Cestode larvae excite host neuronal circuits via glutamatergic signaling.

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Abstract

Neurocysticercosis (NCC) is caused by the presence of *Taenia solium* larvae in the brain and is the leading cause of adult-acquired epilepsy worldwide. However, little is known about how seizures emerge in NCC. To address this knowledge gap we used whole-cell patch-clamp electrophysiology and calcium imaging in rodent hippocampal organotypic slice cultures to identify direct effects of cestode larval products on neuronal activity. We found both whole cyst homogenate and excretory/secretory (E/S) products of *Taenia* larvae have an acute excitatory effect on neurons, which trigger seizure-like events *in vitro*. Underlying this effect was *Taenia*-induced neuronal depolarization, which was mediated by glutamate receptor activation but not by nicotinic acetylcholine receptors, acid-sensing ion channels nor Substance P. Glutamate assays revealed the homogenate of both *Taenia crassiceps* and *Taenia solium* larvae contained high concentrations of glutamate and that larvae of both species consistently produce and release this excitatory neurotransmitter into their immediate environment. These findings contribute towards the understanding of seizure generation in NCC.

Author summary

Brain infection by larvae of the tapeworm *Taenia solium* (neurocysticercosis or NCC) is the leading cause of acquired epilepsy in adulthood. Little is understood about the mechanisms by which larvae cause seizures. To address this, we used electrophysiological and imaging techniques in rodent brain slices to investigate how tapeworm larvae directly impact neuronal function. We discovered that both the homogenate and secretory products of tapeworm larvae excite neurons and can trigger seizure-like events in brain slices. This effect was caused by the activation of glutamate receptors and not by activating other types of receptors in the brain. Finally, we observed that tapeworm larvae both contain and release the neurotransmitter glutamate into their immediate environment. These findings are relevant for understanding how tapeworm larvae cause seizures in NCC.

75 *Introduction*

76 Neurocysticercosis (NCC) is the most prevalent parasitic infection of the central nervous system (CNS)
77 (1,2). It is caused by the presence of *Taenia solium (T. solium)* larvae in the brain (3). The most common
78 symptom of NCC is recurrent seizures (4). As a result, NCC is the leading cause of adult-acquired
79 epilepsy worldwide (5), resulting in significant morbidity and mortality. In endemic areas,
80 approximately 29% of people with epilepsy also have NCC (6). Despite the impact of NCC, there are a
81 paucity of studies investigating the seizure mechanisms involved (7). As a result, precisely how larvae
82 are involved in seizure generation is still relatively poorly understood.

83

84 In NCC seizures may occur at any stage following initial infection (4). It is thought that inflammatory
85 processes in the brain can play an important role in the development of recurrent seizures (8).
86 Previous work exploring seizure development in NCC has therefore typically focused on how the host
87 neuroinflammatory response to larvae might precipitate seizures (7,9). Robinson *et al*. found, for
88 example, that production of the inflammatory molecule and neurotransmitter, Substance P, produced
89 by peritoneal larval granulomas, can precipitate acute seizures (10). In addition to host-derived
90 substances, cestodes themselves are known to excrete or secrete various products that interact with
91 host cells in their vicinity. Cestode larvae derived factors are known to modulate the activation status
92 of immunocytes such as microglia and dendritic cells (11,12). However, comparatively little is known
93 about how factors contained in, or secreted by, cestode larvae might affect neurons and neuronal
94 networks directly, including whether these may have pro-seizure effects.

95

96 To address this, we used whole-cell patch-clamp recordings and calcium imaging in rodent
97 hippocampal organotypic slice cultures to demonstrate the direct effects of larval products on
98 neuronal activity. We find that both the whole cyst homogenate and the excretory/secretory (E/S)
99 products of *Taenia crassiceps (T. crassiceps)* larvae have a strong, acute excitatory effect on neurons.
100 This was sufficient to trigger seizure-like events *in vitro*. Underlying seizure induction was *T. crassiceps*

101 larval induced neuronal depolarization, which was mediated by glutamate receptor activation and not
102 nicotinic acetylcholine receptors, acid-sensing ion channels nor Substance P. Direct measurement of
103 glutamate concentration revealed that the homogenate of both *T. crassiceps* and *T. solium* larvae
104 contain high levels of glutamate. Lastly, we provide evidence that larvae of both species consistently
105 produce and release this excitatory neurotransmitter into their immediate environment; suggesting
106 that parasite released glutamate directly contributes to NCC seizures.
107

108 *Materials and Methods*

109 *Ethics statement*

110 All animal handling, care and procedures were carried out in accordance with South African national
111 guidelines (South African National Standard: The care and use of animals for scientific purposes, 2008)
112 and with approval from the University of Cape Town Animal Ethics Committee (Protocol No: AEC
113 015/015, AEC 014/035).

114

115 *Taenia maintenance and preparation of whole cyst homogenates and E/S products*

116 Larvae of *T. crassiceps* (ORF strain) were donated to us by Dr Siddhartha Mahanty (University of
117 Melbourne, Melbourne, Australia) and propagated *in vivo* by serial intraperitoneal infection of 5-8
118 week old female C57BL/6 mice. Every 3 months parasites were harvested by peritoneal lavage and
119 washed 6 times in phosphate buffered saline (PBS, 1X, pH 7.4; Sigma-Aldrich) before further
120 processing.

121

122 For the preparation of *T. crassiceps* whole cyst homogenate, larvae were stored immediately after
123 harvesting at -80 °C. Later these were thawed (thereby lysing the cells) and suspended in PBS (1X)
124 containing a protease cocktail inhibitor (1% vol/vol, Sigma-Aldrich) at a larval:PBS ratio of 1:3. The
125 larvae were then homogenised on ice using a Polytron homogenizer (Kinematica). The resulting
126 mixture was centrifuged at 4000rpm for 20 minutes at 4 °C. The liquid supernatant (between the white
127 floating layer and solid pellet) was collected and sterile filtered through a 0.22 µm size filter (Millex-
128 GV syringe filter, Merck). This supernatant was then aliquoted and stored at -80 °C until use. To assess
129 whether large or small molecules were responsible for the excitation of neurons, a portion of the
130 whole cyst homogenate was dialysed using a Slide-A-Lyzer™ dialysis cassette (3kDa MWCO,
131 Separations) in 2l of artificial cerebro-spinal fluid (aCSF) at 4 °C. The aCSF solution was changed twice
132 over 24 hours. To determine the ionic composition of *T. crassiceps* whole cyst homogenate a Cobas

133 6000 analyser (Roche) was used, with ion specific electrodes for K^+ and Na^+. A Mettler Toledo
134 SevenCompact™ pH meter S210 (Merck) was used to determine the pH of the homogenate.
135 For the preparation of *T. crassiceps* E/S products, after harvesting, washed larvae were maintained for
136 14-21 days at 37°C in 5% CO_2 in 50ml tissue culture flasks (approximately 7ml volume of larvae per
137 flask) containing culture medium consisting of Earle's Balanced Salt Solution (EBSS) supplemented
138 with glucose (5.3 g/liter), Glutamax (1X), penicillin (50 U/ml), streptomycin (50 ug/ml), gentamicin
139 sulphate (100 ug/ml) and nystatin (11.4 U/ml) (Sigma-Aldrich). Culture medium was replaced after 24
140 hours and the original media discarded. Thereafter, culture media were replaced and collected every
141 48-72 hours, stored at -20 °C and at the end of the culture period, thawed and pooled. The pooled
142 conditioned medium was termed total excretory/secretory products (total E/S). A portion of the total
143 E/S was aliquoted and stored at -80 °C, while another portion was concentrated (about 100X) and
144 buffer exchanged to PBS using an Amicon stirred cell with a 3 kDa MWCO cellulose membrane (Sigma-
145 Aldrich). The concentrated product contained *T. crassiceps* E/S products larger in size than 3kDa (E/S
146 > 3kDa), in PBS (1X). This was aliquoted and stored at -80 °C. The fraction of the total E/S that passed
147 through the 3kDa membrane (E/S < 3 KDa, still in medium) was also retained, aliquoted and stored at
148 -80 °C.
149 For the preparation of *T. solium* whole cyst homogenate, larvae of *T. solium* were harvested from the
150 muscles of heavily infected, freshly slaughtered pig. After extensive washing with sterile 1X phosphate
151 buffered saline (PBS; pH 7.4; Sigma-Aldrich), *T. solium* larvae were suspended in PBS containing
152 phenylmethyl-sulphonyl fluoride (5mM) and leupeptin (2.5 µM) at a larval:PBS ratio of 1:3. Larvae
153 were then homogenised using a sterile hand held homogenizer at 4 °C. The resulting homogenate was
154 sonicated (4 x 60 s at 20 kHz, 1 mA, with 30 s intervals) and gently stirred with a magnetic stirrer for
155 2h at 4°C.Thereafter it was centrifuged at 15,000 g for 60 min at 4 °C and the liquid supernatant
156 (between the white floating layer and solid pellet) was collected. The supernatant was filtered through
157 0.45 µm size filters (Millex-GV syringe filter, Merck), aliquoted and stored at -80 °C. All *T. crassiceps*

158 and *T. solium* larval products were assessed for protein concentration using a BCA protein or
159 Bradford's assay kit respectively.

