor iXon Ultra; ANDOR Technology, Belfast, UK). Image sequences were

220    collected at 33 fps using MetaMorph software (Molecular Devices INC, CA, USA).

221    Additional $Ca^{2+}$ imaging data was acquired with an Eclipse E600FN microscope

222    (Nikon Inc., Melville, NY, USA) equipped with a 60x 1.0 CFI Fluor lens (Nikon

223    instruments INC, NY, USA). In this system, GCaMP3 was excited at 488 nm

224    (T.I.L.L. Polychrome IV, Grafelfing, Germany), as previously described (Baker *et*

225    *al.*, 2013). All $Ca^{2+}$ imaging experiments were performed in the presence of

226    nicardipine (100 nM) to minimize contractile movements.

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**Calcium event analysis**

230    Analysis of $Ca^{2+}$ activity in ICC-DMP was performed, as described

231    previously (Baker et al., 2016). Briefly, movies of $Ca^{2+}$ activity in ICC-DMP were

232    converted to a stack of TIFF (tagged image file format) images and imported into

233    custom software (Volumetry G8c, GW Hennig) for analysis. Tissue movement

234    was stabilized to ensure accurate measurement of $Ca^{2+}$ transients from ICC-

235    DMP. Whole cell ROIs were used to generate spatio-temporal (ST) maps of $Ca^{2+}$

236    activity in individual ICC-DMP. ST maps were then imported as TIFF files into

237    Image    J    (version1.40,    National    Institutes    of    Health,    MD,    USA,

238    http://rsbweb.nih.gov/ij) for post hoc quantification analysis of $Ca^{2+}$ events.

239

**Experimental design and statistical analysis**

241    $Ca^{2+}$ event frequency in ICC-DMP was expressed as the number of

242    events fired per cell per second ($sec^{-1}$). $Ca^{2+}$ event amplitude was expressed as

243    $\Delta F/F_0$, the duration of $Ca^{2+}$ events was expressed as full duration at half

244    maximum amplitude (FDHM), and $Ca^{2+}$ event spatial spread was expressed as

245    $\mu m$ of cell propagated per $Ca^{2+}$ event. Unless otherwise stated, data is

246    represented as mean ± standard error (S.E.M.). Statistical analysis was

247    performed using either a student's *t*-test or with an ANOVA with a Dunnett post

248    hoc test where appropriate. In all statistical analyses, $P<0.05$ was taken as

249    significant. P values  <0.05 are represented by a single asterisk (*), P values

250    <0.01 are represented by two asterisks (**) and P values $<0.001$ are represented

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251    by three asterisks (***). When describing data throughout the text, n refers to the

252    number of animals used in that dataset while c refers to the numbers of cells

253    used in that same data set.

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257 **Results**

258 ***Post-junctional modulation of Ca$^{2+}$ signaling in ICC-DMP by enteric nerve***

259 ***stimulation***

260     ICC-DMP displayed intracellular Ca$^{2+}$ transients that fired in a stochastic

261 manner (Figure 1), as reported previously (Baker et al., 2016). Ca$^{2+}$ transients

262 were generated at multiple sites along the length of individual ICC-DMP and

263 were typically localized, demonstrating no mechanism for active or regenerative

264 propagation of these events within individual cells or between cells and no

265 extrinsic mechanism of entrainment, as has been previously suggested (Huizinga

266 et al., 2014). Ca$^{2+}$ transients in ICC-DMP exhibit a range of frequencies,

267 amplitudes, durations and spatial spread (Baker et al., 2016). ICC are thought to

268 be intermediaries in enteric neurotransmission, relaying signals from enteric

269 neurons to smooth muscle cells, that are electrically coupled to ICC (Daniel et al.,

270 1998; Daniel and Wang, 1999).  Therefore, we investigated how Ca$^{2+}$ transients

271 are modulated by enteric neurons activated by electric field stimulation (EFS).

272     EFS (10 Hz, 0.5ms for 5 sec trains) caused two distinct Ca$^{2+}$ responses: i)

273 an initial inhibitory phase; ii) an excitatory response that occurred largely after

274 cessation of EFS (movie 1). The initial inhibitory response at the onset of EFS

275 lasted about  ~ 2 sec.  During this phase Ca$^{2+}$ transients in ICC-DMP ceased

276 (Figure 1 A-C). In the final 3 sec of EFS Ca$^{2+}$ transients escaped from inhibition

277 leading to an excitatory response that persisted into the period after cessation of

278 the stimulus (Figure 1 A). These effects are illustrated by an ST map and Ca$^{2+}$

279 activity traces in Figure 1 B&C. This example demonstrates that in the final 3 sec

13

280    of EFS and particularly in the 5 sec after cessation of EFS, $Ca^{2+}$ transients were

281    increased relative to the control period, and firing sites within ICC-DMP increased

282    their firing frequency.  We also found that the initiation sites for $Ca^{2+}$ transients

283    varied temporally in response to EFS (Figure 1B). These responses were

284    mediated by neuronal inputs, as they were blocked by tetrodotoxin (TTX, 1 $\mu$M,

285    data not shown). As above, after the onset of EFS, an inhibitory response phase

286    occurred, but in subsequent experiments we concentrated on the excitatory

287    aspects of the neural responses.

288        The excitatory $Ca^{2+}$ response to EFS was quantified during the final 3 sec

289    of EFS (Figure 2 A, blue dashed box) and in the 5 sec immediately following EFS

290    (Post EFS, Figure 2 A, green dashed box). In the pre EFS period, the control

291    frequency of $Ca^{2+}$ transients was 1.04 ± 0.08 events $sec^{-1}$, and this was

292    increased significantly during the final 3 sec period of EFS to 1.8 ± 0.15 events

293    $sec^{-1}$ (Figure 2 B, P<0.0001, n=23, c=56). The frequency of $Ca^{2+}$ transients in the

294    post EFS period was also significantly increased from control, firing on average

295    at 2.1 ± 0.1 events $sec^{-1}$ (Figure 2 B, P<0.0001, n=23, c=56). There was a

296    significant increase in $Ca^{2+}$ transient amplitude in the final 3 sec of EFS from 0.8

297    ± 0.06 to 1.1 ± 0.05 $\Delta F/F_0$ (Figure 2 C, P<0.05, n=23, c=56), although there was

298    no significant increase in amplitude in the post EFS period compared to control

299    (Figure 2 C, P>0.05, n=23, c=56). $Ca^{2+}$ transient duration increased in the final 3

300    sec of EFS from 193 ± 3.7 to 219.6 ± 7.9 ms (Figure 2 D, P<0.01, n=23, c=56)

301    and was also significantly increased in the post EFS period, increasing to 222 ±

302    6.5 ms (Figure 2 D, P<0.001, n=23, c=56). $Ca^{2+}$ transient propagation spread

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303     was also increased in the final 3 sec of EFS from 11 ± 0.6 to 15.4 ± 0.9 $\mu$m

304     (Figure 2 E, P<0.001, n=23, c=56) and was also significantly increased, as

305     compared to control, in the post EFS period, with $Ca^{2+}$ transients propagating an

306     average of 12.9 ± 0.6 $\mu$m (Figure 2 E, P<0.05, n=23, c=56).  The number of $Ca^{2+}$

307     firing sites in ICC-DMP was decreased significantly during the final 3 sec of EFS

308     (P<0.001) and during the post-EFS period (P<0.001) (Figure 2 F, n=23, c=56).

