Title: Almost nontoxic tetrodotoxin analog, 5,6,11-trideoxytetrodotoxin, as an olfactory chemoattractant for the grass puffer

Running title: Grass puffer can smell TTX analog

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Keywords

Tetrodotoxin (TTX), TTX analog, chemoattractant, olfactory sensory neurons, EOG

Summary statement:

Electroolfactogram, behavioral experiments, and activity-dependent labeling of olfactory sensory neurons suggest that an almost nontoxic TTX analog, 5,6,11-trideoxyTTX, acts as an olfactory chemoattractant for grass puffers.

Abstract (217 words; max 250 words)

Toxic puffers contain the potent neurotoxin, tetrodotoxin (TTX). Although TTX is considered to serve as a defense substance, previous behavioral studies have demonstrated that TTX (extracted from the ovary) acts as an attractive pheromone for some toxic puffers. To determine the putative pheromonal action of TTX, we examined whether grass puffers (*Takifugu alboplumbeus*) can detect TTX using electrophysiological, morphological, and behavioral experiments.

Electroolfactogram results suggest that the olfactory epithelium of grass puffers responded in a dose-dependent manner to a type of TTX analog (5,6,11-trideoxyTTX), although it did not respond to TTX. We also examined the attractive action of 5,6,11-trideoxyTTX on grass puffers by recording their swimming behavior under dark conditions. Grass puffers preferred to stay on the side of the aquarium where 5,6,11-trideoxyTTX was administered, and their swimming speed decreased. Additionally, odorant-induced labeling of olfactory sensory neurons using a fluorescent dextran conjugate or immunohistochemistry against phosphorylated extracellular signal regulated kinase (pERK) revealed that labeled olfactory sensory neurons were localized in the region surrounding “islets” where there was abundant cilia on the olfactory lamella.

5,6,11-trideoxyTTX has been known to accumulate in grass puffers, but its toxicity is much lower (almost nontoxic) than TTX. Our results suggest that grass puffers can detect 5,6,11-trideoxyTTX using their nose and may positively use this functionally unknown TTX analog as an olfactory chemoattractant.

Introduction

Toxic puffers accumulate the potent neurotoxin, tetrodotoxin (TTX), which is a well-known antagonist of voltage-gated sodium channels. TTX was first believed to be present only in pufferfish from the family Tetraodontidae. However, it was later detected in taxonomically diverse animals (e.g., teleosts, amphibians, echinoderms, crustaceans, mollusks, and worms) inhabiting terrestrial, marine, freshwater, and brackish water environments (Noguchi and Arakawa, 2008).

Regardless of the abundant studies, the biological origin of TTX and its ecological functions remain unclear. However, the presence of TTX in such a variety of organisms suggests that the ultimate origin of TTX is exogenous or that it is symbiotically produced by marine bacteria (Noguchi et al., 2006a). TTX and its analogs were detected in potential pufferfish prey, including starfish, gastropods, crustaceans, flatworms, and ribbonworms, in addition to several species of bacteria that are free-living or symbiotic with pufferfish or their potential food organisms (Noguchi and Arakawa, 2008). Several shell fragments of the TTX-bearing gastropod *Zeuxis siquijorensis* were detected in the digestive tract of the toxic puffer *Takifugu pardalis* (Noguchi et al., 2011). Additionally, juveniles of the cultivated tiger puffer *T. rubripes* became nontoxic in aquaria or cages that were suspended above the seafloor with a TTX-free diet (Honda et al., 2005; Noguchi et al., 2006b). These results suggest that TTX in pufferfish is a result of its accumulation through the food chain.

Why do toxic puffers accumulate TTX in their bodies? Some toxic puffers have TTX-secreting glands on the skin (Kodama et al., 1986), and electrical stimulation induced TTX secretion from the body surface of these puffers (Kodama et al., 1985). TTX induces repulsive or avoidance reactions in predatory fish (Sakakura et al., 2017); predators that ingested *Takifugu* larvae spat them out promptly (Itoi et al., 2014, 2018a). Thus, in toxic puffers, TTX functions as a biological defense substance against predators.

Although the main function of TTX in pufferfish appears to be defensive, several studies have reported that pufferfish can exploit TTX as a source of communication. Sexually active male grass puffers (*T. alboplumbeus*) were attracted to TTX (Matsumura, 1995). Artificially raised nontoxic tiger puffer (*T. rubripes*) juveniles were also attracted to TTX (Saito et al. 1997; 2000; Okita et al. 2013). These studies suggest that pufferfish may sense TTX. However, the TTX-sensing mechanism in these puffers remains unclear, although the

attractive effect of TTX on *T. rubripes* juveniles disappeared after nose ablation (Okita et al., 2013).

In this study, we found that a TTX analog, 5,6,11-trideoxyTTX, is an olfactory chemoattractant for the grass puffer. We demonstrated the presence of the olfactory sensing ability, specific olfactory sensory neurons (OSNs), and the attraction behavior of grass puffers in response to 5,6,11-trideoxyTTX. To the best of our knowledge, this is the first report that pufferfish positively use functionally unknown TTX analogs for chemical communication.

Materials and Methods

Animals

One hundred fifty-nine grass puffers (body length, 5–12 cm; bodyweight, 6–32 g) were collected from the coastal waters of Ise-bay in Minamichita-Cho on the Pacific coast of central Japan by line-fishing. Grass puffers were fed commercial diets (Tetra Krill-E; Spectrum Brands, Madison, WI, USA) in aerated 300 L tanks (10–25°C, approximately 50 fish per tank) filled with artificial seawater (ASW; Instant Oceans® Sea Salt, Cl^- 543 mM, Na^+ 471 mM, Mg^{2+} 54.3 mM, K^+ 10.7 mM, Ca^{2+} 10 mM Br^- 0.7 mM, Sr^{2+} 0.1 mM, F^- 0.05 mM, I^- 0.002 mM, CO_3^{2-}/HCO_3^- 3.3 mM, pH 8.2; Instant Ocean, Blacksburg, VA, USA) under a 12-h light/12-h dark photoperiod. In this study, both males and females were used without distinction. All animals were maintained and used in accordance with the Nagoya university regulations on animal care and use in research.

Odorant chemicals

TTX (for biochemistry, citrate buffered, cat. No. 206–11071, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in ultra-pure water at 1.5×10^{-3} M. Two types of TTX analogs, 4,9-anhydroTTX and 5,6,11-trideoxyTTX, were synthesized and purified according to the methods described by Adachi et al. (2014a) and Adachi et al. (2014b), respectively. Synthesized TTX analogs were dissolved in 2.5×10^{-2} N acetic acid at 10^{-3} M and stored at −30°C until use. L-Arginine (Arg; a food odor cue for teleosts; FUJIFILM Wako) was prepared as a 10^{-2} M stock in artificial cerebrospinal fluid (ACSF; NaCl 231 mM, KCl 8.1 mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 2.4 mM, D-glucose 10 mM, 4-(2-

112 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM; pH adjusted 7.4 with
113 NaOH). For testing, test odorant solutions were prepared fresh as required by diluting
114 aliquots of concentrated stock in ACSF or in ASW. L-Arginine was always tested at 10^{-5} M.
115 When testing a series of solutions with different concentrations, the lowest dose was tested
116 first, followed by solutions with concentrations that increased by a factor of 10 to 10^{-4} M. A
117 vehicle solution was prepared in an identical manner by diluting 2.5×10^{-2} N acetic acid in
118 ACSF or ASW (final concentration, 2.5×10^{-4} N).