160 For the assessment of daily glutamate production both *T. crassiceps and T. solium* larvae were placed
161 into 6 well plates (+/- 15 per well, of roughly 5mm length) with 2 ml of culture medium (*Taenia solium*
162 medium: RPMI 1640 with 10 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25
163 µg/ml amphotericin B and 2 mM L-glutamine; *Taenia crassiceps* medium: EBSS with 100 U/ml
164 penicillin, 100 µg/ml streptomycin, 11.4 U/ml nystatin and 2 mM glutamax). Every 24 hours 1 ml of
165 culture media were collected from each well, stored at -80 °C, and replaced with fresh culture
166 medium. The concentration of glutamate was measured using a Glutamate assay kit according to the
167 supplier's instructions (Sigma-Aldrich).

168

169 *Hippocampal brain slice preparation*

170 Organotypic brain slices were prepared using 6-8 day old Wistar rats and C57BL/6 mice following the
171 protocol originally described by Stoppini *et al*., (1991). Brains were extracted and swiftly placed in
172 cold (4°C) dissection media consisting of Geys Balanced Salt Solution (Sigma-Aldrich) supplemented
173 with D-glucose (Sigma-Aldrich). The hemispheres were separated and individual hippocampi were
174 removed and immediately cut into 350 µm slices using a McIlwain tissue chopper (Mickle). Cold
175 dissection media was used to rinse the slices before placing them onto Millicell-CM membranes
176 (Sigma-Aldrich). Slices were maintained in culture medium consisting of 25 % (vol/vol) Earles balanced
177 salt solution (Sigma-Aldrich); 49 % (vol/vol) minimum essential medium (Sigma-Aldrich); 25 % (vol/vol)
178 heat-inactivated horse serum (Sigma-Aldrich); 1 % (vol/vol) B27 (Invitrogen, Life Technologies) and
179 6.2 g/l D-glucose. Slices were incubated in a 5 % carbon dioxide (CO_2) humidified incubator at between
180 35 – 37 °C. Recordings were made after 6-14 days in culture.

181

182 *Electrophysiology and calcium imaging*

183 Brain slices were transferred to a submerged recording chamber on a patch clamp rig, which was
184 maintained at a temperature between 28 and 30 °C, and were continuously superfused with standard
185 aCSF bubbled with carbogen gas (95 % O_2: 5 % CO_2) using peristaltic pumps (Watson-Marlow). The
186 standard aCSF was composed of: NaCl (120 mM); KCl (3 mM); $MgCl_2$ (2 mM); $CaCl_2$ (2 mM); NaH_2PO_4
187 (1.2 mM); $NaHCO_3$ (23 mM) and D-Glucose (11 mM) in deionised water with pH adjusted to between
188 7.35 - 7.40 using 0.1 mM NaOH (Sigma-Aldrich). Neurons in the CA3 region of the hippocampus were
189 visualized using a Zeiss Axioskop or Olympus BX51WI upright microscope using 20x or 40x
190 water-immersion objectives and targeted for recording. Micropipettes were prepared (tip resistance
191 between 3 and 7 MΩ) from borosilicate glass capillaries (outer diameter 1.2 mm, inner diameter 0.69
192 mm, Harvard Apparatus Ltd) using a horizontal puller (Sutter). Recordings were made in current clamp
193 mode using Axopatch 200B amplifiers (Axon Instruments) and data acquired using WinWCP
194 (University of Strathclyde) or Igor (Markram Laboratory, Ecole polytechnique fédérale de Lausanne).
195 Matlab (MathWorks) was utilised for trace analysis. Two internal solutions were used: a "standard"
196 internal solution (K-gluconate (126 mM), KCl (4 mM), HEPES (10 mM) Na_2ATP (4 mM), NaGTP (0.3 mM)
197 and Na_2-phosphocreatine (10 mM); Sigma-Aldrich) and a "caesium" internal solution (CsOH (120 mM),
198 Gluconic acid (120 mM), HEPES (40 mM), Na_2ATP (2 mM), NaGTP (0.3 mM) and NaCl (10 mM);
199 Sigma-Aldrich). Experimental substances were puffed onto neurons using an OpenSpritzer, a custom
200 made pressure ejection system (14). Current was injected if required to ensure a neuronal resting
201 membrane potential within 2 mV of -60 mV. In all puffing experiments each data point represents the
202 mean peak puff-induced change in membrane potential from 10 sweeps. In some experiments
203 tetrodotoxin (TTX) (2 µM) was added to the aCSF to block voltage-gated sodium channels.
204 For calcium imaging, organotypic hippocampal mouse brain slices were virally transfected with a
205 genetically-encoded Ca^{2+} reporter (GCAMP6s under the synapsin promoter,
206 AAV1.Syn.GCaMP6s.WPRE.SV40) one day post culture using the OpenSpritzer and imaged 5 days later
207 using an Olympus BX51WI microscope, 20x water-immersion objective, CCD camera (CCE-B013-U,

208 Mightex) and 470 nm LED (Thorlabs). Images were collected using µManager (15) and analysed using
209 Caltracer3 beta scripts in Matlab.
210 Pharmacological manipulations were performed by bath application of drugs using a perfusion system
211 (Watson-Marlow). Mecamylamine, Amiloride, D-AP5 and CNQX were purchased from Tocris.
212 Kynurenic acid and Substance P were acquired from Sigma-Aldrich.
213
214 *Data analysis and statistics*
215 Data was graphed and analysed using Matlab, Microsoft Excel and GraphPad Prism. All data was
216 subjected to a Shapiro-Wilk test to determine whether it was normally distributed. Normally
217 distributed populations were subjected to parametric statistical analyses, whilst skewed data was
218 assessed using non-parametric statistical analyses. Parametric tests included: unpaired student's t-
219 test; paired student's t-test; one-way analysis of variance (ANOVA) with Tukey's post hoc test and
220 repeated measures ANOVA with Tukey's post-hoc test. Non-parametric tests included: Mann-Whitney
221 test; Wilcoxin ranked pairs test; Kruskal Wallis one-way ANOVA with post hoc Dunn's Multiple
222 Comparison test; and Friedman test with post-hoc Dunn's Multiple Comparison test. The confidence
223 interval for all tests was set at 95%.
224

225 *Results*

226 *Taenia crassiceps* homogenate excites neurons and can elicit epileptiform activity.

227 To investigate the potential acute effects of *T. crassiceps* larvae on neurons, *T. crassiceps* larval
228 somatic homogenate was prepared using larvae harvested from the peritonea of mice which were
229 freeze-thawed and homogenized (see Materials and Methods and **Fig. 1A**). Whole-cell patch-clamp
230 recordings were made from CA3 pyramidal neurons in rodent hippocampal organotypic brain slice
231 cultures and pico-litre volumes of *T. crassiceps* homogenate were directly applied to the soma of
232 neurons using a custom built pressure ejection system (**Fig. 1A**) (16). Application of the homogenate
233 (20 ms puff) elicited immediate, transient depolarization of the membrane voltage in recordings from
234 both rat and mouse (**Fig. 1B**) neurons. Increasing the amount of homogenate delivered by increasing
235 the pressure applied to the ejection system resulted in increasingly large membrane depolarization,
236 which could trigger single or multiple action potentials (**Fig. 1B, C**). Similar puffs of artificial
237 cerebrospinal fluid (aCSF) did not affect the neuronal membrane potential, thereby excluding the
238 possibility that mechanical effects might account for the homogenate-induced depolarization
239 observed.

240 To further explore the acute excitatory effect of *T. crassiceps* on neurons, neuronal networks and the
241 propagation of network activity, we performed fluorescence Ca^{2+} imaging in mouse hippocampal
242 organotypic brain slice cultures. Neurons were virally transfected with the genetically-encoded Ca^{2+}
243 reporter, GCAMP6s, under the synapsin promoter and imaged using widefield epifluorescence
244 microscopy (**Fig. 1D** and Materials and Methods). To simulate a pro-ictal environment, a low Mg^{2+}
245 aCSF was used (0.5 mM Mg^{2+}) and neurons in the dentate gyrus were imaged whilst small, spatially
246 restricted puffs of *T.crassiceps* homogenate were delivered every 15 s using a glass pipette (**Fig. 1D**).
247 The cells within the direct vicinity of the puffing pipette showed a sharp increase in fluorescence
248 immediately following the delivery of *T. crassiceps* homogenate (**Fig. 1.1E t_1**) for all 3 puffs, indicating
249 Ca^{2+} entry following membrane depolarization and action potential generation. Interestingly, cells in
250 the periphery showed notable increases in fluorescence at a delayed interval following some (but not