309     This is likely a result of the increased propagation spread of $Ca^{2+}$ transients

310     during these periods, as shown in Figure 2 E. As the frequency of $Ca^{2+}$ transients

311     increased and they propagated over longer distances, individual firing sites may

312     summate to create the increase in propagation distances observed during the

313     final seconds of EFS and post EFS.  This could lead to an apparent reduction in

314     firing sites, as the underlying sites were masked by propagating $Ca^{2+}$ waves. A

315     small increase in $Ca^{2+}$ transient propagation velocity, that did not reach

316     significance, was also observed during the final 3 sec of EFS and during the

317     post-EFS period (P<0.05, Figure 2 G, n=23, c=56).

318

319     ***EFS evoked frequency-dependent excitatory $Ca^{2+}$ responses in ICC-DMP***

320         We examined whether the $Ca^{2+}$ responses in ICC-DMP were dependent

321     upon the frequency of EFS.  EFS was applied to muscles from 1-20 Hz (0.5ms, 5

322     sec trains).  No changes in $Ca^{2+}$ transient parameters were resolved during 1Hz

323     stimulation (Figure 3 A, n=5, c=16), although a significant increase in the

324     frequency of $Ca^{2+}$ transients occurred in the post-EFS period (Figure 3 A, P<0.05,

325     n=5, c=16).  Higher EFS frequencies (5, 10 and 20 Hz) increased $Ca^{2+}$ transients

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326    significantly during EFS (final 3 sec) and during the post stimulus period (Figure

327    3 B&C). For example, 5 Hz EFS increased the firing frequency (final 3 sec) to 2.5

328    ± 0.6 events $sec^{-1}$, which was significantly greater than control values of 1.6 ± 0.4

329    events $sec^{-1}$ (P<0.05, n=5, c=16).  EFS 5 Hz also increased $Ca^{2+}$ transient

330    frequency during the post- EFS period to 2.5 ± 0.4 events $sec^{-1}$, as compared to

331    1.6 ± 0.4 events $sec^{-1}$ in control (P<0.01, n=5, c=16). During EFS, the amplitude

332    and duration of $Ca^{2+}$ transients were not significantly changed at all frequencies

333    tested (P>0.05).  However, $Ca^{2+}$ transient duration increased during the post-

334    EFS period at 5 Hz from 198 ± 10 ms to 228 ± 13.1 ms (P<0.05, n=5, c=16).  The

335    spread of $Ca^{2+}$ transients was not significantly affected by 1 Hz EFS, but

336    increased significantly at 5 Hz during EFS (final 3 sec; increased from 10.1 ± 0.9

337    to 18.6 ± 2.6 $\mu$m (P<0.05, n=5, c=16).  At 20 Hz the spatial spread increased

338    from 8.5 ± 0.6 to 11.9 ± 1.2 $\mu$m during EFS (P<0.05, n=5, c=14). The change in

339    firing frequency (% change) for each stimulus 1, 5 10 and 20 Hz was calculated

340    and plotted in Figure 3 D&E during EFS (final 3 sec, Figure 3 D) and after the

341    stimulus period (Figure 3 E).  The firing of $Ca^{2+}$ transients was dependent upon

342    the stimulus frequency during both periods (Figure 3 D&E).

343

344    **Expression of excitatory cholinergic and neurokinin receptors in ICC**

345        Excitatory neurotransmitters mediate responses by binding to specific post

346    junctional receptors.  In the case of excitatory enteric neurotransmission,

347    responses have been attributed to muscarinic (M2 and M3) receptors and

348    neurokinin (NK1 and NK2) receptors expressed in small intestinal muscles (Lavin

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349    et al., 1998; Stadelmann et al., 1998; Iino et al., 2004; Faussone-Pellegrini and

350    Vannucchi, 2006). In this study we sorted ICC (CopGFP-Kit$^+$ cells) from small

351    intestinal muscles of Kit$^{+/copGFP}$ mice by FACS from, as previously described

352    (Baker et al., 2016), and characterized the expression of *Chrm2* and *Chrm3*

353    transcripts and *Tacr1* and *Tacr2* transcripts. We noted higher expression of

354    *Chrm3* in ICC in comparison to *Chrm2* normalized to the housekeeping gene

355    *Gapdh* (*Chrm3:* 0.043 ± 0.001; *Chrm2:* 0.029 ± 0.002, P= 0.001, n=4).  *Chrm3*

356    transcripts were also higher in ICC relative to unsorted cells (total cell population).

357    *Tacr1* was also highly expressed in ICC (*Tacr1:* 0.06 ± 0.01, n=4), and

358    expression of *Tacr2* was not resolved in ICC. Thus, the dominant receptor

359    transcripts in ICC were *Chrm3* and *Tacr1*.

360

361    ***Cholinergic regulation of Ca$^{2+}$ transients in ICC-DMP***

362         Atropine (1 $\mu$M) decreased the frequency of basal Ca$^{2+}$ transients from 1.9

363    ± 0.31 events sec$^{-1}$ to 1.2 ± 0.2 events sec$^{-1}$ (Figure 4 A&B, P=0.0005, n=5,

364    c=13).  No significant effects on the other parameters of Ca$^{2+}$ transients were

365    noted: amplitude (P=0.39), duration (P=0.83) or spatial spread (P=0.53; Figure 4

366    B-E; n=5, c=13). When cholinergic stimulation was initiated by exogenous

367    acetylcholine (ACh, 10 $\mu$M; in the presence of TTX, 1 $\mu$M), Ca$^{2+}$ transients

368    increased markedly. ACh increased the frequency of Ca$^{2+}$ transients from 0.85 ±

369    0.2 events sec$^{-1}$ to 1.85 ± 0.4 events sec$^{-1}$ (Figure 4 F&G, P=0.003, n=5, c=9),

370    and Ca$^{2+}$ transient amplitude increased from 0.3 ± 0.04 to 0.6 ± 0.1 $\Delta$F/F$_0$ (Figure

371    4 H, P=0.042, n=5, c=9).  ACh increased the duration of Ca$^{2+}$ transients from 240

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372  ± 16.1 ms to 296 ± 15.3 ms (Figure 4 I, P=0.008, n=5, c=9). The spatial spread of

373  $Ca^{2+}$ transients also increased in response to ACh, sometimes leading to

374  propagating $Ca^{2+}$ waves in contrast to more spatially limited events.  Spatial

375  spread increased from 7.1 ± 0.7 to 11 ± 1.3 $\mu$m (Figure 4 F&J, P=0.017, n=5,

376  c=9).

377

378  ***The effects of atropine on EFS evoked excitatory $Ca^{2+}$ response in ICC-DMP***

379  Next we investigated the extent of regulation exerted by cholinergic

380  neurotransmission on $Ca^{2+}$ transients in ICC-DMP.  EFS (10Hz, for 5 sec) in the

381  presence of atropine (1 $\mu$M) resulted in a decrease in the frequency of $Ca^{2+}$

382  transients during stimulation (final 3 sec), from 1.7 ± 0.4 to 0.9 ± 0.2 events $sec^{-1}$

383  (Figure 5 A-C, P=0.042, n=5, c=21).   Atropine also decreased the $Ca^{2+}$ transient

384  frequency during the post stimulus period, from 2.4 ± 0.3 to 1.6 ± 0.2 events $sec^{-1}$

385  (Figure 5 A-C, P=0.037, n=5, c=21). This suggests that cholinergic

386  neurotransmission can also affect the post-stimulus excitatory period in ICC-DMP.