119

120 Electroolfactogram recordings

121 Electroolfactogram (EOG) recordings were performed on secured grass puffers on a holding
122 apparatus that was placed into a plastic experimental trough. Before recording, the fish was
123 anesthetized by immersion in tricaine methanesulfonate (MS–222; 0.02%). The anesthetized
124 grass puffer was mounted on the center groove of a hand-made plastic holder by wrapping it
125 in a paper towel and aluminum foil after attaching an Ag–AgCl plate (1 × 1 cm) with the
126 body wall as a reference electrode. The gills were perfused with ASW containing 0.01% MS–
127 222 through a tube inserted into the mouth throughout the experiments (Flow rate: 2.5
128 mL/min). The skin and cartilage covering the right olfactory cavity were opened, and the
129 exposed olfactory cavity was continuously perfused with ACSF using an 18 G stainless
130 needle (NN–1838S; Terumo, Tokyo, Japan). The perfusion rate of ACSF was 1.0 mL/min. A
131 computer-controlled solenoid valve (UMG1–T1; CKD, Komaki, Japan) delivered 10-s pulses
132 of test solutions by switching between the ACSF and test solutions (diluted appropriately in
133 ACSF) at 3-min intervals.

134 EOG responses were recorded using a saline–agar (0.6%)-filled glass capillary (tip
135 diameter, 100 μm) that was bridged with an Ag–AgCl wire filled with ACSF. The
136 indifference electrode was placed lightly on the head skin surface near the perfused olfactory
137 cavity, and the recording electrode was positioned over the olfactory rosette using
138 micromanipulators (M-152 and MM-3; Narishige, Tokyo, Japan). The electrodes were
139 insulated with a paraffin–Vaseline paste that was placed on the surface of the fish's head.
140 Electrical signals were differentially amplified (gain 1,000×; Band-pass 0.8–100 Hz) using a
141 DC preamplifier (AVH-11 in VC-11 digital oscilloscope; Nihon-Kohden, Tokyo, Japan) and
142 notch filtered through a 60 Hz noise eliminator (Humbug; Quest Scientific, North Vancouver,
143 Canada). The amplified EOG signal was digitized (10 kHz) and stored on a laptop computer

144 (ThinkPad T61; Lenovo, Beijing, China) using a USB-6251 data acquisition device (National
145 Instruments, Austin, TX, USA) and WinEDR V3.2.7 software
146 (http://spider.science.strath.ac.uk/sipbs/software_ses.htm; Strathclyde University, Glasgow,
147 UK). In this study, we showed negative potential changes in the EOG as upward deflections.

148

149 Behavioral experiments

150 Behavioral experiments were performed using adult male and female grass puffers. For the
151 behavioral assay, a grass puffer was transferred to a test aquarium (50 × 20 × 20 cm) that was
152 filled with 5 L of fresh artificial seawater. The test aquarium was covered with a black curtain
153 and illuminated by an infrared LED-array illuminator (940 nm, ca. 2 W; AE-LED56V2,
154 Akizuki Denshi Tsusho Co., Tokyo, Japan) so that the experimenter was invisible to the fish.
155 A video camera (XC-ST50, Sony, Tokyo, Japan) equipped with a low distortion C-mount
156 lens (f = 4.4 mm; LM4NC1M, Kowa, Nagoya, Japan) was mounted vertically above the
157 aquarium, and the camera system was adjusted so that the aquarium filled the camera frame.
158 The fish was allowed to acclimate to a dark environment for 90–180 min.

159 Time-lapse image acquisitions from the top of the test aquarium were started (1 s
160 intervals). The time-lapse images were acquired on a laptop computer (ThinkPad T410;
161 Lenovo, Beijing, China) using image acquisition software (Micro-Manager 1.4.22;
162 https://micro-manager.org) and a USB-video capture unit (PCA-DAV, Princeton, Tokyo,
163 Japan) at a resolution of 640 × 480 pixels. After 6 min of image acquisition, 5 mL of 10^{-5} M
164 5,6,11-trideoxyTTX or vehicle (5 mL of 2.5×10^{-4} N acetic acid) was gently applied to one
165 side of the test aquarium through a silicone tube attached to a 10 mL syringe. Then, image
166 acquisitions were continued for up to 6 min. The numeric x–y coordinates representing the
167 movement of the fish over time (one coordinate in a frame) were extracted from each video
168 using the UMA tracker software (http://ymnk13.github.io/UMATracker/; Yamanaka and
169 Takeuchi, 2018). We first binarized the grayscale images using appropriate filters that were
170 produced by the UMATracker. We then tracked the fish's position using the "Group tracker
171 GMM" algorithm and exported the coordinates as a text file.

172

173 Activity-dependent labeling of ORNs

174 To study the ligand specificity of olfactory receptor neurons, we used odor-induced uptake of
175 fluorescent dye-conjugated dextran (Døving et al., 2011). The fish was anesthetized using

176 0.02% MS-222 and secured on the holding apparatus used in EOG experiments. The gills
177 were continuously perfused with artificial seawater containing 0.01% MS-222 through a tube
178 inserted into the mouth throughout the procedure. The skin and cartilage covering the
179 olfactory cavity were opened, and the exposed olfactory cavity was continuously perfused
180 with ACSF using an 18G stainless needle. The olfactory cavity of anesthetized fish was
181 perfused with L-Arginine (Arg; 10^{-5} M) or 5,6,11-TrideoxyTTX (10^{-5} M) together with a
182 dextran conjugated with Alexa Fluor® 555 (M.W. 10,000, 10^{-6} M; D34679, Thermo Fisher
183 Scientific, MA, USA) for 5 min. In the control experiments, the olfactory cavity of
184 anesthetized fish was exposed to only Alexa Fluor 555-dextran (10^{-6} M) for 5 min. The flow
185 rate was approximately 1.0 mL/min. After the odorant application, the olfactory cavity was
186 rinsed with a continuous flow of ACSF for at least 5 min. Fish were kept alive for 3 to 4 h in
187 aerated artificial seawater at room temperature. Then, the fish was re-anesthetized using 0.1%
188 MS-222 and decapitated. The decapitated head was immersed in 4% paraformaldehyde in 0.1
189 M PBS (pH 7.2, 4°C, 1 h). After fixation, the olfactory lamellae were dissected and rinsed
190 with PBS. The olfactory lamellae were then counterstained with 4′,6-diamidino-2-
191 phenylindole (DAPI; 1 ng/mL, 5 min) and mounted on a hole glass slide with mounting
192 medium (10% Mowiol 4-88, 25% glycerol, 0.2 M Tris-HCl (pH 9.0), and 2.5%-3.0% 1,4-
193 diazabicyclo [2,2,2] octane; diluted with ultrapure water).

194 For immunohistochemistry, we excised the olfactory lamellae from anesthetized fish.
195 The exposed olfactory lamella was washed three times for 5 min in ACSF and stimulated
196 with odorant solutions (vehicle, arginine, TTX, or 5,6,11-trideoxyTTX; 10^{-6} M, 1 mL, diluted
197 with ACSF) for 5 min in a 1.5 mL tube. After odor stimulation, olfactory epithelium (OE)
198 was fixed in 4% paraformaldehyde and 15% picric acid in 0.1 M PBS overnight at 4°C,
199 rinsed in PBS, and cryoprotected in 30% sucrose in PBS overnight at 4ºC. OE was embedded
200 in Tissue-Tek® O.C.T. compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan). Cross
201 cryosections (14–20 μm) of OE mount onto gelatin-coated slides and dried. After rehydration
202 in PBS, the sections were blocked in 2% normal goat serum (NGS) in PBS with 0.05%
203 Triton-X100 (PBST) for 3 h at 28°C.