251 all) puffs (**Fig. 1E t_2**). The excitation of these cells could only be as a result of being synaptically
252 connected to the cells that were exposed to the puff itself, as they were not positioned close enough
253 to the puffer pipette to be directly excited by the homogenate. Indeed, a current-clamp recording
254 from a neuron in the same slice (**Fig. 1E, inset**) indicates that a single, spatially restricted puff of
255 *T. crassiceps* homogenate can elicit the onset of a regenerative seizure-like event lasting far longer
256 than the puff itself. Together, these results indicate that *T. crassiceps* larvae homogenate results not
257 just in a transient depolarization of cells in the immediate vicinity of application, but can trigger a wave
258 of excitation that propagates through the brain slice in both space and time. This demonstrates that
259 *T. crassiceps* homogenate can initiate seizure-like activity under suitable conditions.
260
261 Potassium contributes modestly to *Taenia crassiceps* induced neuronal depolarization.
262 The extracellular concentration of K^+ ($[K^+]_e$, typically 3 - 4mM) in the brain is considerably lower than
263 the intracellular concentration of K^+ ($[K^+]_i$, typically 100 mM) of neurons (17), which means that even
264 small changes in $[K^+]_e$ result in relatively large changes to the transmembrane K^+ gradient and
265 consequently the reversal potential for K^+ (E_{K+}). Due to the fact that constitutively activated K^+
266 conductances, or K^+ "leak" channels, are the major contributor to the resting membrane potential,
267 addition of extracellular K^+ and accompanying changes to E_{K+} result in membrane depolarization (18).
268
269 As most cell types tend to have a high $[K^+]_i$, it is conceivable that the *T. crassiceps* homogenate could
270 have a high K^+ concentration, which could potentially account for its depolarizing and excitatory
271 effects on neurons. To address this, we first directly measured the ionic composition of the
272 *T. crassiceps* homogenate using a Roche Cobas 6000. The K^+ concentration of the *T. crassiceps*
273 homogenate was 11.4 mM as compared to 3.0 mM in our standard aCSF. The *T. crassiceps*
274 homogenate Na^+ concentration was 123.9 mM, and 144.2 mM for the aCSF. Although the K^+
275 concentration is higher in the homogenate than the aCSF, the ionic composition more closely reflects
276 that of the extracellular as opposed to intracellular compartment. Nonetheless, we set out to

277 determine what effect application of 11.4 mM K^+ would have on neurons in our system. Whole-cell
278 patch-clamp recordings in current clamp mode using a standard internal solution and aCSF (see
279 Materials and Methods) were made from CA3 pyramidal neurons in rat hippocampal organotypic
280 brain slice cultures. If necessary, current was injected to ensure that the baseline membrane potential
281 was within 2 mV of -60 mV. To remove membrane potential fluctuations due to synaptic noise, and to
282 prevent regenerative Na^+ conductances due to voltage-gated sodium channels, 2 µM TTX was added
283 to the aCSF. Puffs of aCSF containing 11.4 mM K^+ were directed toward the soma of recorded neurons.
284 Whilst this did cause a depolarization of the membrane potential, the effect was modest, a median
285 positive shift of only 0.72 mV (IQR 0.51 - 1.04 mV, N = 8) in the membrane potential, which was
286 significantly less than the depolarization caused by puffs of *T. crassiceps* homogenate (median 10.12
287 mV, IQR 9.93 - 12.49 mV, N = 5, p = 0.0016, Mann-Whitney U test, **Fig. 2A, B, D**). Next, to isolate
288 neuronal depolarization induced by *T. crassiceps* homogenate, but not mediated by K^+, we performed
289 whole cell patch clamp recordings using a caesium based internal solution (see Materials and
290 Methods), which blocks K^+ channels. The addition of caesium did not greatly change the positive shift
291 in membrane potential caused by *T. crassiceps* homogenate (median 13.76, IQR 10.87 - 17.24 mV,
292 N = 49, p ≤ 0.0489, Mann-Whitney U test, **Fig. 2C, D**). Together, this indicates that although K^+ in the
293 *T. crassiceps* homogenate does contribute to membrane depolarization, the majority of the effect is
294 mediated by a different component.
295
296 <u>*Taenia crassiceps* homogenate induced neuronal depolarization is mediated by a small molecule.</u>
297 Next, to determine the fraction of the *T. crassiceps* homogenate, which underlies the acute excitatory
298 effect on neurons we observed, we used a dialysis membrane to separate the fraction of the
299 homogenate bigger than 3 kDa from the total homogenate. To do so we used a 3 kDa dialysis cassette
300 incubated overnight in standard aCSF. This effectively removed molecules smaller than 3 kDa from the
301 homogenate. When this dialysed *T. crassiceps* was puffed onto the cells in the same recording
302 conditions as in **Fig. 2B** and **C**, the depolarizing response was greatly reduced (p ≤ 0.0003,

303 Mann-Whitney U test, **Fig. 3 A-C**). The median positive shift in membrane potential for dialysed
304 *T. crassiceps* homogenate was only 0.27 mV (IQR 0.20 - 0.85 mV, N = 5) as compared to a median of
305 13.76 mV (IQR 10.87 - 17.24 mV, N = 49) for the total homogenate (**Fig. 3**). This suggests that the
306 excitatory component of the *T. crassiceps* homogenate is a molecule smaller than 3 kDa.
307
308 *Taenia crassiceps* excretory/secretory products also cause neuronal depolarization.
309 Helminths are well known to excrete or secrete products into their immediate environment.
310 Therefore, having confirmed that a small molecule within the *T. crassiceps* homogenate causes acute
311 neuronal depolarization we next sought to determine whether E/S products generated by *T. crassiceps*
312 larvae could also result in neuronal depolarisation. *T. crassiceps* larvae were cultured in media. The
313 media was collected over a period of 21 days and either used as is (*T. crassiceps* total E/S products) or
314 separated into two fractions and buffer exchanged to PBS using an Amicon stirred cell with a 3 kDa
315 cellulose membrane. The first fraction contained E/S products larger than 3 kDa (*T. crassiceps*
316 E/S > 3 kDa) in PBS, and the second fraction contained E/S products smaller than 3 kDa (*T. crassiceps*
317 E/S < 3 kDa) in media. To control for possible effects of molecules in the buffers, both media and PBS
318 were independently puffed onto neurons.
319
320 Again, CA3 neurons in rat hippocampal organotypic slice cultures were whole-cell patched using a
321 caesium based internal in the presence of 2 µM TTX. Brief application of total *T. crassiceps* E/S
322 products using a soma directed puff was sufficient to cause a neuronal depolarization (mean
323 12.29 mV, SEM 1.74 mV, N = 7) significantly larger than that of the media control (mean -0.48 mV,
324 SEM 0.40 mV, N = 7, $p \leq 0.001$, One-way ANOVA, Tukey's post hoc test, **Fig. 4A, D, F**). Similarly, *T.*
325 *crassiceps* E/S products containing components smaller than 3 kDa evoked a significantly larger shift
326 in membrane potential than the media control (mean 16.09 mV, SEM 1.56 mV, N = 7, $p \leq 0.001$, One-
327 way ANOVA, Tukey's post hoc test, **Fig. 4B, D, F**). The response evoked by the *T. crassiceps* E/S

328 products containing components smaller than 3 kDa did not differ significantly than that evoked by
329 the total E/S ($p > 0.05$, One-way ANOVA, Tukey's post hoc test, **Fig. 4A, B, F).**
330
331 However, the *T. crassiceps* E/S product fraction larger than 3 kDa (mean 1.43 mV, SEM 0.43 mV, N = 8)
332 did not generate significant neuronal depolarization when compared to the PBS control (mean
333 1.10 mV, SEM 0.22 mV, N = 8, $p \leq 0.001$, One-way ANOVA, Tukey's post hoc test, **Fig. 4C, E, F**). Both
334 the total E/S and the E/S products < 3 kDa evoked a depolarization larger than that of the E/S products
335 > 3 kDa ($p \leq 0.001$, One-way ANOVA, Tukey's post hoc test, **Fig. 4A, B, D, F).** Together this set of
336 experiments demonstrated that *T. crassiceps* E/S products also contain an excitatory component,
337 which is a small molecule.
338
339 *Taenia crassiceps* homogenate induced neuronal depolarization is not mediated by nicotinic
340 acetylcholine receptors, ASIC receptors, or Substance P.
341 Our results demonstrate that the *T. crassiceps* derived small molecule induces neuronal depolarization
342 very rapidly (within ms), as such, it is highly likely that this molecule is an agonist of a neuronal
343 ionotropic receptor. Therefore, we utilized pharmacological methods to determine the ionotropic
344 receptor system via which the *T. crassiceps* derived small molecule acts.
345
346 Nicotinic acetylcholine receptors (nAchRs) are well described ionotropic receptors in the nervous
347 system, which mediate depolarization via a mixed Na^+, K^+ conductance (19). Again, using a caesium
348 based internal solution in the presence of TTX in the aCSF, CA3 neurons were whole-cell patched and
349 the peak voltage during *T. crassiceps* homogenate induced neuronal depolarisation measured before,
350 during and after bath application of the non-competitive nicotinic acetylcholine receptor antagonist
351 mecamylamine hydrochloride (10µM) to block nAchRs (**Fig. 5A**). The blockade of nAchRs did not
352 significantly alter the *T. crassiceps* homogenate induced depolarisation. The median *T. crassiceps*
353 homogenate induced depolarization was 16.28 mV (IQR 13.54 - 23.63 mV) during baseline, 16.58 mV