387  There were no significant changes in $Ca^{2+}$ transient amplitude (Figure 5 D,

388  P=0.46, P=0.19), duration (Figure 5 E, P=0.63, P=0.42) or spatial spread (Figure

389  5 F, P=0.44, P=0.56) during either the final 3 sec of EFS or during the post-

390  stimulus period in the presence of atropine (n=5, c=21).

391

392  ***Neurokinins control over basal $Ca^{2+}$ signaling in ICC-DMP***

393  NK1 receptors are the major neurokinin receptors expressed in ICC, and

394  results from this study confirmed previous reports (Sternini et al., 1995; Iino et al.,

18

395    2004). Therefore, contributions of neurokinins to EFS responses in ICC-DMP

396    were first evaluated with neurokinin 1 (NK1) receptor antagonists.  RP 67580 (1

397    $\mu$M) dramatically reduced basal $Ca^{2+}$ transients in ICC-DMP from 1.2 ± 0.2 to 0.5

398    ± 0.1 events $sec^{-1}$ (Figure 6 A&B, P=0.0003, n=11, c=27). The amplitude

399    (P=0.0039), duration (P=0.002) and spatial spread (P=0.005) of $Ca^{2+}$ transients

400    were also significantly depressed by RP 67580 (Figure 6 C-E n=11, c=27).  SR

401    140333 (1 $\mu$M), another NK1 receptor antagonist, also inhibited basal $Ca^{2+}$

402    transients in ICC-DMP, reducing frequency from 0.9 ± 0.1 to 0.4 ± 0.06 events

403    $sec^{-1}$ (Figure 6 F-J, P=0.006, n=4, c=14).  Amplitudes (P=0.042) and spatial

404    spread (P=0.003) of $Ca^{2+}$ transients were also significantly decreased by SR

405    140333 (Figure 6 H&J, n=4, c=14).  A selective NK2 receptor antagonist, MEN

406    10376 (1 $\mu$M), had no effect on $Ca^{2+}$ transients in ICC-DMP (i.e. frequency

407    (P=0.081), amplitude (P=0.67), duration (P=0.24) or spatial spread (P=0.21),

408    (n=5, c=9, data not shown).

409            After inhibition of $Ca^{2+}$ transients with RP 67580 (Figure 7 A&B), carbachol

410    (CCh; 10 $\mu$M) persisted in enhancing $Ca^{2+}$ transient firing frequency from 0.6 ±

411    0.2 to 2.4 ± 0.5 events $sec^{-1}$ (Figure 7 C&D, P=0.018, n=3, c=6).  The duration of

412    $Ca^{2+}$ transients was increased by CCh from 183.4 ± 37.4 to 308.3 ± 18.9 ms

413    (Figure 7 F, P=0.035, n=3, c=6) and the spatial spread of $Ca^{2+}$ transients was

414    also increased from 6.2 ± 1.3 to 12.1 ± 1.3 $\mu$m (Figure 7 G, P=0.016, n=3, c=6).

415    CCh also increased $Ca^{2+}$ transients after treatment with SR 140333 (data not

416    shown).  These results show that the effects of the NK1 antagonists were not due

417    to off-target effects, such as inhibition of $IP_3$-dependent signaling or $Ca^{2+}$ release

418    from intracellular stores.

419            The observations above suggest that $Ca^{2+}$ signaling in ICC-DMP can be

420    modulated by neurokinins via NK1, but not NK2 receptors. Regulation by

421    neurokinins was further tested by application of NK1 agonists.  Substance P (1

422    $\mu$M, in the presence of TTX) increased $Ca^{2+}$ transients significantly (Figure 8 A-

423    E); frequency increased from 1.2 ± 0.3 to 2 ± 0.3 events $sec^{-1}$ (Figure 8 B,

424    P=0.042, n=4, c=10), duration increased from 207 ± 15.9 to 342 ± 21.8 ms

425    (Figure 8 D, P<0.0001, n=4, c=10) and spatial spread increased from 7 ± 0.6 to

426    10.9 ± 1.2 $\mu$m (Figure 8 E, P=0.0199, n=4, c=10). A more selective NK1 agonist,

427    GR 73632 (1 $\mu$M, in the presence of TTX) also increased the frequency of $Ca^{2+}$

428    transients from 0.3 ± 0.03 to 1.2 ± 0.2 events $sec^{-1}$ (Figure 8 F&G, P=0.0014, n=4,

429    c=9), but effects on amplitude (P=0.92), duration (P=0.78) or spatial spread

430    (P=0.42) were not changed significantly (Figure 8 H-J, n=4, c=9). These data

431    suggest that neurokinins are released tonically in small intestinal muscles, and

432    responses of ICC-DMP to neurokinins are mediated largely by NK1 receptors.

433

434    ***The effects of RP 67580 on $Ca^{2+}$ responses evoked by EFS in ICC-DMP***

435            We also tested whether NK1 receptors mediate $Ca^{2+}$ responses in ICC-

436    DMP evoked by EFS.  RP 67580 (1 $\mu$M; Figure 9 A&B) caused a dramatic

437    decrease in the $Ca^{2+}$ responses to EFS (Figure 9 C-F). The frequency of $Ca^{2+}$

438    transients during the final 3 sec of EFS period was reduced from 1.5 ± 0.3 to 0.2

439    ± 0.17 events $sec^{-1}$ (Figure 9 C, P=0.0015, n=4, c=11). During the post EFS

20

440  period, the frequency of $Ca^{2+}$ transients was also significantly reduced from 1.8 ±

441  0.3 to 0.1 ± 0.05 events $sec^{-1}$ (Figure 9 C, P=0.002, n=4, c=11).  $Ca^{2+}$ transient

442  amplitude, duration and spatial spread during the final 3 sec EFS and post EFS

443  periods were also inhibited (Figure 9 D-F, n=4, c=11).

444      When cholinergic and NK1 receptors were both antagonized by adding

445  both  atropine (1 $\mu$M) and RP 67580 (1 $\mu$M), pronounced inhibition of $Ca^{2+}$

446  transients persisted during the final 3 sec of EFS and during the post stimulus

447  period, as shown in Figure 10 A-F (n= 4, c=20).

448

449  ***Cholinergic and neurokinin mediated excitatory responses after blocking***

450  ***nitrergic and purinergic transmission***

451      Nitrergic and purinergic antagonists N-omega-nitro-L-arginine (L-NNA,

452  100 $\mu$M) and MRS 2500 (1 $\mu$M) were used to examine excitatory neural

453  regulation of $Ca^{2+}$ transients in ICC-DMP after blocking major inhibitory pathway

454  of neurotransmission. In the presence of L-NNA, MRS 2500 and atropine (1 $\mu$M)

455  $Ca^{2+}$ transient frequency (Figure 11 C, P=0.29, n=7, c=26), amplitude (Figure 11

456  D, P=0.57, n=7, c=26) and spatial spread (Figure 11 F, P=0.3, n=7, c=26) in the

457  final 3 sec period were not significantly affected.  $Ca^{2+}$ transient duration

458  decreased significantly from 266 ± 14.15 to 199 ± 18.9 ms (Figure 11 E, P=0.007,

459  n=7, c=26) in the final 3 sec period.  In the presence of L-NNA, MRS 2500 and

460  atropine, $Ca^{2+}$ transients in the post-stimulus period were not reduced in

461  amplitude (Figure 11 D, P=0.64, n=7, c=26) or spatial spread (Figure 11 F,

462  P=0.088, n=7, c=26). However, the frequency of $Ca^{2+}$ transients was reduced

21

463  during the post-stimulus period from 2.6 ± 0.25 to 1.9 ± 0.2 events sec$^{-1}$ (Figure

464  11 C, P=0.03, n=7, c=26) and duration decreased from 237 ± 11.6 to 177 ± 11.9

465  ms (Figure 11 E, P=0.0008, n=7, c=26).