204 The sections were then incubated with anti-phosphorylated-p44/42 mitogen-activated
205 protein kinase (MAPK; extracellular regulated kinase, ERK1/2) rabbit monoclonal antibody
206 (#4370, Cell Signaling Technology, Danvers, MA, USA; 1:500), which was used in zebrafish
207 olfactory epithelium as validated in Biechl et al. (2016), as a neural activity marker that was
208 diluted with 0.2% NGS in PBST for 2 days at 4°C. The sections were washed three times for

209 5 min with 0.2% NGS in PBST. Sections were then incubated with Alexa Fluor® 488-
210 conjugated secondary antibody (goat anti-rabbit IgG (H+L); A-11008, Thermo Fisher
211 Scientific, 1:500) that was diluted with 0.2% NGS in PBST for 1 day at 4°C. The sections
212 were washed three times for 5 min in PBST. Finally, sections were counterstained with DAPI
213 (1:1000) for 5 min and mounted using mounting medium.

214 A confocal microscope (FluoVIEW 1000D, IX81, Olympus, Tokyo, Japan) was used
215 to observe the details of olfactory lamellae. Pictures were taken in planes and were separated
216 by 0.45 μm z-axis steps. Confocal microscopy images were analyzed using FluoRender
217 (http://www.sci.utah.edu/software/fluorender.html; Wan et al., 2012) for three-dimensional
218 (3D) reconstruction and visualization of dextran-labeled cells. Pre-processing of images
219 before 3D reconstruction (median filtering [radius: 1 pixel] and trimming) was performed by
220 Fiji (https://fiji.sc/).

221

222 Data analysis

223 All animal experiments were performed on fish of both sexes. Statistical analyses were
224 performed using Kyplot5 (Kyence, Tokyo, Japan) and Igor Pro 6 software (WaveMetrics
225 Inc., Lake Oswego, OR, USA). Data are presented as a dot plot or a box–whisker plot (box:
226 median and quartiles; whiskers: 10%–90% range) or mean ± standard error of the mean
227 (SEM). The statistical test that was used in each experiment is shown in the corresponding
228 figure legend. No statistical methods were used to predetermine the sample size because
229 many of the outcomes were unknown.

230

231 **Results**

232 EOG response of grass puffers to TTX

233 We first performed EOG recordings of adult grass puffers in response to a commercially
234 available TTX. The EOG response to a 10-s stimulus with an amino acid solution (10^{-5} M L-
235 Arg) was a slow monophasic potential with a steep rising phase followed by an exponential
236 decay toward the baseline (Fig.1A, bottom trace). However, TTX (10^{-7} and 10^{-5} M) did not
237 induce significant changes in the EOG (Fig. 1A upper and middle traces, respectively). The
238 EOG amplitude of 10^{-7} M TTX (0.027 ± 0.007 mV, n = 4; Fig. 1B○) and 10^{-5} M TTX (0.026

239 ± 0.007 mV, n = 7; Fig. 1B●) were significantly lower than that of 10^{-5} M L-Arg (0.074 ±
240 0.008 mV, n = 11; Fig. 1B■).

241

242 EOG response to TTX analogs

243 We then examined the EOG responses of the grass puffer to the two synthesized intrinsic
244 TTX analogs. A TTX analog, 4,9-anhydroTTX, did not induce significant changes in EOG
245 traces (10^{-5} M; Fig. 2A, second trace) when the same concentration of L-Arg showed clear
246 EOG responses (Fig. 2A, bottom trace). However, the same concentration of another TTX
247 analog, 5,6,11-trideoxyTTX, induced a clear increase in EOG response (Fig. 2A, third trace).
248 Vehicle control (2.5×10^{-5} N acetic acid solution in ASW) did not induce any changes in
249 EOG traces (Fig. 2A, top trace). EOG amplitude of 4,9-anhydroTTX, 5,6,11-trideoxyTTX,
250 and L-Arg (all 10^{-5} M) was 0.027 ± 0.004 mV [n = 4], 0.197 ± 0.029 mV [n = 7], and 0.128 ±
251 0.017 mV [n = 7], respectively. EOG amplitudes of 5,6,11-trideoxyTTX and L-Arg were
252 significantly larger than those of the vehicle (0.032 ± 0.007 mV [n = 5]; Fig.2B).

253 We also examined the dose–response relationship of the EOG response to 5,6,11-
254 trideoxyTTX. Threshold EOG response concentration for 5,6,11-trideoxyTTX ranged
255 between 10^{-7} and 10^{-8} M (Fig. 3A). The response amplitude increased with a logarithmic
256 increase in the concentration, and no response saturation was observed over the concentration
257 range tested (Fig. 3B). These results suggest that the olfactory epithelium of the grass puffers
258 can detect 5,6,11-trideoxyTTX.

259

260 Behavioral experiments

261 We next examined the behavioral response of adult grass puffers to 5,6,11-trideoxyTTX. One
262 mature grass puffer was put in a test aquarium filled with 5 L of fresh artificial seawater (50
263 × 20 × 20 cm) and acclimated to the fish for the test environment in the dark (>1 h). Then, 5
264 mL of Vehicle or 5,6,11-trideoxyTTX (10^{-5} M) was administered to one side of the aquarium
265 (the final estimated concentration of 5,6,11-trideoxy TTX was 10^{-8} M). Before
266 administration, the fish intermittently swam left and right and occasionally stopped in one
267 place in the aquarium (Fig.4 and 5, A–C). Vehicle administration did not change the
268 swimming patterns of the fish (Fig. 4D–F). This swimming pattern was reflected in the time
269 vs. X-axis position plot (Fig. 4G) and the time vs. swimming velocity plot (Fig. 4H).
270 Conversely, administration of 5,6,11-trideoxyTTX caused the fish to approach and frequently

271 stop on the administered side (Fig. 5D and E). Although the time vs. x-axis plot did not show
272 an apparent change in the swimming pattern (Fig. 5G), 5,6,11-trideoxyTTX administration
273 decreased the swimming velocity of the fish (Fig. 5H).

274 To quantify the degree of attraction, we calculated the time spent on the administered
275 sides and the mean swimming velocity of the individual grass puffers for every 1 min period.
276 Time spent by the grass puffers on the administered side was longer with the 5,6,11-
277 trideoxyTTX treatment than with the vehicle treatment ($p<0.01$, two-way ANOVA; Fig. 6A
278 and B), and the swimming velocity of grass puffers was decreased by 5,6,11-trideoxyTTX
279 compared to the vehicle treatment ($p<0.01$, two-way ANOVA; Fig. 6C and D). These results
280 suggest that 5,6,11-trideoxyTTX worked as a chemoattractant for grass puffers.

281

282 Activity-dependent labeling of 5,6,11-trideoxyTTX responding OSNs

283 To identify OSN that responded to 5,6,11-trideoxyTTX, we first observed the gross
284 morphology of the olfactory epithelium from the grass puffer. Differential interference
285 microscopy images of live olfactory lamellae showed that the surface of olfactory lamella had
286 repeated structures in which the elliptical "islets" were bordered by oval cells and exposed on
287 the surface (arrowheads in Fig. 7A). The cross-sectional structure of the olfactory lamellae
288 showed that oval cells (arrowheads in Fig. 7B, C, and F) surrounded small islets where there
289 were many cilia (SE in Fig. 7A and B).

290 Exposing the olfactory organ of the grass puffer to 5,6,11-trideoxyTTX (10^{-6} M) with
291 10^{-6} M of 10 kDa dextran conjugated with the fluorescent dye, Alexa Fluor 555, resulted in
292 the labeling of several cells (arrows in Fig. 7G). The labeled cells were primarily found in the
293 surrounding region of the islets (Fig. 7H, in the rectangular region). Application of Alexa
294 Fluor 555-dextran without 5,6,11-trideoxyTTX did not lead to the labeling of any cells in the
295 epithelium (Fig. 7D). Activity-dependent labeling of Alexa Fluor 555 was frequently seen as
296 bright granular aggregations within the cell (Fig. 7I, J, and K). Additionally, an axon-like
297 protrusion that extended from the basal membrane side of the labeled cell could be observed
298 (arrow in Fig. 7K).