354 (IQR 11.21 - 24.50 mV) in the presence of mecamylamine hydrochloride and 13.20 mV (IQR = 10.37 -
355 23.48 mV) following washout (N = 5, p = 0.6914, Friedman test, **Fig. 5A,B**).
356
357 Acid-sensing ion channels (ASICs) are proton-gated sodium channels known to be expressed by
358 hippocampal neurons and result in neuronal depolarization when activated. We therefore considered
359 that the pH of the *T. crassiceps* homogenate may be important as acidic conditions could activate
360 ASICS and cause neuronal depolarization. We measured the pH of the *T. crassiceps* homogenate
361 (mean = 7.99), which was too alkaline to activate ASICs, activated maximally at a pH of 5 (20).
362 However, certain compounds can activate ASICs directly (21). Therefore, to determine whether
363 activation of ASICs could underlie *T. crassiceps* homogenate induced depolarization we used the non-
364 specific ASIC blocker amiloride (2 mM). The presence of amiloride did not significantly attenuate the
365 effect of the homogenate (**Fig. 5C**), with the mean *T. crassiceps* homogenate induced depolarization
366 being 14.55 mV (SEM 1.41 mV) during baseline and 12.12 mV (SEM 2.25 mV) in the presence of
367 amiloride, whilst 17.06 mV (SEM 2.20 mV) following washout (N = 10, p = 0.1124, repeated measures
368 ANOVA, **Fig. 5C, D**).
369
370 A further possible candidate for our excitatory small molecule was Substance P, an abundant
371 neuropeptide and neurotransmitter (22,23). Robinson *et al* (2012) have found Substance P in close
372 vicinity to human NCC granulomas. Garza *et al*. (2010) found that substance P signalling contributes
373 to granuloma formation and Substance P enhanced NMDA channel function (25). We therefore
374 investigated whether Substance P could elicit a similar neuronal depolarizing response to that of *T.*
375 *crassiceps* homogenate. However, we found that 100 µM Substance P had no acute effect on the
376 membrane potential of CA3 hippocampal pyramidal neurons (median = 0.08 mV, IQR 0.01 – 0.12 mV,
377 N = 5, Wilcoxon signed rank test with theoretical median = 0.00, p = 0.0625, **Fig. 5E, F**). Together these
378 data indicate that *T. crassiceps* homogenate induced neuronal depolarization is not mediated by
379 nicotinic acetylcholine receptors, ASIC receptors, nor by substance P.

380

381 The depolarizing effects of *Taenia crassiceps* are mediated by glutamate receptor activation.

382 The predominant excitatory neurotransmitter in the nervous system is glutamate (19). Glutamate
383 activates a number of ionotropic glutamate receptors (GluRs) including AMPA, Kainate and NMDA
384 receptors (19), therefore it is likely that *T. crassiceps* homogenate could cause neuronal depolarization
385 by activation of GluRs. To test this possibility, whole cell current clamp recordings from CA3
386 hippocampal pyramidal cells were acquired using a caesium based internal in the presence of TTX to
387 block voltage-gated sodium channels. *T. crassiceps* homogenate induced neuronal depolarization was
388 measured before, during and after the application of 10µM CNQX, 50µM D-AP5 and 2mM kynurenic
389 acid (in the aCSF) to block all three classes of GluRs. We found that GluR blockade significantly reduced
390 the median *T. crassiceps* homogenate induced neuronal depolarization from 14.72 mV (IQR 13.39 -
391 15.68 mV) to 1.90 mV (IQR 1.09 - 2.21 mV, N = 9, $p \leq 0.01$, Friedman test with Dunn's multiple
392 comparison test, **Fig. 6A, B**), which recovered to a median value of 14.37 mV (IQR 7.32 - 16.38 mV)
393 following washout (N = 9, $p \leq 0.01$, Friedman test with Dunn's multiple comparison test, **Fig. 6A, B**).
394 This indicated that activation of GluRs is the primary mediator of the depolarizing effect of *T.*
395 *crassiceps* homogenate we observed.

396

397 Thereafter, we confirmed whether the depolarizing effect of *T. crassiceps* E/S products is also
398 mediated by GluRs. Here, too, we found that application of GluR antagonists reduced the median total
399 *T. crassiceps* E/S products induced depolarization from 16.35 mV (IQR 15.17 - 19.03 mV) to 2.19 mV
400 (IQR 1.67 - 4.16 mV, N = 10, $p \leq 0.01$, Friedman test with Dunn's multiple comparison test, **Fig. 6C, D**),
401 which recovered to a median value of 16.43 mV (IQR 14.41 - 23.14 mV) following washout (N = 10,
402 $p \leq 0.01$, Friedman test with Dunn's multiple comparison test, **Fig. 6C, D**). These results indicate that
403 the acute excitatory effect of both *T. crassiceps* homogenate and *T. crassiceps* E/S products is
404 mediated through GluRs.

405

406 *Taenia crassiceps* and *Taenia solium* larvae contain and produce glutamate.

407 Glutamate is the major excitatory neurotransmitter in the brain and the prototypical agonist of GluRs
408 (19). We therefore directly measured the concentration of glutamate in the *T. crassiceps* homogenate
409 we had been puffing onto neurons using a glutamate assay (see Materials and Methods). We found
410 that the mean concentration of glutamate in the *T. crassiceps* homogenate was 6.47 µg/mg (SEM 0.97
411 µg/mg, N = 5, **Fig. 7A**) **–** corresponding to approximately 112 µM for puffing experiments. Glutamate
412 at this concentration robustly activates GluRs in hippocampal pyramidal neurons (Forman *et al*.,
413 2017). *T. crassiceps*, whilst a closely related species to *T. solium*, is not the causative pathogen in
414 humans. We therefore also measured the glutamate concentration in homogenate prepared from
415 larvae of *T. solium*, the predominant pathogen responsible for human NCC. We recorded a glutamate
416 concentration of 4.56 µg/mg (N = 1), which indicates that the concentration of this excitatory
417 neurotransmitter is also high within larvae of *T. solium* (**Fig. 7A**).

418

419 Whilst glutamate is a neurotransmitter in the CNS, it is also an ubiquitous metabolite present in the
420 cytoplasm of the majority of cells (26). Therefore, it is possible that the *T. crassiceps* and *T. solium*
421 homogenate only contain glutamate due to cell lysis during homogenate preparation. To determine
422 whether both these larvae actively produce and excrete/secrete glutamate into their environments
423 we measured the *de novo* daily production of glutamate by larvae following harvest of live larvae from
424 the intermediate host (mice for *T. crassiceps* and pigs for *T. solium*, see Materials and Methods). Both
425 species of larvae were observed to generate glutamate *de novo* and release this into the culture media
426 (**Fig. 7B**). *T. crassiceps* larvae released a relatively constant daily amount of glutamate (mean 64.07
427 µg/15 larvae per day, SEM 4.31 µg/15 larvae per day, N = 3, **Fig. 7B**). *T. solium* larvae released a large
428 amount of glutamate on the first day post-harvest, none on the 2nd and 3rd day *in vitro,* but again begin
429 to produce large amounts of glutamate on days 4, 5 and 6 (mean 137.06 µg/15 larvae per day, SEM
430 58.49 µg/15 larvae per day, N = 1, **Fig. 7B**). Collectively these results demonstrate that larvae of both

431 *T. crassiceps* and *T. solium* contain and continually release glutamate into their immediate

432 surroundings.

433

434 *Discussion*

435 Here we used patch-clamp electrophysiology, and calcium imaging in hippocampal organotypic brain
436 slice cultures to demonstrate that *Taenia* larval products cause neuronal depolarization and can
437 initiate seizure-like events via glutamate receptor activation. Glutamate assays revealed that both *T.*
438 *crassiceps* and *T. solium* larvae contain and release considerable quantities of glutamate.

439

440 Clinical evidence and animal models conclusively demonstrate that the presence of larvae in the brain
441 can result in the generation of seizures (9,27). Previous work has focused on the involvement of the
442 host inflammatory response in seizure generation (10,28). Whilst this is likely important, it does not
443 preclude the involvement of additional or exacerbating pathogenic mechanisms for seizure
444 generation and epileptogenesis in NCC. In this study we have identified that *Taenia* larvae have a
445 direct excitatory effect on neurons via glutamatergic signaling. This is important, as the central role of
446 glutamatergic signaling in epileptogenesis has conclusively been shown using multiple cell culture (29–
447 31), slice (32–34), and *in vivo* models of epilepsy (35–37).

448

449 Clinically, in addition to NCC, the other major causes of adult acquired epilepsy are stroke, traumatic
450 brain injury and CNS tumors (38,39). In these other causes of acquired epilepsy, glutamatergic
451 signaling and glutamate excitotoxicity are thought to be central to the pathogenic process. Glutamate
452 excitotoxicity occurs when depolarized, damaged or dying neurons release glutamate, which activate
453 surrounding neurons via NMDA and AMPA receptors, resulting in sustained neuronal depolarization,
454 Ca^{2+} influx and the subsequent activation of signaling cascades and enzymes. These in turn lead to cell
455 death via necrosis and apoptosis (40), the further release of intracellular glutamate and the
456 propagation of the excitotoxic process to neighboring cells. In the neurons that survive, the prolonged
457 exposure to glutamate has been shown to cause hyperexcitability and seizures (29,31) via multiple
458 mechanisms including enhanced intrinsic excitability and NMDA receptor dependent disruption of
459 GABAergic inhibition (41–43). Given our finding that *Taenia* larvae contain and release significant