466      We next examined the neurokinin input into EFS-mediated excitatory

467  responses in ICC-DMP in the presence of blockers of nitrergic and purinergic

468  neurotransmission. With L-NNA, MRS 2500 and RP 67580 present, responses to

469  EFS were significantly reduced in amplitude, duration and spatial spread of Ca$^{2+}$

470  transients during the final 3 sec of EFS (Figure 12 D-F, n=4, c=13). The

471  amplitude decreased from 1.4 ± 0.2 to 0.42 ± 0.8 $\Delta F/F_0$ (P=0.002, Figure 12 D,

472  n=4, c=13), the duration decreased from 214.4 ± 16.9 to 122 ± 22 ms (P=0.004,

473  Figure 12 E, n=4, c=13) and the spatial spread decreased from 15.4 ± 3.2 to 5.8

474  ± 1 $\mu$m (P=0.029, Figure 12 F, n=4, c=13). Overall the frequency of Ca$^{2+}$

475  transients in the final 3 sec of the EFS period was not significantly affected

476  (Figure 12 A-C, P=0.21, n=4, c=13).  In the presence of L-NNA, MRS 2500 and

477  RP 67580, the frequency of Ca$^{2+}$ transients in the post-EFS period was

478  significantly reduced from 1.8 ± 0.3 to 0.4 ± 0.1 events sec$^{-1}$ (Figure 12 C,

479  P=0.006, n=4, c=13). The amplitude of Ca$^{2+}$ transients during this period was not

480  significantly affected (Figure 12 D, P=0.059, n=4, c=13), but the duration of Ca$^{2+}$

481  transients was reduced from 237 ± 23.9 to 121 ± 19.7 ms (Figure 12 E, P=0.0029,

482  n=4, c=13), and spatial spread decreased from 12.61 ± 1.6 to 6.8 ±1.2 $\mu$m

483  (Figure 12 F, P=0.014, n=4, c=13).

484      Next we inhibited cholinergic and neurokinin transmission with atropine

485  and RP 67580 in the presence of L-NNA and MRS 2500. Under these conditions

22

486    all Ca$^{2+}$ transients were significantly diminished across all parameters tabulated,

487    as shown in (Fig.13 A-F n=5, c=32).

488

489

490 **Discussion**

491       Innervation of GI muscles by enteric motor nerves and the integrated firing

492 of these neurons is essential for generating archetypal motility patterns (Spencer

493 et al., 2016).  ICC are innervated by enteric motor neurons, and their responses

494 to neurotransmitters contribute to complex post-junctional responses of the SIP

495 syncytium (Ward et al., 2000; Iino et al., 2004).  In the case of the small intestine

496 ICC-DMP are an intramuscular type of ICC that are closely associated with and

497 innervated by motor neurons (Rumessen *et al.*, 1992; Zhou & Komuro, 1992;

498 Wang *et al.*, 2003b; Iino *et al.*, 2004; Ward *et al.*, 2006).  We recently described

499 the properties of spontaneous $Ca^{2+}$ transients that occur in the absence of

500 extrinsic stimuli in these cells (Baker et al., 2016).  In the present study we

501 investigated the effects of excitatory enteric motor neurotransmission on $Ca^{2+}$

502 transients in ICC-DMP, because these events mediate activation of CaCC, the

503 ion channels responsible for the electrophysiological post-junctional excitatory

504 responses to nerve stimulation in small intestinal muscles (Zhu et al., 2011). EFS

505 of intrinsic neurons resulted in three-component effects on $Ca^{2+}$ transients: a

506 brief inhibitory period (about 2s), a period of escape from inhibition during

507 sustained EFS, and a period of strong excitation after cessation of the stimuli

508 (post-stimulus or 'rebound' excitation).  The complexity of these responses is

509 likely due to the fact that the enteric nervous system contains both inhibitory and

510 excitatory motor neurons (Furness, 2012), and EFS can be expected to activate

511 both classes of neurons.

24

512        In the mouse small intestine, the neurokinin component of the excitatory

513    neural inputs to ICC-DMP was dominant. Our experiments also suggest that

514    tonic release of neurokinins and binding to NK1 receptors is responsible for

515    significant drive in generating the $Ca^{2+}$ transients observed under basal

516    conditions in ICC-DMP (Baker et al., 2016).  Thus, the $Ca^{2+}$ transients observed

517    in the absence of applied stimuli are not 'spontaneous' and do not appear to be

518    driven intrinsically within ICC-DMP.  Excitatory neurotransmitters greatly

519    increased $Ca^{2+}$ transients in ICC-DMP, and this mechanism likely underlies a

520    portion of the post-junctional electrophysiological response to excitatory neural

521    regulation (Zhu et al., 2011; Zhu et al., 2015).

522        ICC-DMP are plentiful and in close contact with varicosities of enteric

523    motor neurons in the deep muscular plexus region of the small intestine

524    (Rumessen et al., 1992; Zhou and Komuro, 1992).  We confirmed that ICC

525    express receptors required for excitatory motor neurotransmission (e.g.

526    muscarinic and neurokinin receptors), and transcripts for M3 (*Chrm3*) and NK1

527    (*Tacr1*) were enriched in ICC-DMP vs. unsorted cells.  However, transcripts of

528    *Chrm2* were also present, suggesting these receptors and coupling to effectors

529    via Gi/Go may also have a role in transduction or modulation of excitatory

530    neurotransmission.  Our findings are consistent with previous studies showing

531    muscarinic receptors and NK1 receptor expression in ICC with

532    immunohistochemical techniques (Sternini et al., 1995; Vannucchi et al., 1997;

533    Stadelmann et al., 1998; Iino et al., 2004; Iino and Nojyo, 2006; Ward et al.,

534    2006; Sanders et al., 2014b).

535    This study demonstrates that ICC-DMP receive and transduce excitatory

536    neural inputs in the small bowel.  Previous studies predicted this finding from

537    morphological observations (Rumessen et al., 1992; Zhou and Komuro, 1992;

538    Wang et al., 2003a; Iino et al., 2004; Faussone-Pellegrini, 2006; Shimizu et al.,

539    2008) and by showing that cholinergic excitatory neural responses develop in

540    phase with the development of ICC-DMP and blocking Kit receptors causes

541    parallel loss of ICC and cholinergic neural responses (Ward et al., 2006).

542    Excitatory neurotransmission caused PKCε translocation in ICC-DMP that was

543    blocked by atropine (Wang et al., 2003b), demonstrating functional cholinergic

544    innervation and muscarinic responses in these cells.  ACh binding to M3

545    receptors can enhance $Ca^{2+}$ release in ICC-DMP via generation of inositol 1,4,5-

546    trisphosphate ($IP_3$) which activates $Ca^{2+}$ release from the endoplasmic reticulum

547    (ER).  All of the molecular components of this pathway are expressed in ICC, as

548    shown by transcriptome analyses (Chen et al., 2007; Lee et al., 2017). Previous

549    direct observation of ICC-DMP *in situ* has shown that $Ca^{2+}$ transients are due to

550    $Ca^{2+}$ release from intracellular stores (e.g. ER), mediated, in part, by $IP_3R$ (Baker

551    et al., 2016).  Increasing $Ca^{2+}$ release in ICC leads to activation of CaCC, and the

552    inward current generated by thousands of ICC-DMP in whole muscles would

553    provide a net depolarizing influence that would summate with slow wave

554    depolarizations, increase the likelihood of action potentials being generated

555    during the plateau phase of slow waves (i.e. period of peak depolarization), and

556    enhance the amplitude of phasic contractions (Zhu et al., 2011).