299 We also performed immunohistochemistry of OE with the pERK antibody as another
300 activity-dependent labeling of 5,6,11-trideoxyTTX-sensitive OSNs. Excised OE from grass
301 puffers was exposed to 5,6,11-trideoxyTTX (10^{-6}M), L-Arg (10^{-6}M), or vehicle for 1 min.
302 Administration of 5,6,11-trideoxyTTX activated oval cells clustering on the surface of the

303 OE (Fig. 8A), whereas L-Arg administration labeled elongated cells with strongly labeled
304 cilia whose somata were in the middle layer of the OEs (close to the basal membrane; Fig.
305 8B). Additionally, 5,6,11-trideoxyTTX-sensitive OSNs were localized in the NSE area of
306 Fig. 7A. However, Vehicle administration did not activate any type of OSNs (Fig. 8C).

307

308 **Discussions**

309 In this paper, we electrophysiologically, morphologically, and behaviorally demonstrated that
310 one of the TTX analogs, 5,6,11-trideoxyTTX, worked as an olfactory chemoattractant for
311 grass puffers, *T. alboplumbeus*. To the best of our knowledge, this is the first report that
312 shows that pufferfish positively use a functionally unknown, almost nontoxic TTX analog for
313 chemical communications.

314

315 <u>5,6,11-trideoxyTTX, but not TTX, is an odorant for grass puffers</u>

316 We first examined whether commercially available TTX induces EOG responses in grass
317 puffers. A previous behavioral study reported that TTX attracts male grass puffers during the
318 spawning period (Matsumura 1995). TTX also attracted juvenile tiger puffers regardless of
319 sex (Okita et al., 2013; Saito et al., 1997, 2000), and this attraction effect was blocked by
320 nose ablation (Okita et al., 2013). However, TTX did not induce any EOG responses in our
321 study. Previous studies used crude TTX that was extracted from the pufferfish ovary or liver.
322 Okita et al. (2013) reported that they purified their crude TTX from the ovary of wild-caught
323 adult tiger puffers, using the method of Ikeda et al. (2009). They reported that their crude
324 TTX from pear puffer (*T. vermicularis*) ovaries contained both TTX and TTX analogs,
325 including 5,6,11-trideoxyTTX, 4-epi TTX, and 4,9-anhydroTTX. We used a commercially
326 available TTX in this study. The manufacturer's datasheet indicated that this TTX was also
327 extracted from pufferfish ovary and liver, but the species were not specified. However, our
328 electrospray ionization mass spectrometry analysis indicated that the commercially available
329 TTX that we used did not contain detectable levels of these TTX analogs (except 4,9-
330 anhydroTTX, a chemically equilibrium analog of TTX; data not shown). Thus, we
331 hypothesized that the crude TTX extract from previous studies might contain TTX analogs,
332 and some of these TTX analogs may act as olfactory chemoattractants for the pufferfish.

333 To investigate this hypothesis, we examined the effects of two types of synthesized
334 TTX analogs (4,9-anhydroTTX and 5,6,11-trideoxyTTX) using EOG and a behavioral assay.

Our EOG recordings showed that 5,6,11-trideoxyTTX induced an EOG response in grass puffers. In our recordings, the EOG amplitude of 10^{-5} M for 5,6,11-trideoxyTTX was significantly greater than that at the same concentration of L-Arg, which is a well-known odorant for many teleosts. Furthermore, 5,6,11-trideoxyTTX induced EOG responses that were lower than 10^{-8}M. Because the stimulation threshold of amino acids on the fish olfactory system is 10^{-9}M to 10^{-7}M (Hara, 1994), 5,6,11-trideoxyTTX is a potent, powerful odorant for grass puffers.

5,6,11-trideoxyTTX attracts grass puffers

We examined whether adult grass puffers are attracted to 5,6,11-trideoxyTTX. In response to 5,6,11-trideoxyTTX administration to one side of the aquarium, grass puffers were attracted to the administered side of the aquarium and showed the behavior of slowing down and swimming in that area. A preliminary test indicated that diffusion indicator dye reached half of the experimental aquarium within 2 min after administration and had spread throughout the aquarium at the end of the observation period. Thus, we estimated that the grass puffers could recognize 5,6,11-trideoxyTTX ranging 2×10^{-8} to 1×10^{-5} M within 2 minutes after administration. These estimated 5,6,11-trideoxyTTX concentrations correspond to the threshold concentration of the EOG response, although the concentration is much higher than those of the previous behavioral studies. Mature male grass puffers were attracted to 1.5×10^{-11} M TTX (Matsumura, 1995). Both male and female juvenile tiger puffers also could recognize 3.7×10^{-9} (Saito et al., 2000) and 2.6×10^{-8} M (Okita et al., 2013) of TTX, respectively. In these studies, they started their “choice” experiments after diffusion of TTX from agar or gelatin on one side of the Y-maze aquarium. These differences in experimental paradigms seem to contribute to the difference in the effective TTX concentration. Alternatively, TTX analogs other than 5,6,11-trideoxyTTX may also involve chemoreception, and multiple forms of TTX analogs other than TTX and 4,9-anhydroTTX may act synergistically. In olfaction, a combination of several compounds can be perceived as a specific odor, or a series of structurally similar compounds can be detected by a single olfactory receptor. Most pheromone specificity is achieved using a combination of compounds (Wyatt, 2014). For example, female sex pheromones in moths usually consist of multicomponent blends of five to six hydrocarbons including unbranched fatty acids, alcohols, acetates, or aldehydes in particular combinations and ratios (de Bruyne and Baker, 2008). In goldfish and carp, a combination of species-specific metabolites of prostaglandin

368 F2α (PGF2α), a well-known releaser pheromone that induces sexual behavior, strongly
369 induces species-specific sexual behavioral responses (Lim and Sorensen, 2011). Thus, it is
370 possible that other metabolites in pufferfish may play a synergistic action with 5,6,11-
371 trideoxyTTX as a chemical signal to attract the opposite sex.

372

373 5,6,11-trideoxyTTX sensitive OSNs on the olfactory epithelium of grass puffers

374 The fluorescent dextran-labeled OSNs were sparsely distributed in the border area around the
375 ciliary "islets" on the olfactory lamella of grass puffers by the co-administration of 5,6,11-
376 trideoxyTTX. Comparative histological studies reported that the sensory epithelium of grass
377 puffers was dispersed in many discrete islets on the surface of the olfactory lamella, and the
378 border area of the sensory islet was classified as undifferentiated epithelium (Yamamoto and
379 Ueda, 1979). However, our fluorescent dextran-labeling experiments suggested that oval cells
380 that were activated by 5,6,11-trideoxyTTX were present in the border area around the sensory
381 islets. These oval cells are also labeled with pERK antibody, a neuronal activity marker, after
382 the exposure of 5,6,11-trideoxyTTX.