460 quantities of glutamate, it is possible that should homeostatic mechanisms for taking up and
461 metabolizing glutamate fail to compensate for larval-derived glutamate in the extracellular space,
462 similar glutamate-dependent excitotoxic and epileptogenic processes that occur in stroke, traumatic
463 brain injury and CNS tumors are likely to also occur in NCC.
464
465 Glioma, a common adult primary brain tumor, which typically presents with seizures in over 80% of
466 patients (44), is an intriguing condition for comparison to NCC. Here, the tumor cells themselves have
467 been shown to release glutamate into the extracellular space via the system x_c^-cystine-glutamate
468 antiporter (43). Interestingly, there is compelling evidence that tumoral release of glutamate via this
469 mechanism both causes seizures and favours glioma preservation, progression and invasion in cases
470 of malignant glioma (45–47). Analogously it is possible that *Taenia* larvae in the brain utilize the
471 release of glutamate and the induction of glutamate excitotoxicity to facilitate their growth and
472 expansion, with the accompanying effect of seizure generation. In addition, the death of *Taenia* larvae,
473 or larval-derived cells would also result in the release of metabolic glutamate, further contributing to
474 glutamate release and excitotoxicity. In the case of glioma, where the mechanism of glutamate release
475 by tumor cells is known, pharmacological agents (e.g. sulfasalazine), which block glutamate release
476 have considerable potential as therapeutic agents for reducing seizure burden in this condition.
477 Therefore, it is important that future work attempts to identify the molecular mechanisms underlying
478 the *Taenia*-specific production of glutamate in order to inform the development of new therapeutic
479 strategies to potentially reduce larval expansion and treat seizures in NCC.
480
481
482 An important consideration is how we might reconcile our findings of continuous glutamate release
483 by *Taenia* larvae with the clinical picture of delayed symptom onset in NCC. In people with NCC, some
484 experience acute seizures immediately following infection whilst others present with seizures months
485 to years following initial infection (48,49). This suggests that multiple different, or possibly interacting

486 mechanisms might be involved in the epileptogenic process in NCC. Neuroinflammation has long been
487 thought to be central to the development of seizures in NCC. In support of this, larval suppression of
488 host inflammatory responses could explain why many patients may remain asymptomatic for years
489 following infection. Our findings are not at odds with this line of thinking when considering the effect
490 of neuroinflammation on extracellular glutamate uptake. In the healthy brain parenchymal astrocytes
491 are optimized to maintain extracellular glutamate at low concentrations (50). However, the
492 inflammatory transition of astrocytes to a reactive phenotype is known to impede their ability to
493 buffer extracellular uptake (51). It is therefore conceivable that cyst-associated reactive astrocytosis
494 may gradually erode the ability of homeostatic mechanisms to compensate for larvae derived
495 extracellular glutamate resulting in delayed symptom onset in NCC.
496
497 It is clear that there is still considerable uncertainty regarding the precise sequences of events leading
498 to seizure onset in NCC. Nonetheless our findings provide the first evidence that as is the case with
499 the other common causes of adult acquired epilepsy (i.e. stoke, traumatic brain injury and glioma),
500 increased extracellular, parasite-derived glutamate likely also plays an important role in the
501 development of seizures in NCC.
502

503 *Figure Legends*

504 **Figure 1: *Taenia crassiceps* homogenate excites neurons and can elicit epileptiform activity.** A)
505 Schematic showing the experimental set up in which whole cell patch clamp recordings were made
506 from CA3 hippocampal pyramidal neurons in rodent organotypic slice cultures while a puffer pipette
507 delivered pico-litre volumes of homogenised *Taenia crassiceps* larvae (*T. crassiceps* hom.) targeted at
508 the cell soma. B) Current clamp recording from a rat pyramidal neuron while increasing amounts of *T.*
509 *crassiceps* homogenate was applied via the puff pipette (left to right, orange arrows). Small amounts
510 of *T. crassiceps* homogenate resulted in depolarization (left), increasing amounts elicited single
511 (middle left) or even bursts of action potentials (middle right) whilst a similar puff of aCSF had no
512 effect on the neuronal membrane potential (right, blue arrow), showing that potential mechanical
513 disruption of the cell by a puff is not sufficient to depolarise it. C) As in 'B', identical effects of *T.*
514 *crassiceps* homogenate could be elicited in current clamp recordings from a CA3 hippocampal
515 pyramidal neuron in a mouse organotypic brain slice culture. D) Top: widefield fluorescence image of
516 neurons in the dentate gyrus of a mouse hippocampal organotypic brain slice culture expressing the
517 genetically-encoded Ca^{2+} reporter GCAMP6s under the synapsin promoter in aCSF containing 0.5 mM
518 Mg^{2+}. A subset of neurons used to generate the Ca^{2+} traces in 'E' are indicated by different colours.
519 The orange dotted circle indicates where *T. crassiceps* homogenate was delivered using the puff
520 pipette. Bottom, schematic of the experimental setup including puffer pipette and CCD camera for
521 Ca^{2+} imaging using a 470 nm LED. E) Top, dF/F traces representing Ca^{2+} dynamics from the GCAMP6s
522 expressing neurons labelled in 'D' concurrent with 3 puffs (30 ms duration) of *T. crassiceps*
523 homogenate 15 s apart. Bottom: two images of raw Ca^{2+} fluorescence at two time points t_1 and t_2.
524 Note how at time point t_2 neurons distant to the site of *T. crassiceps* homogenate application are also
525 activated, indicating spread of neuronal activity. Inset, top-right: current clamp recording from a
526 neuron demonstrates that *T. crassiceps* homogenate application was able to evoke a seizure-like
527 event.

528

529 **Figure 2: Potassium contributes modestly to *Taenia crassiceps* induced neuronal depolarization.** A)
530 Whole-cell patch clamp recording using a standard internal in current clamp mode from a CA3
531 pyramidal neuron in a rat organotypic hippocampal slice culture. A 20 ms puff of *T. crassiceps*
532 homogenate (orange arrow) directed at the cell soma is delivered via a puffer pipette, causing
533 depolarization. B) As in 'A' but puffing aCSF containing 11.39 mM K^+ (equivalent to the K^+
534 concentration of *T. crassiceps* homogenate) caused modest depolarization. 2 µM TTX was added to
535 the aCSF to reduce synaptic noise in the voltage trace. C) As in 'A' but with a caesium based internal
536 electrode solution to block K+ channels and 2 µM TTX in the aCSF. Puffs of *T. crassiceps* homogenate
537 (orange arrow) resulted in sizeable depolarization. D) Population data showing that the response from
538 the 11.39 MM K^+ puff was significantly smaller than the *T. crassiceps* homogenate (with and without
539 caesium internal). Values with means ± SEM; *** $p \leq 0.001$.
540
541 **Figure 3: *Taenia crassiceps* homogenate induced neuronal depolarization is mediated by a small**
542 **molecule.** Whole-cell patch recordings in current clamp mode were made from CA3 pyramidal
543 neurons in rat organotypic hippocampal slice cultures using a caesium based internal in the presence
544 of 2 µM TTX. A) Delivery of a puff of *T. crassiceps* homogenate caused a depolarizing shift in
545 membrane potential. B) The depolarizing response to *T. crassiceps* homogenate was largely abolished
546 by dialysing out all molecules smaller than 3 kDa. C) Population data demonstrating that the *T.*
547 *crassiceps* homogenate response is significantly reduced by dialysis, indicating that the excitatory
548 component is a small molecule (< 3 kDa). Values with means ± SEM; *** $p \leq 0.001$.
549
550 **Figure 4: *Taenia crassiceps* excretory/secretory products cause neuronal depolarization.** A) Current
551 clamp, whole-cell patch recording from a rat CA3 pyramidal neuron using a caesium based internal
552 and 2 µM TTX in the aCSF. A 20 ms puff of *T. crassiceps* excretory/secreted (E/S) products caused a
553 marked depolarizing shift in voltage. B) Similar application of *T. crassiceps* E/S products smaller than
554 3 kDa also resulted in a large depolarizing response. C) In contrast, a puff of *T. crassiceps* E/S products

555 larger than 3 kDa resulted in a negligible shift in membrane potential. D) No change in membrane
556 voltage occurred in response to a puff of growth media, a control for the E/S products < 3 kDa. E) A
557 negligible depolarization of the membrane voltage occurred in response to a puff of phosphate-
558 buffered saline (PBS), a control for the E/S products > 3kDa. F) Population data demonstrating that *T.*
559 *crassiceps* E/S products as a whole or when separated to E/S products < 3kDa cause significant
560 depolarization when compared to either the larger constituent of E/S products or either of the control
561 substances. Values with means ± SEM; *** $p \leq 0.001$; ns = not significant, $p > 0.05$.
562
563 **Figure 5: *Taenia crassiceps* homogenate induced neuronal depolarization is not mediated by**
564 **nicotinic acetylcholine receptors, ASIC receptors, or Substance P.** Whole-cell patch recordings were
565 made from CA3 pyramidal neurons in rat organotypic hippocampal slice cultures using a caesium
566 based internal in the presence of 2 µM TTX. If necessary, current was injected to maintain the
567 membrane potential within 2 mV of -60 mV. A) The depolarizing response to a *T. crassiceps*
568 homogenate puff (orange arrow) before (black trace), during (blue trace) and after (grey trace) the
569 addition of a nicotinic acetylcholine receptor (nAchR) blocker (mecamylamine hydrochloride, 10µM).
570 B) Population data demonstrating that nAchR blockade has no significant effect on the *T. crassiceps*
571 homogenate induced depolarization. C) The depolarizing response to a *T. crassiceps* homogenate puff
572 (orange arrow) before (black trace), during (blue trace) and after (grey trace) the addition of an ASIC
573 receptor blocker (amiloride, 2 mM) D) Population data demonstrating that ASIC receptor blockade has
574 no significant effect on the *T. crassiceps* homogenate induced depolarization. E) Current clamp trace
575 showing that a puff of 100 µM Substance P (blue arrow) does not affect the membrane potential of
576 CA3 pyramidal neurons. F) Population data for Substance P application. ns = not significant, $p > 0.05$.
577
578 **Figure 6: The depolarizing effects of *Taenia crassiceps* are mediated by glutamate receptor**
579 **activation.** A) Whole-cell current clamp recording from a rat CA3 pyramidal neuron in a hippocampal
580 organotypic slice culture. Neuronal membrane potential in response to a 20 ms puff of *T. crassiceps*