26

557    While our observations suggest innervation and contributions from

558    cholinergic nerves to post-junctional excitatory responses, our data also suggest

559    that neurokinins are the dominant excitatory neurotransmitters affecting $Ca^{2+}$

560    transients in ICC-DMP in the mouse small intestine.  ICC-DMP are closely

561    associated with substance P containing nerve fibers, and ICC-DMP express NK1

562    receptors (Iino et al., 2004; Faussone-Pellegrini, 2006; Shimizu et al., 2008)

563    which is consistent with our observation that excitatory transmission to ICC-DMP

564    was mediated through NK1 receptors.  Previous studies have shown that

565    exposure of small intestinal muscles to substance P or stimulation of motor

566    neurons causes internalization of NK1 receptors in ICC (Lavin et al., 1998; Iino et

567    al., 2004).  Our experiments showed that two NK1 receptor antagonists greatly

568    attenuated basal $Ca^{2+}$ transients and suppressed responses of ICC-DMP to EFS.

569    The strong inhibitory effects of NK1 antagonists on $Ca^{2+}$ transients could possibly

570    be due to off-target effects on $Ca^{2+}$ stores or $Ca^{2+}$ release mechanisms, however

571    non-specific effects do not appear to be significant because responses to CCh on

572    $Ca^{2+}$ transients were intact in the presence of the NK1 antagonist, RP 67580.

573    Taken together these findings support the importance of neurokinin signaling in

574    shaping motility patterns in the small intestine.

575    The degree to which basal $Ca^{2+}$ transients were affected by NK1

576    antagonists in the present study was somewhat surprising.  These results

577    suggest ongoing release of neurokinins (i.e. tonic excitation), similar in concept to

578    the tonic inhibition phenomena observed in many GI muscles (Wood, 1972;

579    Lyster et al., 1995).  Although this phenomenon has not been described

27

580    previously in the small intestine, tonic activation of NK1 receptors has been

581    proposed in other systems (Henry et al., 1999; Jasmin et al., 2002).  In the

582    present study attenuation of $Ca^{2+}$ transients by the NK1 receptor antagonists may

583    be caused by continuous release of neurokinins or persistence of the ligand in

584    the spaces between motor nerve varicosities and ICC-DMP.

585         The enhanced relative reliance on neurokinins for excitatory effects may

586    be due, in part, to the high expression of NK1 receptors by ICC-DMP which does

587    not appear to be true for intramuscular ICC in the colon (Lee et al., 2017).   NK1

588    receptors also couple to cellular responses through activation of phospholipase C

589    and generation of $IP_3$ (Steinhoff et al., 2014).  Thus, there is a signaling pathway

590    available for the enhancement of $Ca^{2+}$ transients in ICC-DMP.  However, it

591    should also be noted that transfection of neurokinin receptors in model cells has

592    also been associated with activation of adenylate cyclase and production of

593    cAMP (Steinhoff et al., 2014), a pathway not typically linked to enhanced release

594    of $Ca^{2+}$.  Generation of cAMP and stimulation of cAMP-dependent protein kinase

595    is known to enhance $Ca^{2+}$ sequestration into stores by phosphorylation of

596    phospholamban (highly expressed in ICC; (Lee et al., 2017) and stimulation of

597    SERCA (Stammers et al., 2015).  Perhaps increased loading of $Ca^{2+}$ stores

598    contributes to augmentation of $Ca^{2+}$ transient amplitude and spatial spread by

599    neurokinins, and enhancing the rate of recovery of $Ca^{2+}$ into stores after a

600    release event reduces the time required for a given store to generate another

601    $Ca^{2+}$ transient.

28

602    In summary, this study supports the idea that significant neural regulation

603    occurs in the intramuscular class of ICC in the small intestine (ICC-DMP).  As

604    discussed above, much of the excitatory response was mediated through NK1

605    receptors that are expressed largely by ICC-DMP (Sternini et al., 1995;

606    Vannucchi et al., 1997; Iino et al., 2004).  Responses to EFS were attenuated by

607    NK1 antagonists.  Previous studies have shown that electrophysiological

608    responses in ICC-DMP are linked to $Ca^{2+}$ release events (Zhu et al., 2011; Zhu et

609    al., 2015), suggesting that $Ca^{2+}$ transients in ICC-DMP couple to generation of

610    inward currents and depolarizing effects on the SIP syncytium.  NK2 receptors,

611    expressed largely by SMCs (Cipriani et al., 2011), were apparently not involved

612    in responses of ICC-DMP to neurokinins released from nerve terminals, because

613    an NK2 antagonist had no effect on responses.  The effectiveness of neurokinins

614    as neurotransmitters in the *tunica muscularis* of the small intestine may be

615    spatially confined by concentrations achieved in post-junctional spaces to a

616    subset of neurokinin receptors expressed by ICC-DMP.

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792 **Figure legends**

793

794 **Figure 1. ICC-DMP Ca$^{2+}$ transient responses to nerve stimulation.**

795 **A** Time-lapse montage showing post-junctional Ca$^{2+}$ responses to EFS (10 Hz;

796 0.5 ms duration; 5 sec) on an ICC-DMP *in situ*. An image of the GCaMP3 signal

797 in the cell is shown in the leftmost panel. Scale bar is 25 $\mu$m and pertains to all

798 panels. A color-coded overlay and calibration scale was imported to depict

799 fluorescence intensity (*F*/*F*$_0$) and enhance visualization of Ca$^{2+}$ sites. Low

800 fluorescence areas are indicated in dark blue or black. High intensity

801 fluorescence areas are indicated in red and orange. The 'pre stimulation' panel

802 shows a summed image of Ca$^{2+}$ activity within the cell for 5 seconds before the

803 onset of EFS, Ca$^{2+}$ firing sites are marked with red asterisks. Panels showing the

804 summed Ca$^{2+}$ activity for the initial 2 seconds of EFS, the final 3 seconds of EFS

805 and 5 seconds post EFS are also shown. **B** Representative ST map of Ca$^{2+}$

806 transients in ICC-DMP shown in panel *A*. EFS duration is indicated by the

807 dashed white box. The firing activities of 3 sites highlighted on the ST map are

808 plotted in panel **C**, and the timing of EFS is indicated by the dashed red box.

809

810 **Figure 2. Effects of nerve stimulation (EFS) on Ca$^{2+}$ transients in ICC-DMP.**

811 **A** Representative traces representing Ca$^{2+}$ transients in ICC-DMP in response to

812 EFS (10Hz; 5 sec). The period of EFS is indicated by the red arrowed line).

813 Excitatory responses during the final 3 sec of EFS, indicated by the dashed blue

814 box, and during the post EFS period (5 sec), highlighted by the green box. **B – G**

34

815     Summary data quantifying the effects of EFS on ICC-DMP:  $Ca^{2+}$ transient

816     frequency (*E*), amplitude (*F*), duration (*G*), spatial spread (*H*), number of $Ca^{2+}$

817     firing sites (*I*) and $Ca^{2+}$ transient velocity (*J*) were analyzed and shown; n = 23, c

818     = 56. All statistical analyses shown are compared to control values. ns = P > 0.05,

819     * = P < 0.05, ** = P < 0.01, *** = P < 0.001, *** = P < 0.0001.