383 It is widely known that teleost fish are equipped with three types of OSNs that are
384 intermingled in the OE but show distinct morphology (Bazáes et al., 2013). First, there are
385 the ciliated cells with long dendrites and a few cilia, and second, there are microvillous cells
386 with shorter dendrites than the ciliated cells, which have microvilli extending from their
387 apical surface. The third type is the crypt cells. Crypt cells have an oval shape, are located
388 close to the epithelial surface, and have few cilia and microvilli (Hansen and Eckart, 1998).
389 Cells that were labeled with fluorescent dextran by 5,6,11-trideoxyTTX co-administration
390 had no cilia or microvilli and were located on the epithelial surface around the islets of
391 ciliated epithelium. Based on these morphological characteristics, the 5,6,11-trideoxyTTX-
392 sensitive OSNs are presumed to be crypt or another unknown type of OSN. Our recent study
393 using the green spotted puffers (Suzuki et al., 2021) suggested that cells that were
394 immunopositive for S-100, a marker protein of crypt-type OSNs, were also labeled by
395 another neuronal activity marker, phosphorylated ribosomal protein S6, antibody that is
396 formed after 5,6,11-trideoxyTTX administration. This result further supports that the 5,6,11-
397 trideoxyTTX sensitive OSNs are crypt-type olfactory sensory neurons.

398

399 Biological significance of 5,6,11-trideoxyTTX for grass puffers

400 On the basis of these experimental data, we concluded that adult grass puffers can detect
401 5,6,11-trideoxyTTX by olfaction. 5,6,11-trideoxyTTX has been reported to be present in the
402 body of toxic pufferfish. The distribution of TTX and its analogs throughout the body of
403 grass puffers was reported, and 5,6,11-trideoxyTTX accumulated mainly in the ovary (Jang
404 et al., 2010). Remarkably, 5,6,11-trideoxyTTX accumulates more in the ovary than TTX. A
405 receptor binding assay study of voltage-gated sodium channels in rat brain suspensions
406 reported that the dissociation equilibrium constant of 5,6,11-trideoxyTTX (> 5000 nM) was
407 extremely high compared with that of TTX (1.8 ± 0.1 nM; Yotsu-Yamashita et al., 1999).
408 These studies suggest that 5,6,11-trideoxyTTX is almost nontoxic and that it does not act as a
409 defense substance against predators. Kono et al. (2008) reported that the transformation from
410 TTX to its deoxy analogs or oxidized analogs does not occur in the body of grass puffers.
411 This result suggests that 5,6,11-trideoxy-TTX is a precursor to TTX, but it remains unclear
412 whether 5,6,11-trideoxy-TTX is converted to TTX inside the toxic puffers or other organisms
413 (Ueyama et al., 2018; Yotsu-Yamashita et al., 2013). Therefore, the biological significance of
414 why toxic puffers accumulate such nontoxic TTX analogs remains unclear.

415 In the previous study, TTX was treated as an attractive sex pheromone for toxic
416 puffers. Pheromones are defined as follows: "an odor or mixture of odorous substances,
417 released by an individual (the sender) and evoking in conspecifics (the receivers) adaptive,
418 specific, and species-typical response(s), the expression of which needs not require prior
419 experience or learning" (Sorensen and Stacey, 2004) or "molecules that are evolved signals,
420 in defined ratios in the case of multiple component pheromones, which are emitted by an
421 individual and received by a second individual of the same species, in which they cause a
422 specific reaction, for example, a stereotyped behavior or a developmental process" (Wyatt,
423 2014). In fish, 17,20β-dihydroxy-4-pregnen-3-one and prostaglandin F2α (goldfish), a type of
424 sex steroid that is found in ovarian cavity fluid and urine, and a bile acid that is released from
425 gills, urine, and feces (jawless sea lamprey, rainbow trout, and lake char) have been reported
426 to act as attractive sex pheromones (Sorensen and Stacey, 2004). TTX is secreted from the
427 secretory glands on the skin of *T. pardalis, T. poecilonotus, T. vermicularis*, and grass puffers
428 (Kodama et al., 1985) and is released from ovulated oocytes in the grass puffers (Matsumura
429 1995). Because 5,6,11-trideoxyTTX is particularly abundant in pufferfish ovaries (Puilingi et
430 al., 2015), 5,6,11-trideoxyTTX may also be released from the ovarian luminal fluid during
431 spawning periods.

These chemicals, known as fish pheromones, are synthesized by conspecific fish. However, current circumstantial evidence suggests that 5,6,11-trideoxyTTX, unlike other pheromones, is acquired from TTX-bearing organisms by eating them (Lago et al., 2015; Noguchi et al., 2011). In some animals, molecules made by other organisms are harvested and used as pheromones. For example, male neotropical orchid bees can unsuccessfully display and attract females without a species-specific pheromone mix, such as limonenes, from the right kinds of orchid flowers (Zimmermann et al., 2009). Animals may also use molecules that are produced by bacteria as pheromones. Phase-change pheromones of the locust are guaiacol (2-methoxyphenol) and phenol, and they are produced by locust gut bacteria (Pener and Simpson, 2009).

Noguchi et al. (2006a) stated that wild toxic puffers tend to feed on benthic organisms, such as flatworms, starfish, small gastropods, and skeleton shrimp that are known to possess TTX. A recent study reported that a TTX-bearing planocerid flatworm was found in the gut content of grass puffers (Itoi et al., 2018b) and *Chelondon patoca* (Itoi et al., 2020). Furthermore, some carnivorous gastropods have 5,6,11-trideoxyTTX in addition to TTX (*Charonia lampas lampas*, Rodriguez et al., 2008; *Nassarius spp*., Luo et al., 2012), and toxic snails (*Polinices didyma, Natica lineata, Natica vitellus, Zeuxis sufflatus, Niotha clathrata, Oliva miniacea, Oliva mustelin*a, and *Oliva hirasei*) have been reported to be attracted to crude TTX extracts (Hwang et al., 2004). Additionally, grass puffers ingested toxic eggs of another pufferfish, *T. pardalis,* which contained 5,6,11-trideoxyTTX (Itoi et al., 2015). All these studies support that the pufferfish seems to be attracted by 5,6,11-trideoxyTTX and that it ingest TTX-bearing organisms as food, resulting in accumulating TTX as a possible defense mechanism and attractive pheromone.

In conclusion, the grass puffers seem to use 5,6,11-trideoxyTTX as an olfactory cue to capture TTX-bearing organisms and use the accumulated 5,6,11-trideoxyTTX as an attractive pheromone to attract other conspecific puffers. Thus, this study revealed that pufferfish might actively use functionally unknown TTX analogs for chemical communication. Further studies are needed to determine whether other TTX analogs can also be detected by olfaction and whether multiple TTX analogs synergistically induce such effects. However, we believe that our results provide a new perspective on the use of TTX by toxic puffers.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: H.A.; Methodology: Y.N., T.S., K.M., R.N., Y.S., M.A., H.A.; Validation: T.S., H.A.; Formal analysis: Y.N.; Investigation: Y.N., H.A.; Resources: T.N., H.A.; Data curation: Y.N., T.S., K.M., H.A.; Writing - original draft: Y.N., H.A.; Writing - review & editing: T.S., T.N., H.A.; Visualization: Y.N., T.S., H.A.; Supervision: T.N., H.A.; Project administration: H.A.; Funding acquisition: T.N., H.A.

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633 Figure 1: **Grass puffer did not respond to TTX.**

634 A: EOG responses of the olfactory epithelium of *T. alboplumbeus* to TTX (10^{-7} M, upper
635 trace; 10^{-5} M, middle trace) and L-Arg (10^{-5} M, under trace).

636 B: EOG amplitudes of TTX (10^{-7} M, n = 6, open circle; 10^{-5} M, n = 7, filled circle) and L-
637 Arg (10^{-5} M, n = 11, filled square). Box plots show the median, quartiles (boxes), and
638 10%–90% range (whiskers), and dot plots show raw EOG amplitudes. Steel's multiple
639 comparison test: ** $P<0.01$.

640

641 Figure 2: **5,6,11-trideoxyTTX induced EOG response of the grass puffer.**

642 A: EOG responses to Vehicle (top trace), 4,9-anhydroTTX (10^{-5} M, second trace), 5,6,11-
643 trideoxyTTX (10^{-5} M, third trace), and L-Arg (10^{-5} M, bottom trace) from same grass
644 puffer.