581 homogenate (orange arrow) before (black trace), during (blue trace) and following wash out (grey
582 trace) of a pharmacological cocktail to block glutamate receptors (10µM CNQX, 50µM D-AP5 and 2mM
583 kynurenic acid). B) Population data shows that the depolarization response to *T. crassiceps*
584 homogenate is significantly reduced in the presence of glutamate receptor blockers and returns upon
585 wash out. C) The depolarization response to a puff of *T. crassiceps* E/S products is also markedly
586 reduced during glutamate receptor blockade. D) Population data for glutamate receptor blockade
587 during *T. crassiceps* E/S products application. Values with means ± SEM; ***$p \leq 0.001$; **$p \leq 0.01$.
588
589 **Figure 7: *Taenia crassiceps* and *Taenia solium* larvae contain and produce glutamate.** A) Glutamate
590 levels in whole cyst hom. of *T. crassiceps* and *T. solium* larvae. B) Glutamate production per day per
591 15 larvae for 6 days *in vitro* for *T. crassiceps* and *T. solium* larvae. Note that both species of larvae
592 produce glutamate *de novo*, particularly on days 4 – 6.

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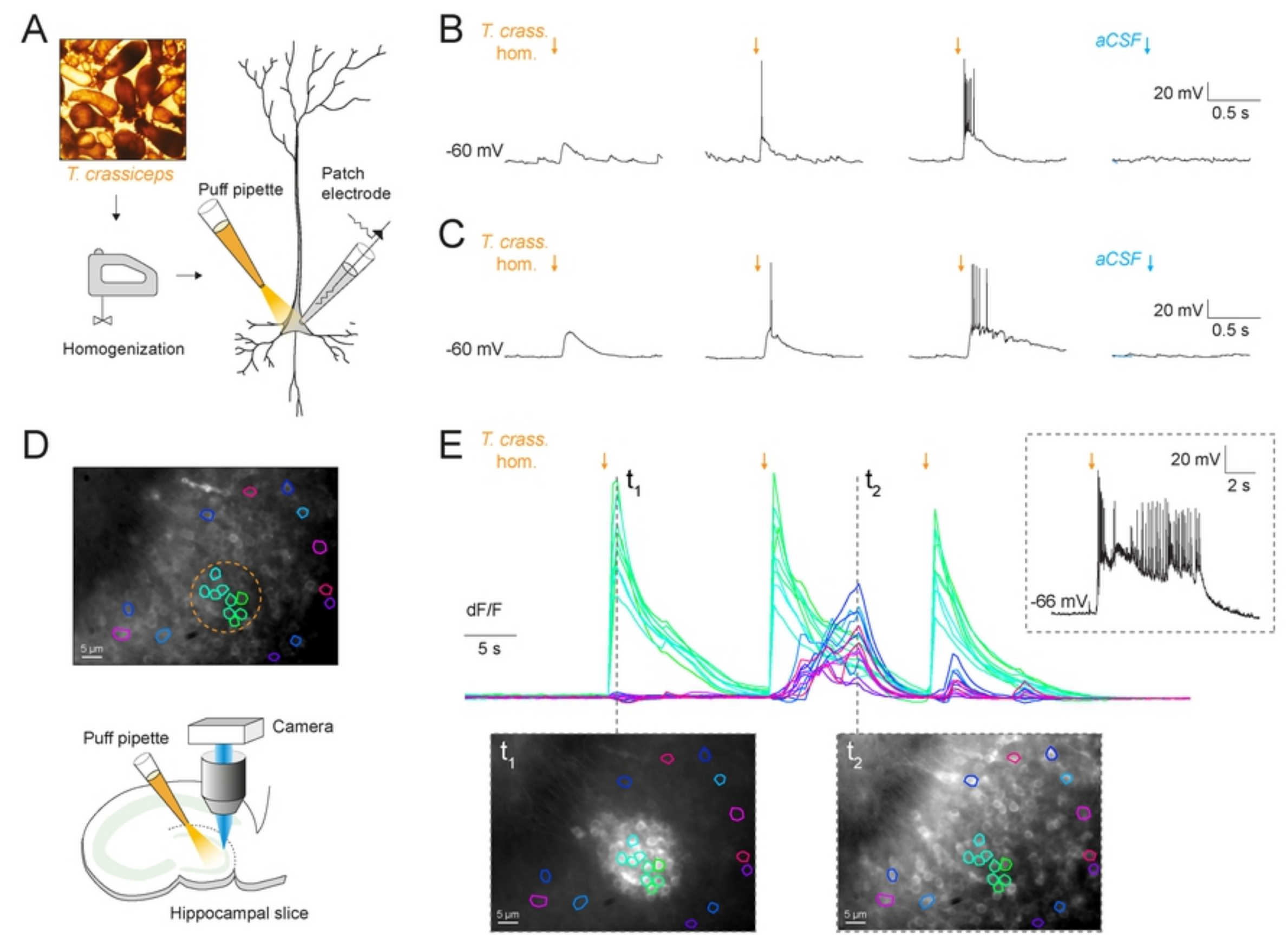

Figure 1: *Taenia crassiceps* homogenate excites neurons and can generate epileptiform activity

Figure 1

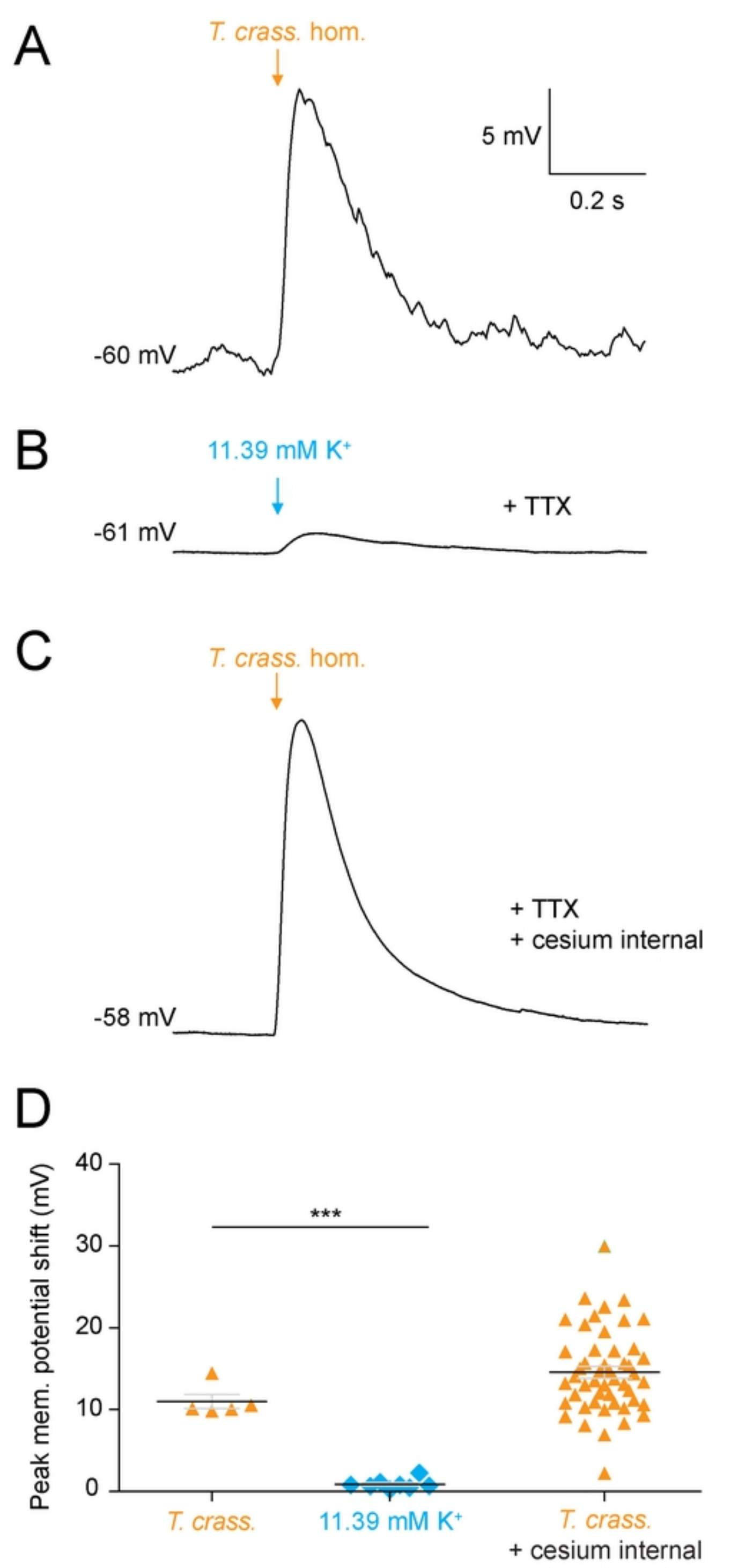

Figure 2: K^+ contributes modestly to *Taenia crassiceps* induced neuronal depolarization