820

821     **Figure 3. Frequency dependence of $Ca^{2+}$ transient responses to EFS.**

822     *A* Summary data showing the excitatory effects of EFS (1Hz for 5 sec) on $Ca^{2+}$

823     transients in ICC-DMP during the final 3s of EFS and during the post-stimulus

824     period (5s following termination of EFS). $Ca^{2+}$ transient parameters shown

825     include: frequency ($sec^{-1}$), amplitude ($\Delta F/F_0$), duration (FDHM) and spatial spread

826     ($\mu$m) of $Ca^{2+}$ transients. *B* Summary data showing the effects of EFS (5Hz; 5

827     sec) on $Ca^{2+}$ transient parameters. *C* Summary data showing the effects of EFS

828     (20Hz; 5 sec) on $Ca^{2+}$ transient parameters. *D* Percentage (%) change of $Ca^{2+}$

829     transient firing frequency at all frequencies of EFS tested (1-20 Hz; net

830     percentage change normalized to control) during the final 3 sec of EFS and

831     during the post-stimulus period *E*. Note the frequency dependent effects of EFS

832     on $Ca^{2+}$ transient responses. Summary data in all panels shows the include 5

833     secs before EFS, the final 3 secs during EFS and 5 seconds post-EFS. ns = P >

834     0.05, * = P < 0.05, ** = P < 0.01.

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839 **Figure 4. Modulation of basal Ca$^{2+}$ transients by cholinergic input.**

840 *A* Representative ST maps showing the effects of atropine (1 $\mu$M) on basal Ca$^{2+}$

841 transient activity in ICC-DMP. *B – E* Summary graphs showing the effect of

842 atropine on the frequency (*B*), amplitude (*C*), duration (*D*) and spatial spread (*E*)

843 of basal Ca$^{2+}$ transients in ICC-DMP (n=5, c=13). *F* Representative ST maps

844 showing the effects of ACh (10 $\mu$M; in the presence of TTX) on Ca$^{2+}$ transients in

845 ICC-DMP. *G – J* Summary graphs showing the effect of ACh (in the presence of

846 TTX) on the frequency (*G*), amplitude (*H*), duration (*I*) and spatial spread (*J*) of

847 Ca$^{2+}$ transients in ICC-DMP (n=5, c=9). ns = P > 0.05, * = P < 0.05, ** = P < 0.01,

848 *** = P < 0.001.

849

850 **Figure 5. Effects of atropine on Ca$^{2+}$ transient responses to EFS.**

851 *A - B* Representative ST maps showing the effects of atropine (1 $\mu$M) on Ca$^{2+}$

852 transients in ICC-DMP in response to nerve stimulation (EFS; 10 Hz; 5 sec;

853 indicated by the red line and dotted white box in ST maps). *C – F* Summary data

854 showing the effects of atropine (1 $\mu$M) on Ca$^{2+}$ transients during EFS:  frequency

855 (*C*), amplitude (*D*), duration (*E*) and spatial spread (*F*) in ICC-DMP during control

856 conditions, during the excitatory period of EFS (final 3 sec), and during the post

857 EFS period (5 sec), n=5, c=21. ns = P > 0.05, * = P < 0.05, ** = P < 0.01.

858

859 **Figure 6. Effects of neurokinin receptor (NK1) antagonists on basal Ca$^{2+}$**

860 **transients.**

36

861    *A* Representative ST maps showing the inhibitory effects of the NK1 receptor

862    antagonist, RP 67580 (1 $\mu$M), on $Ca^{2+}$ transients in ICC-DMP. *B − E* Summary

863    graphs showing the effects of RP 67580 on the frequency (*B*), amplitude (*C*),

864    duration (*D*) and spatial spread (*E*) of $Ca^{2+}$ transients in ICC-DMP (n=11, c=27).

865    *F* Representative ST maps showing the inhibitory effects of the NK1 receptor

866    antagonist, SR 140333 (1 $\mu$M), on $Ca^{2+}$ transients in ICC-DMP. *G − J* Summary

867    graphs showing the effects of 1 $\mu$M SR 140333 on the frequency (*G*), amplitude

868    (*H*), duration (*I*) and spatial spread (*J*) of $Ca^{2+}$ transients in ICC-DMP (n=4, c=14).

869    ns = P > 0.05, * = P < 0.05, ** = P < 0.01, *** = P < 0.001.

870

871    **Figure 7. NK1 antagonist does not interfere with $Ca^{2+}$ release mechanisms.**

872    *A - B* Representative ST maps showing the effect of the NK1 antagonist RP

873    67580 (1 $\mu$M) on $Ca^{2+}$ transients in ICC-DMP. *C* ST map showing that in the

874    presence of RP 67580, CCh (10 $\mu$M) strongly activates $Ca^{2+}$ transients. *D − G*

875    Summary graphs showing the effects of CCh on $Ca^{2+}$ transient parameters:

876    frequency (*D*), amplitude (*E*), duration (*F*) and spatial spread (*G*) in ICC-DMP in

877    the presence of RP 67580 (n=3, c=6). * = P < 0.05.

878

879    **Figure 8. Neurokinin receptor (NK1) agonists activate $Ca^{2+}$ transients.**

880    *A* Representative ST maps showing the excitatory effects of substance P (1 $\mu$M;

881    in the presence of TTX) on $Ca^{2+}$ transients in ICC-DMP. *B − E* Summary graphs

882    showing the effects of substance P (in the presence of TTX) on the frequency (*B*),

883    amplitude (*C*), duration (*D*) and spatial spread (*E*) of $Ca^{2+}$ transients in ICC-DMP.

884      **F** Representative ST maps showing the excitatory effects of the NK1 receptor

885      agonist GR 73632 (1 $\mu$M; in the presence of TTX) on $Ca^{2+}$ transients in ICC-DMP

886      (n=4, c=10). **G – J** Summary graphs quantifying the effect of GR 73632 on the

887      frequency (*G*), amplitude (*H*), duration (*I*) and spatial spread (*J*) of basal $Ca^{2+}$

888      transient activity in ICC-DMP (n=4, c=9). ns = P > 0.05, * = P < 0.05, ** = P <

889      0.01, **** = P < 0.0001.

890

891      **Figure 9. Effects of  NK1 receptor antagonist on $Ca^{2+}$ transient responses to**

892      **EFS.**

893      **A - B** Representative ST maps showing the inhibitory effects of NK1 antagonist,

894      RP 67580 (1 $\mu$M), on $Ca^{2+}$ transients in response to nerve stimulation (EFS at 10

895      Hz for 5 sec; indicated by the red line and dotted white box in ST maps). **C – F**

896      Summary data showing the inhibitory effects of RP 67580 (1 $\mu$M) on $Ca^{2+}$

897      transient frequency (*C*), amplitude (*D*), duration (*E*) and spatial spread (*F*) in ICC-

898      DMP during the control period, during the final 3 sec of EFS, and during the post-

899      EFS period (5 sec), n=4, c=11. Note: RP 67580 reduced all $Ca^{2+}$ transient

900      parameters significantly. ns = P > 0.05, * = P < 0.05, ** = P < 0.01.