645 B: EOG amplitudes of Vehicle (n = 5, open circles), 4,9-anhydroTTX (n = 4, filled circles),
646 5,6,11-trideoxyTTX (n = 7, filled triangles), and L-Arg (n = 7, filled square). Box plots
647 show the median, quartiles (boxes), and 10%–90% range (whiskers) of EOG
648 amplitudes, and dot plots show the raw value of EOG amplitudes. Steel's multiple
649 comparison test: * $P < 0.05$.

650

651 Figure 3: **EOG response to 5,6,11-trideoxyTTX was dose-dependent.**

652 A: Representative EOG responses by a grass puffer were evoked by increasing the
653 concentration of 5,6,11-trideoxyTTX. The period of 5,6,11-trideoxyTTX administration
654 is shown by the black bar.

655 B: Dose-response relationship of 5,6,11-trideoxyTTX obtained from n = 5–7 fish. Peak
656 EOG responses are plotted as a function of the concentrations that were delivered.
657 Black circles show the individual EOG amplitudes and open circles show the mean
658 EOG amplitude at given concentrations. Error bars represent the standard error. The
659 dotted line represents a Hill equation fitted to the data (EC_{50}: 9.62×10^{-6} M, Hill
660 coefficient: 1.21).

661

662

Figure 4: **Swimming activity of a grass puffer before and after Vehicle administration.**

Tracked location of a grass puffer (2-min periods) before (A–C) and after Vehicle administration (D–F).

Positions of the fish are plotted along the horizontal axis of the aquarium every 1 s. Vehicle (5 mL of 2.5×10^{-4} N acetic acid solution) was applied at time 0 and remained in the aquarium thereafter (H). Swimming velocity of the fish is presented in G (H).

Figure 5: **Swimming activity of a grass puffer before and after the 5,6,11-trideoxyTTX administration.**

Tracked location of a grass puffer (2-min periods) before (A–C) and after 5,6,11-trideoxyTTX application (D–F).

Positions of the fish are plotted along the horizontal axis of the aquarium every 1 s. 5,6,11-trideoxyTTX (5 mL of 10^{-5} M dissolved in 2.5×10^{-4} N acetic acid solution) was applied at time 0 and remained in the aquarium thereafter (G). Swimming velocity of the fish is presented in G (H).

Figure 6: **5,6,11-trideoxyTTX attracts grass puffers.**

A and B: Changes in the time (1-min period) spent by grass puffers in response to an odorant administered on one side of the experimental aquarium (A: Vehicle, B: 5,6,11-trideoxyTTX). The dotted line represents the chance level (= No odorant preference). Open or hatched boxes in the plot represent the time periods of the odorant administrations. Dot plots show the value from individual fish, and box plots show the median, quartiles (boxes), and 10%–90% range (whiskers).

C and D: Changes in the swimming speed (mm/s) in response to Vehicle (C) or 5,6,11-trideoxyTTX (D). The results of the statistical analysis are shown in the text.

Figure 7: **Activity-dependent fluorescent dextran uptake labeled 5,6,11-trideoxyTTX responding olfactory sensory neurons on the olfactory epithelium of the grass puffer.**

Differential interference contrast photomicrographs of the surface (A) and the edge (B) of the olfactory lamella of grass puffer. Arrowheads represent the non-ciliated epithelium

693 that surrounds the “islets” of the sensory epithelium (SE). Sensory cilia are visible on the
694 surface of the sensory epithelium. Scale bar = 20 μm.

695 Confocal microscopy images of the olfactory lamella after Vehicle administration (C–E).
696 Nuclear staining using DAPI (C) visualized the cells surrounding the islets whose nuclei
697 were located on the surface of the olfactory lamella. Co-administration of Alexa Fluor555-
698 dextran and Vehicle did not label any cell-like structures (D). Superimposed image of C
699 and D (E). The image is a z-projection of 41 pictures (z-step, 0.45 μm).

700 Confocal microscopy image of the olfactory lamella after 5,6,11-trideoxyTTX
701 administration (F–H). Nuclear staining using DAPI (F), Alexa Fluor555-dextran (G), and
702 their composite image (H). Co-administration of Alexa Fluor555-dextran labeled the large
703 oval cells that were located in the surrounding region of the islets (arrows in G). Scale bar
704 = 20 μm. The image is a z-projection of 28 pictures (z-step, 0.45 μm).

705 Enlarged image of the rectangular region in H (I). Alexa Fluor 555 labeling was frequently
706 seen as bright granular aggregations within the cell. Scale bar = 5 μm.

707 Another z-projection image (17 planes with a z-step of 0.48 μm) of Alexa Fluor555-
708 dextran labeled cells by 5,6,11-trideoxyTTX administration (surface side view, J; and
709 basal membrane side view, K). The arrow indicates an axon-like structure that extends
710 from the Alexa Fluor555-dextran-labeled cell. Scale bar = 5 μm.

711

712 Figure 8: **Activity-dependent labeling by pERK antibody labeled oval-shaped cells on**
713 **the surface of the olfactory epithelium.**

714 pERK-immunopositive OSNs after exposure to 5,6,11-trideoxyTTX (A) or L-Arg (B). No
715 labeled cells were observed with the Vehicle control (C). Scale bar: 5 μm.

716

717 Movie S1: Attractive swimming behavior of grass puffers toward 5,6,11-trideoxyTTX
718 observed under infrared illumination.

719 Vehicle or 5,6,11-trideoxyTTX solutions were added from the right side of the test aquarium
720 6 minutes after the start of image acquisitions.

721

722 Movie S2: Reconstructed 3D view of the 5,6,11-trideoxyTTX-sensitive OSNs on the surface
723 of olfactory epithelium shown in Fig. 8A.

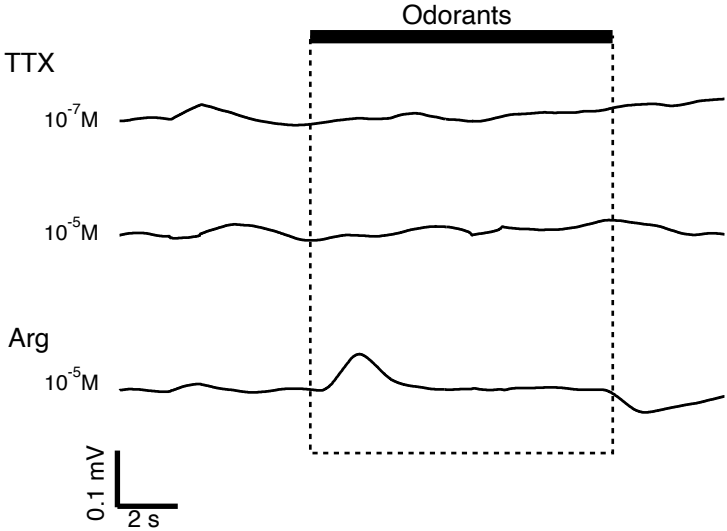
724 pERK-immunopositive OSNs after exposure to 5,6,11-trideoxyTTX. The same confocal
725 microcopy data as in Fig.8A was used. Scale bar: 10 μm.