Figure 2

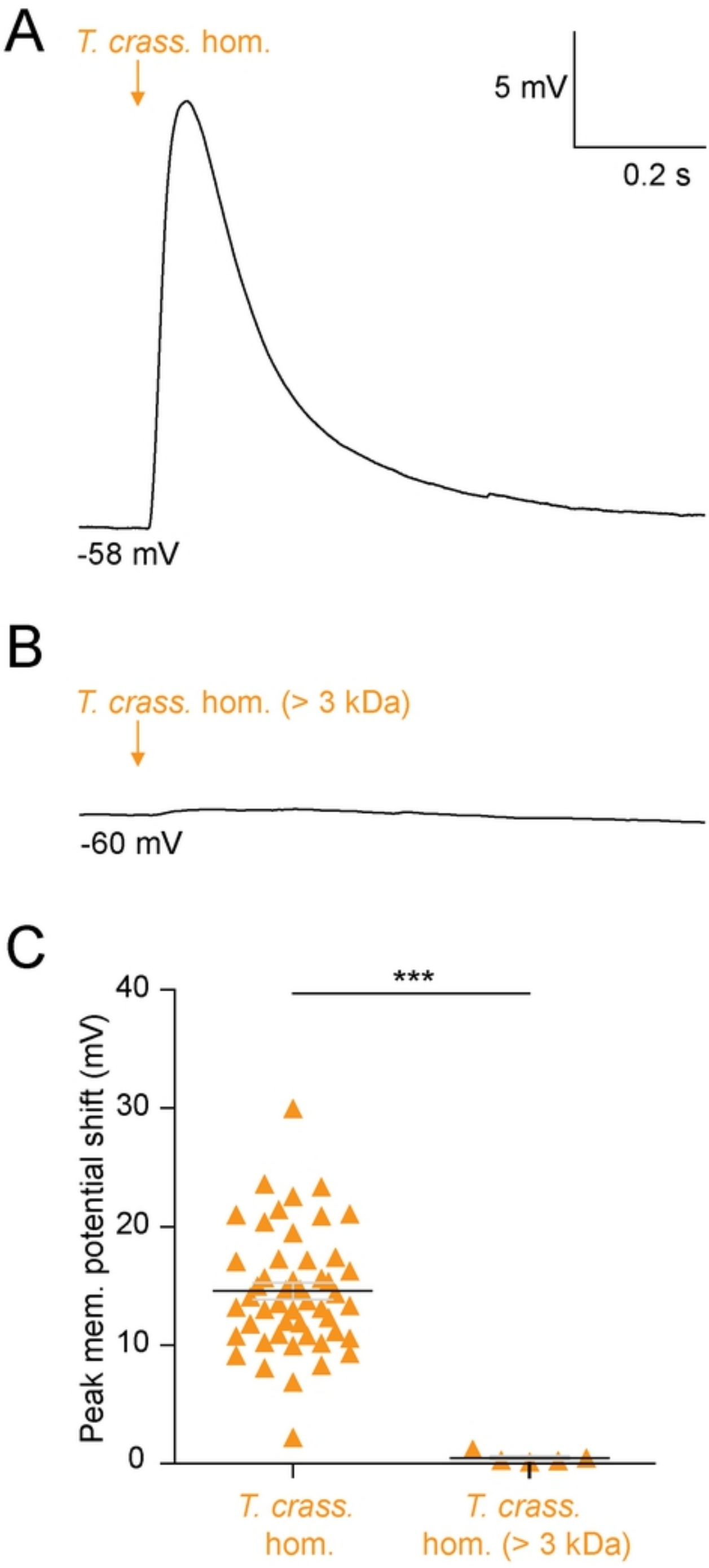

Figure 3: *Taenia crassiceps* homogenate induced neuronal depolarization is mediated by a small molecule

Figure 3

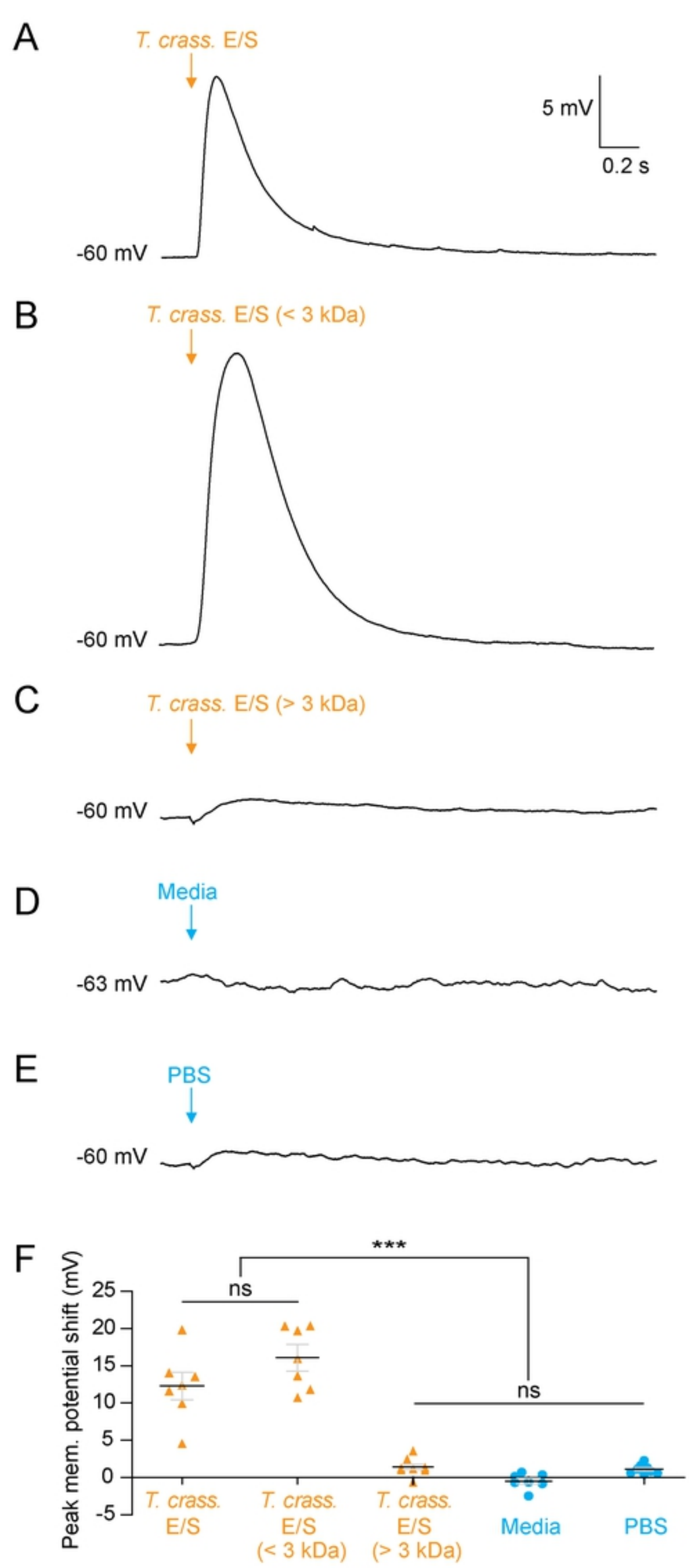

Figure 4: *Taenia crassiceps* excreted / secreted products cause neuronal depolarization