901

902      **Figure 10. Cholinergic and NK1 receptor antagonists inhibit $Ca^{2+}$ transients**

903      **elicited by EFS in ICC-DMP.**

904      **A - B** Representative ST maps showing the inhibitory effects of combining

905      cholinergic and neurokinin antagonists (atropine and RP 67580; both 1 $\mu$M) on

906      $Ca^{2+}$ transients in ICC-DMP during EFS (10 Hz; 5 sec). **C – F** Summary data of

907   $Ca^{2+}$ transient parameters showing the inhibitory effects of atropine and RP

908   67580 on $Ca^{2+}$ transient frequency (*C*), amplitude (*D*), duration (*E*) and spatial

909   spread (*F*) in ICC-DMP during the control period, during the final 3 sec of EFS,

910   and during the post-EFS period (5 sec), n=4, c=20. Note: combination of atropine

911   and RP 67580 abolished all $Ca^{2+}$ transient parameters significantly.  ns = P >

912   0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001.

913

914   **Figure 11. Excitatory responses are modestly reduced by atropine**

915   *A - B* Representative ST maps showing the inhibitory effects of atropine (1 $\mu$M)

916   on responses to EFS (10 Hz; 5 sec; indicated by the red line and dotted white

917   box in ST maps).  In this experiment L-NNA (100 $\mu$M) and the P2Y1 receptor

918   antagonist, MRS 2500 (1 $\mu$M), were present. *C – F* Summary data showing the

919   effects of a combination of L-NNA (100 $\mu$M), MRS 2500 (1 $\mu$M) and atropine (1

920   $\mu$M) on $Ca^{2+}$ transient frequency (*C*), amplitude (*D*), duration (*E*) and spatial

921   spread (*F*) in ICC-DMP during the control period, during the final 3 sec of EFS,

922   and during the post-EFS period (5 sec), n=7, c=26.  ns = P > 0.05, ** = P < 0.01,

923   *** = P < 0.001.

924

925   **Figure 12. Excitatory responses are strongly attenuated by NK1 antagonist**

926   *A - B* Representative ST maps showing the inhibitory effects of RP 67580 (1 $\mu$M),

927   in the presence of nitric oxide synthase inhibitor L-NNA (100 $\mu$M) and purinergic

928   P2Y1 receptor antagonist (MRS 2500; 1 $\mu$M) on $Ca^{2+}$ transients in response to

929   nerve stimulation (EFS at 10 Hz 5 sec; indicated by the red line and dotted white

39

930    box in ST maps). **C – F** Summary data showing the effects of a combination of L-

931    NNA, MRS 2500 and RP 67580 on $Ca^{2+}$ transient frequency (*C*), amplitude (*D*),

932    duration (*E*) and spatial spread (*F*) in ICC-DMP during the control period, during

933    the final 3 sec of EFS, and during the post-EFS period (5 sec), n=4, c=13.  ns = P

934    > 0.05, ** = P < 0.01.

935

936    **Figure 13. Excitatory responses to EFS are abolished by atropine and NK1**

937    **receptor antagonist**

938    **A - B** Representative ST maps showing inhibition of $Ca^{2+}$ transients by atropine

939    (1 $\mu$M) and RP 67580 (1 $\mu$M).   These experiments were conducted in the

940    presence of L-NNA (100 $\mu$M) in the presence of MRS 2500 (1 $\mu$M) during and

941    post nerve stimulation periods (EFS at 10 Hz 5 sec; indicated by the red line and

942    dotted white box in ST maps). **C – F** Summary data showing the effects of a

943    combination of L-NNA, MRS 2500 and atropine and RP 67580 on $Ca^{2+}$ transient

944    frequency (*C*), amplitude (*D*), duration (*E*) and spatial spread (*F*) in ICC-DMP

945    during control conditions, Excitatory periods during EFS (final 3 sec) and in the

946    post EFS period, n=5, c=32.  ns = P > 0.05, ** = P < 0.01, *** = P < 0.001.

947

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949    **Movie 1: ICC-DMP $Ca^{2+}$ transient responses to enteric neuronal stimulation**

950    Movie of intracellular $Ca^{2+}$ transients in ICC-DMP labeled with the genetically

951    encoded $Ca^{2+}$ indicator GCaMP3 in response to electrical field stimulation (EFS

952    10Hz, for 5 sec; real-time playback).  The top left FOV shows typical elongated

953   ICC-DMP using a 60x objective (original recordings).  Note that $Ca^{2+}$ transients

954   fired in stochastic fashion the blue bit-masked cell.  The right window shows $Ca^{2+}$

955   transient particles thresholded (SNR >= 25 dB, to facilitate visualization of active

956   signals) after differentiation ($\Delta t$= 0.5s) and smoothing (Gaussian 1.0sd, box size

957   = 3.3μm) as shown in the middle window. Scale bar in top left window is 15 $\mu$m

958   and pertains to all windows.

959   The blue overlay of ICC-DMP in the FOV (blue bit-masked cell) was used to

960   construct an ST map of $Ca^{2+}$-induced fluorescence intensity across the diameter

961   of the cell, which better displays the firing and propagation of $Ca^{2+}$ transients

962   along the length of the cell in response to EFS (lower panel; EFS duration is

963   indicated with the yellow box). The bottom panel shows active area of $Ca^{2+}$

964   transients across the FOV (area of active particles). Note the caseation of $Ca^{2+}$

965   transients in response to EFS and enhanced $Ca^{2+}$ firing during post stimulus

966   period.  Scale bar in the lower ST map and bottom active area map  is 50 $\mu$m.

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976 **Tables**

977 **Table 1:** Summary table of cholinergic and neurokinin receptors primer

978 sequences.

979 Table lists muscarinic (M2, M3) and neurokinin (NK1, NK2) receptor gene

980 transcripts that were measured in this study including their name, primer

981 sequences and gene bank accession numbers.

982

| Gene | Sequence | GenBank accession number |
|---|---|---|
| *mGapdh-F* | AGACGGCCGCATCTTCTT | NM_008084 |
| *mGapdh-R* | TTCACACCGACCTTCACCAT | |
| *mChrm2-F* | GGTGTCTCCCAGTCTAGTGCAAGG | NM_203491 |
| *mChrm2-R* | ATGTCTGCCTAGAGTTGTCATCTTTGGA | |
| *mChrm3-F* | TGTGGCCAGCAATGCTTCTGTCATGA | NM_033269 |
| *mChrm3-R* | CCACAGGACAAAGGAGATGACCCAAG | |
| *mTacr1-F* | GTGGTGAACTTCACCTACGCAGTC | NM_009313 |
| *mTacr1-R* | GCCATGTATGCTTCAAAGGCCACAG | |
| *mTacr2-F* | CCATCGCCGCTGACAGGTACA | NM_009314 |
| *mTacr2-R* | GGCCCCCTGGTCCACAGTGA | |

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**A**

Pre Stimulation
5 sec

10 Hz Stimulation
0-2 sec

10 Hz Stimulation
2-5 sec

Post Stimulation
5 sec

25 μm

F/F₀

2

0

**B**

F/F₀

2

0

80 μm

Site 1

Site 2

Site 3

3 sec

10 Hz 5 sec

**C**

Site 1

Site 2

Site 3

0.5 F/F₀

3 sec

*A*



*B Frequency*

*C Amplitude*

*D Duration*

*E Spatial Spread*

*F No. of Firing Sites*

*G Velocity*

## A  EFS (1 Hz, 5 Sec)

■ Pre EFS   ■ EFS Final 3 sec   ■ Post EFS

**Frequency**

**Amplitude**

**Duration**

**Spatial Spread**

## B  EFS (5 Hz, 5 Sec)

**Frequency**

**Amplitude**

**Duration**

**Spatial Spread**

## C  EFS (20 Hz, 5 Sec)

**Frequency**

**Amplitude**

**Duration**

**Spatial Spread**

## D  Frequency change during EFS (final 3 sec)

■ Control
□ 1 Hz
■ 5 Hz
■ 10 Hz
□ 20 Hz
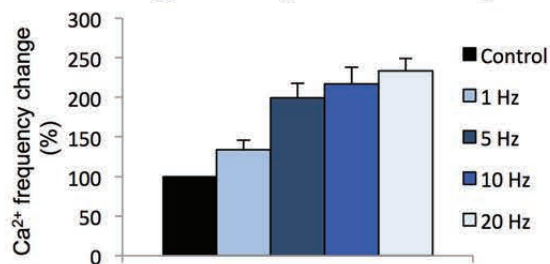
## E  Frequency change Post stimulus