726

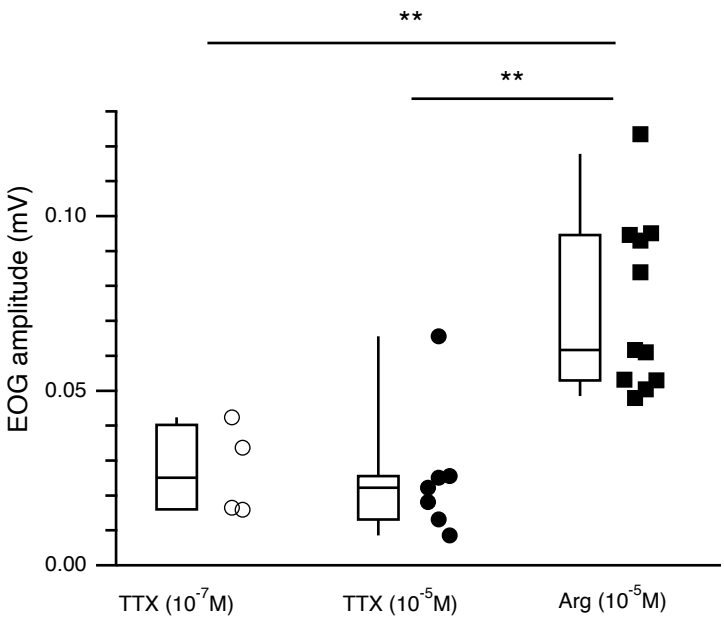
727 Movie S3: Reconstructed 3D view of the L-Arg-sensitive OSNs in the middle layer of
728 olfactory epithelium shown in Fig. 8B.

729 pERK-immunopositive OSNs after exposure to L-Arg. The same confocal microcopy data as
730 in Fig.8B was used. Scale bar: 10 μm.