Figure 4

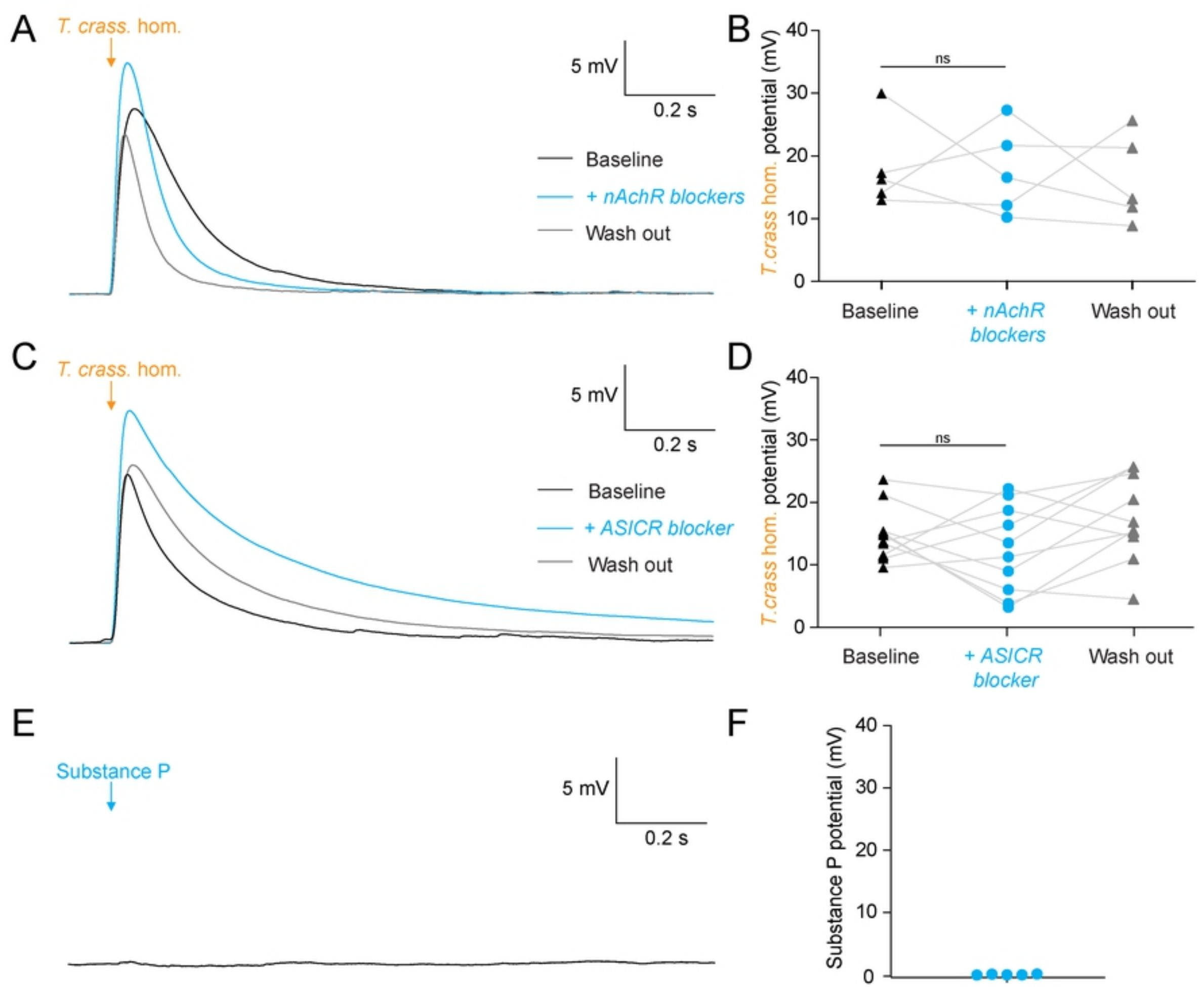

Figure 5: *Taenia crassiceps* homogenate induced neuronal depolarization is not mediated by acetylcholine receptors, ASIC receptors nor Substance P

Figure 5

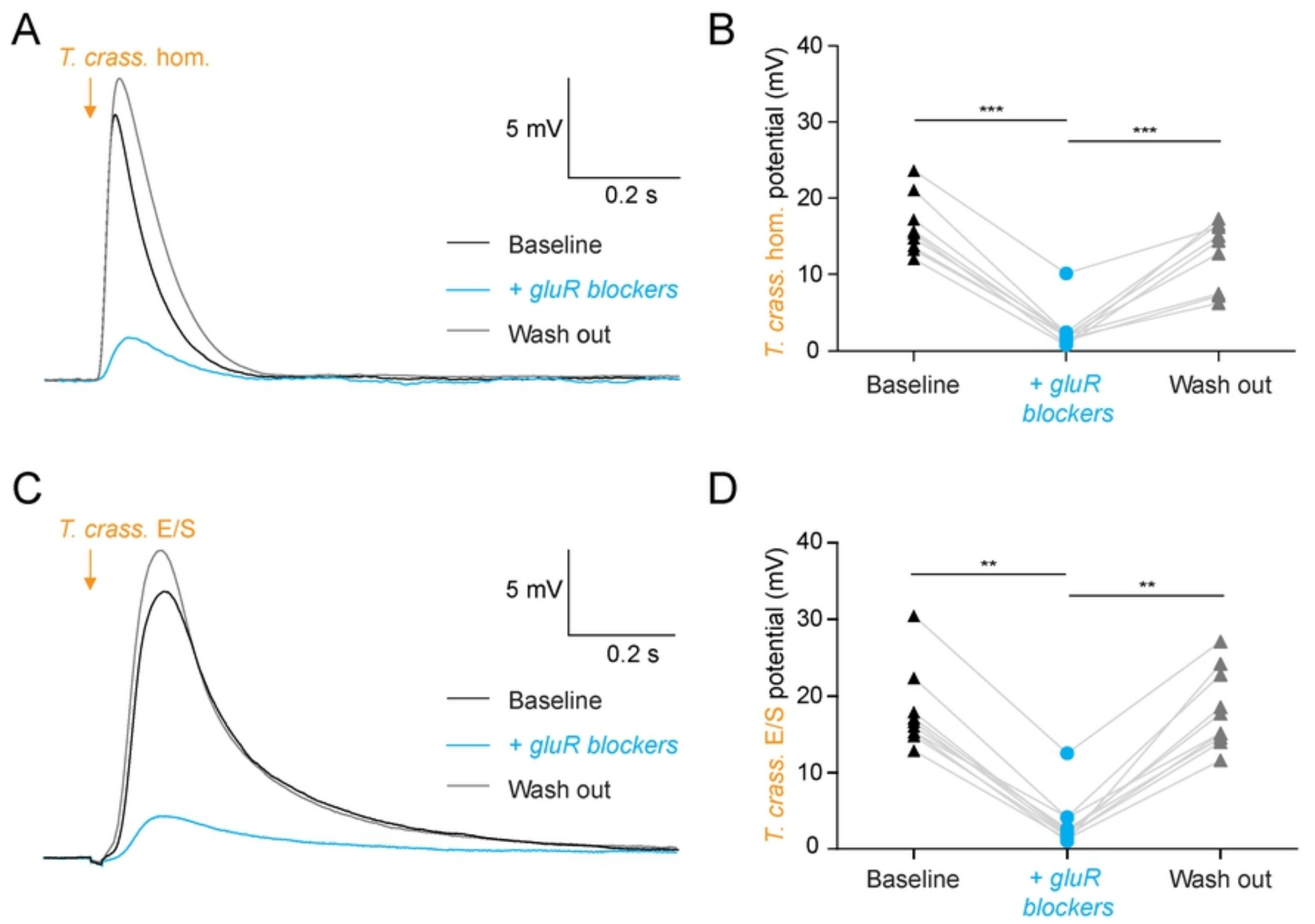

Figure 6: The excitatory effects of *Taenia crassiceps* are mediated by glutamate receptor activation

Figure 6

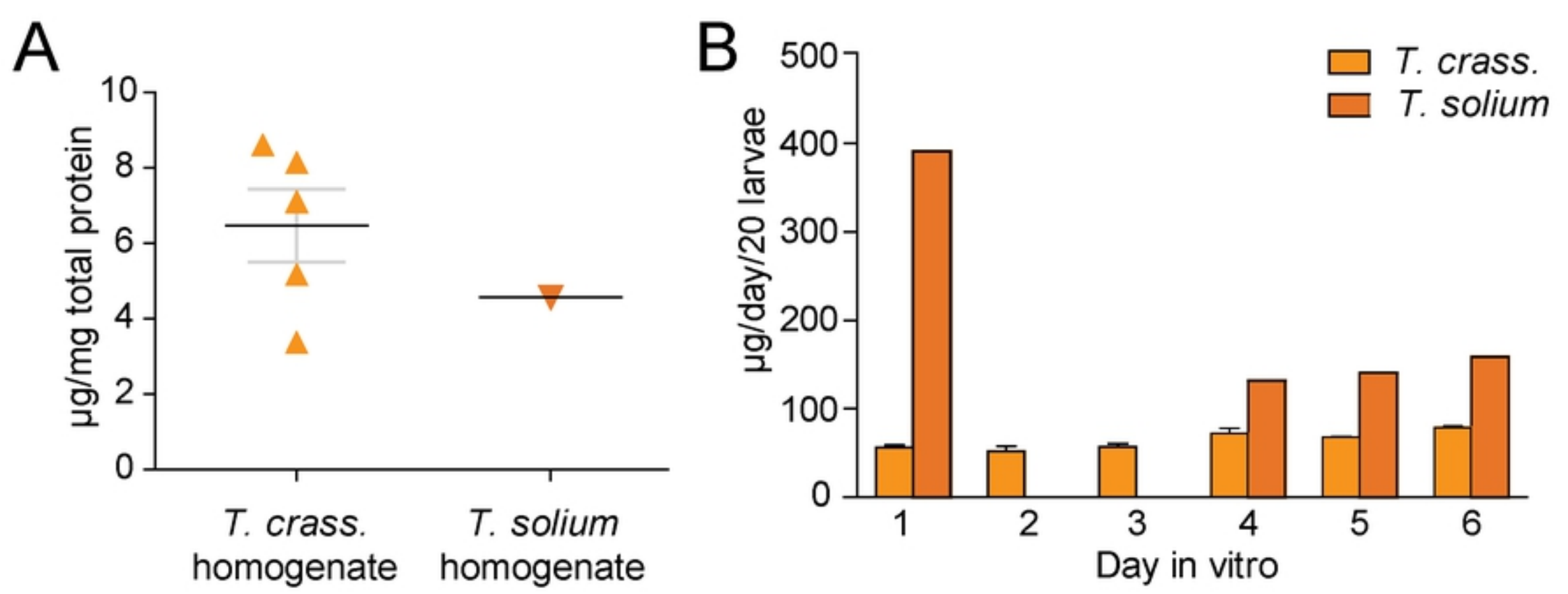

Figure 7: *Taenia crassiceps* and *Taenia solium* larvae contain and produce glutamate.

Figure 7