■ Control
□ 1 Hz
■ 5 Hz
■ 10 Hz
□ 20 Hz

**A**

Control



Atropine (1 $\mu M$)



3 sec

**B Frequency**

**C Amplitude**

**D Duration**

**E Spatial Spread**



□ Control
■ Atropine

Ca²⁺ Events (sec⁻¹)

***

$\Delta F/F_0$

ns

FDHM (ms)

ns

Distance ($\mu m$)

ns

**F**

TTX



TTX + Ach (10 $\mu M$)



3 sec

**G Frequency**

**H Amplitude**

**I Duration**

**J Spatial Spread**



□ TTX
■ ACh

Ca²⁺ Events (sec⁻¹)

**

$\Delta F/F_0$

*

FDHM (ms)

**

Distance ($\mu m$)

*

**A**

**Control**

**EFS**

F/F₀

100 μm

2

0

5 sec

**B**

**Atropine (1 μM )**

F/F₀

100 μm

2

0

5 sec

**C Frequency**

Control
Atropine

Ca²⁺ Events (sec⁻¹)

3

2

1

0

Control          Final 3 s          Post EFS

**          *          *

**D Amplitude**

ΔF/F₀

1.5

1.0

0.5

0.0

Control          Final 3 s          Post EFS

ns          ns          ns

**E Duration**

FDHM (ms)

300

200

100

0

Control          Final 3 s          Post EFS

ns          ns          ns

**F Spatial Spread**

Distance (μm)

25

20

15

10

5

0

Control          Final 3 s          Post EFS

ns          ns          ns

**A**

*Control*

**RP 67580**

F/F₀ — 2 ... 0

45 μm

3 sec

*RP 67580 (1 μM)*

F/F₀ — 2 ... 0

45 μm

**B Frequency**

Ca²⁺ Events (sec⁻¹)

☐ Control
■ RP 67580

***

**C Amplitude**

ΔF/F₀

**

**D Duration**

FDHM (ms)

***

**E Spatial Spread**

Distance (μm)

***

**F**

*Control*

**SR 140333**

F/F₀ — 2 ... 0

65 μm

3 sec

*SR 140333 (1 μM)*

F/F₀ — 2 ... 0

65 μm

**G Frequency**

Ca²⁺ Events (sec⁻¹)

☐ Control
■ SR 140333

**

**H Amplitude**

ΔF/F₀

*

**I Duration**

FDHM (ms)

ns

**J Spatial Spread**

Distance (μm)

**

**A** *Control*

**B** *RP 67580*

**C** *RP 67580 + CCh*

**D Frequency**  **E Amplitude**  **F Duration**  **G Spatial Spread**

**A** *Substance P*

TTX

$F/F_0$

70 μm

3 sec

Substance P (1 μM)

$F/F_0$

70 μm

**B** Frequency

TTX
Substance P

Ca²⁺ Events (sec⁻¹)

*

**C** Amplitude

$\Delta F/F_0$

ns

**D** Duration

FDHM (ms)

****

**E** Spatial Spread

Distance (μm)

*

**F** *GR 73632*

TTX

$F/F_0$

50 μm

3 sec

GR 73632 (1 μM)

$F/F_0$

50 μm

**G** Frequency

TTX
GR 73632

Ca²⁺ Events (sec⁻¹)

**

**H** Amplitude

$\Delta F/F_0$

ns

**I** Duration

FDHM (ms)

ns

**J** Spatial Spread

Distance (μm)

ns

**A** *Control*

EFS

F/F₀



**B** *RP 67580 (1 μM )*

F/F₀



5 sec

**C** *Frequency*



**D** *Amplitude*



**E** *Duration*



**F** *Spatial Spread*

**A** *Control*

EFS

$F/F_0$

85 μm

2

0

5 sec

**B** *Atropine + RP 67580*

$F/F_0$

85 μm

2

0

5 sec

**C** *Frequency*

□ Control  ■ Atropine + RP 67580

$Ca^{2+}$ Events ($sec^{-1}$)

3

2

1

0

***

****

****

Control    Final 3 s    Post EFS

**D** *Amplitude*

$\Delta F/F_0$

1.0

0.8

0.6

0.4

0.2

0.0

ns

****

****

Control    Final 3 s    Post EFS

**E** *Duration*

FDHM (ms)

300

200

100

0

**

****

****

Control    Final 3 s    Post EFS

**F** *Spatial Spread*

Distance (μm)

18

12

6

0

****

**

****

Control    Final 3 s    Post EFS

**A**

*Control*

EFS

2

F/F$_0$

35 μm

0

5 sec

**B**

*L-NNA + MRS 2500 + Atropine*

2

F/F$_0$

35 μm

0

5 sec

**C**  *Frequency*

☐ Control  ■ L-NNA + MRS 2500  + Atropine

Ca$^{2+}$ Events (sec$^{-1}$)

4

3

2

1

0

ns    ns    *

Control    Final 3 s    Post EFS

**D**  *Amplitude*

ΔF/F$_0$

1.2

0.9

0.6

0.3

0.0

ns    ns    ns

Control    Final 3 s    Post EFS

**E**  *Duration*

FDHM (ms)

300

200

100

0

*    **    ***

Control    Final 3 s    Post EFS

**F**  *Spatial Spread*

Distance (um)

20

15

10

5

0

ns    ns    ns

Control    Final 3 s    Post EFS

**A**

Control

EFS

F/F₀

40 μm

2

0

5 sec

**B**

L-NNA + MRS 2500 + RP 67580

F/F₀

40 μm

2

0

5 sec

**C** *Frequency*

Ca²⁺ Events (sec⁻¹)

☐ Control  ■ L-NNA + MRS 2500 + RP 67580

3

2

1

0

Control     Final 3 s     Post EFS

\*           ns           \*\*

**D** *Amplitude*

ΔF/F₀

2

1

0

Control     Final 3 s     Post EFS

\*           \*\*           ns

**E** *Duration*

FDHM (ms)

300

200

100

0

Control     Final 3 s     Post EFS

\*           \*\*           \*\*

**F** *Spatial Spread*

Distance (μm)

20

15

10

5

0

Control     Final 3 s     Post EFS

\*           \*           \*

**A** *Control*     EFS

F/F₀   105 μm

5 sec

**B** *L-NNA + MRS 2500 + Atropine + RP 67580*

F/F₀   105 μm

5 sec

**C** *Frequency*

Ca²⁺ Events (sec⁻¹)

Control    Atropine + RP 67580 + L-NNA + MRS 2500

Control    Final 3 s    Post EFS

**    ***    ****

**D** *Amplitude*

ΔF/F₀

Control    Final 3 s    Post EFS

***    ****    ****

**E** *Duration*

FDHM (ms)

Control    Final 3 s    Post EFS

****    ****    ****

**F** *Spatial Spread*

Distance (μm)

Control    Final 3 s    Post EFS

****    ****    ****