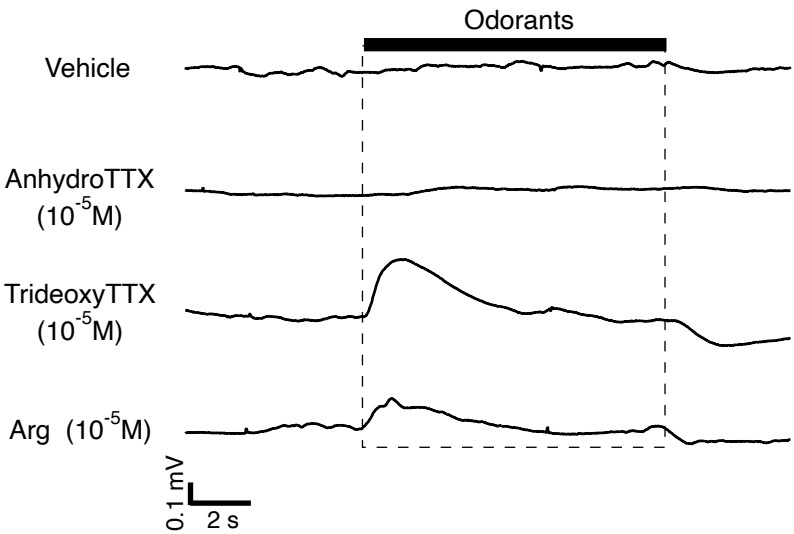
A


Odorants
TTX
10-7M
10-5M
Arg
10-5M
0.1 mV
2 s

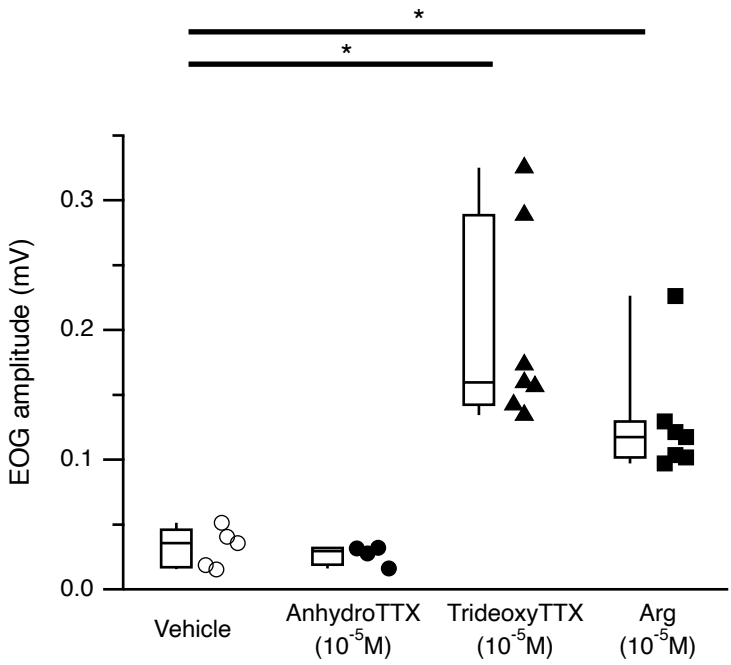
B

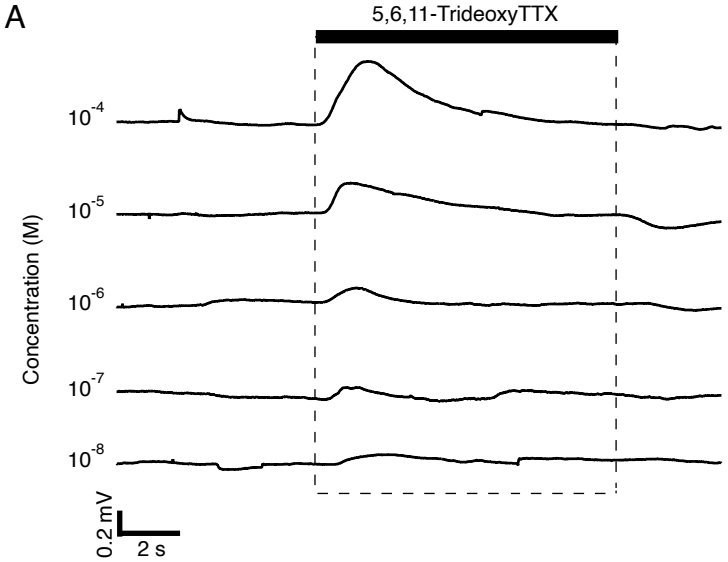

**
**
EOG amplitude (mV)
0.10
0.05
0.00
TTX (10-7M)
TTX (10-5M)
Arg (10-5M)

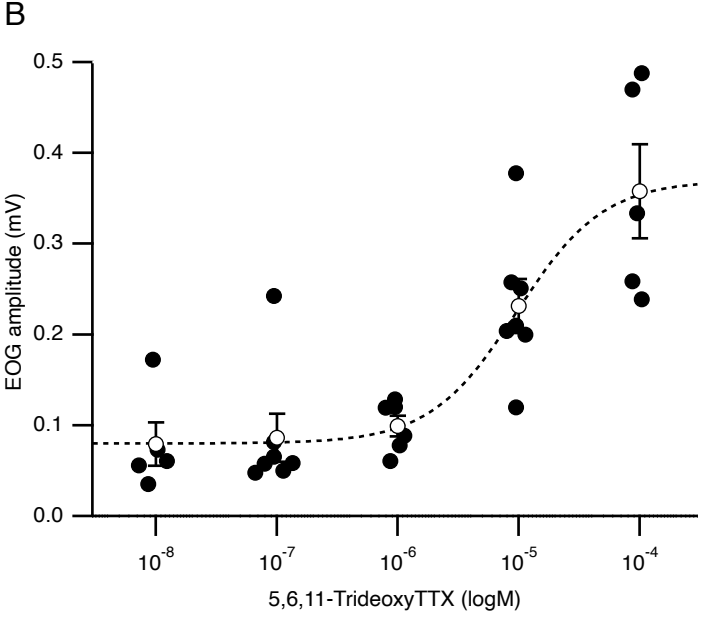
A

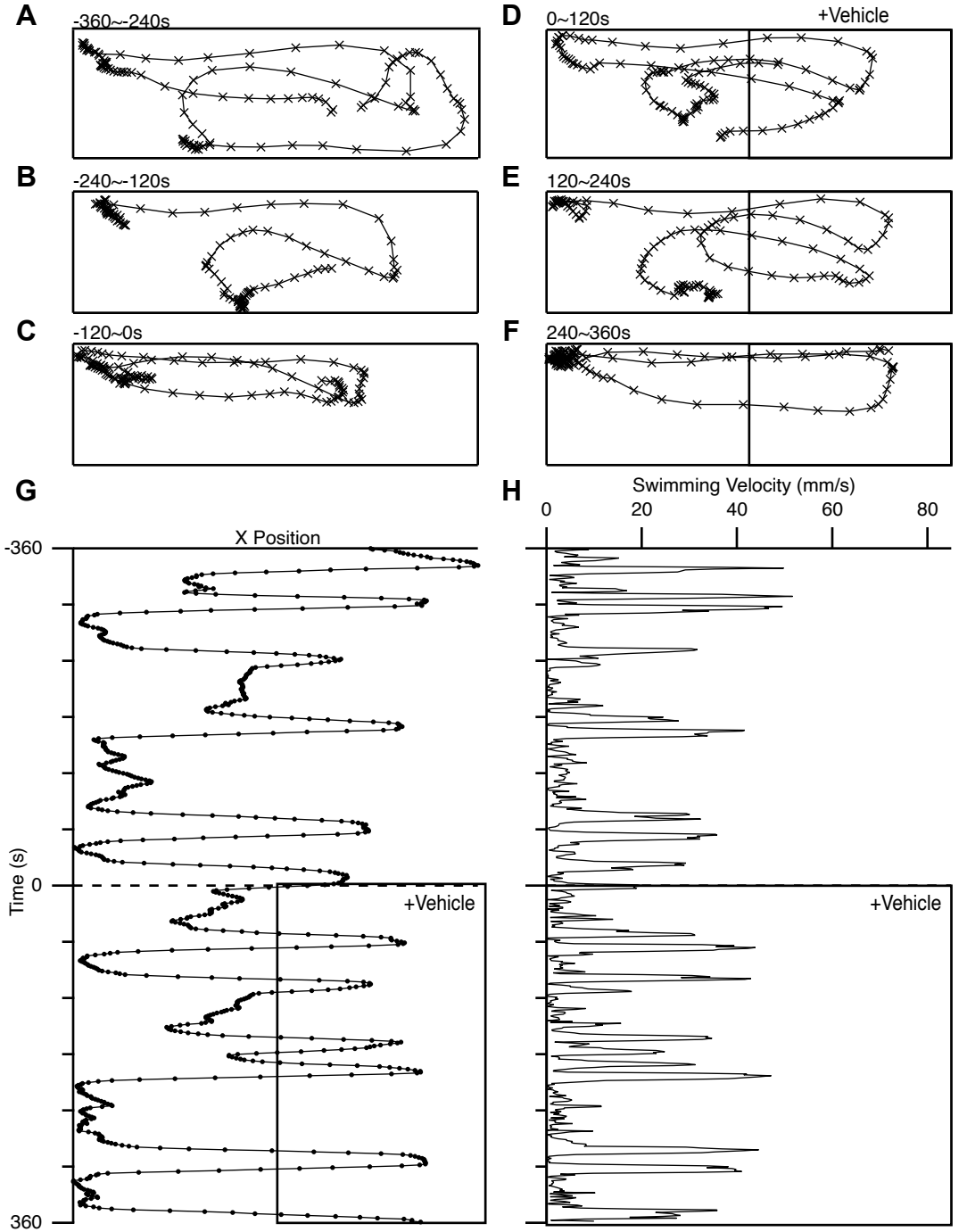

Odorants
Vehicle
AnhydroTTX
(10-5M)
TrideoxyTTX
(10-5M)
Arg (10-5M)
0.1 mV
2 s

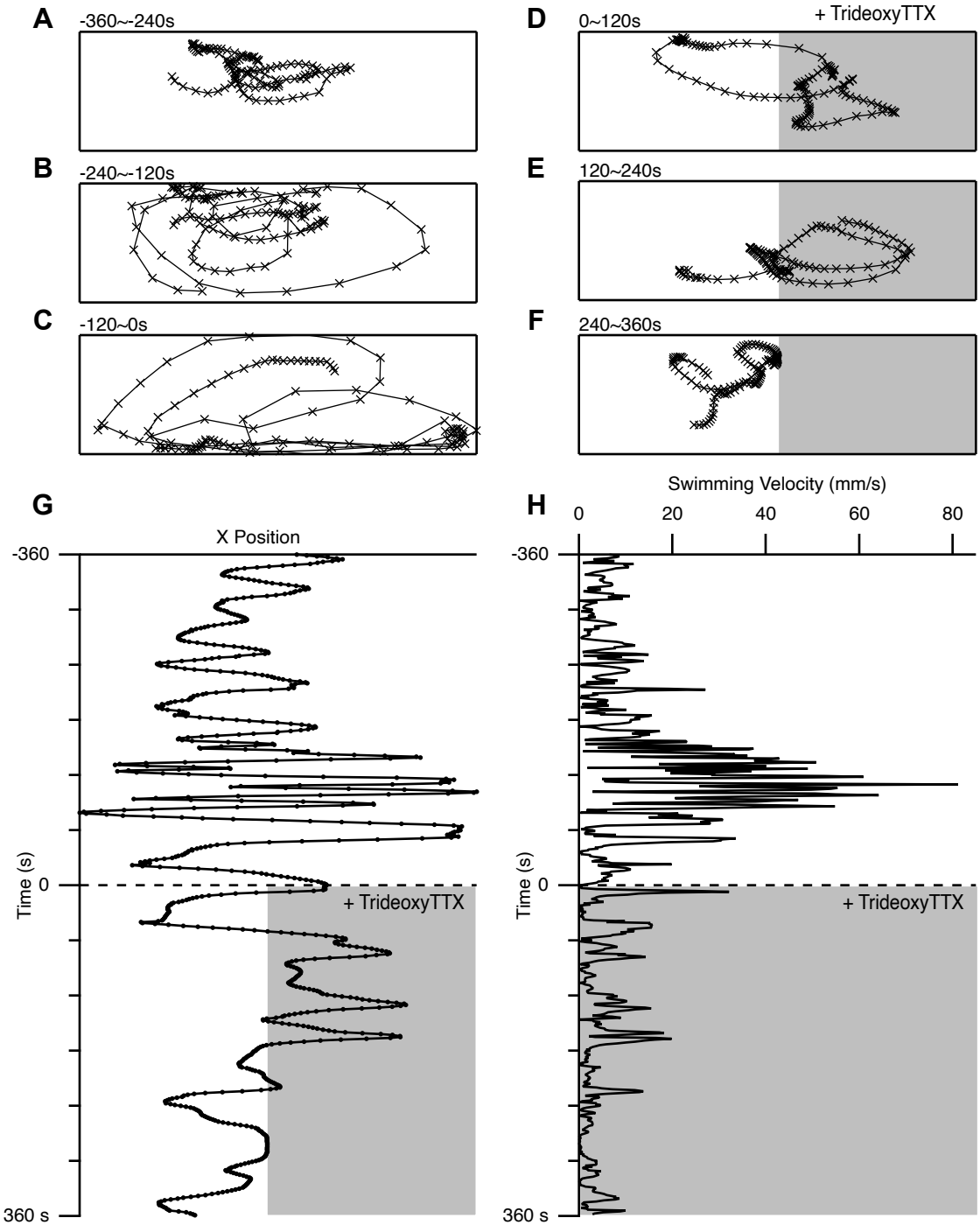
B

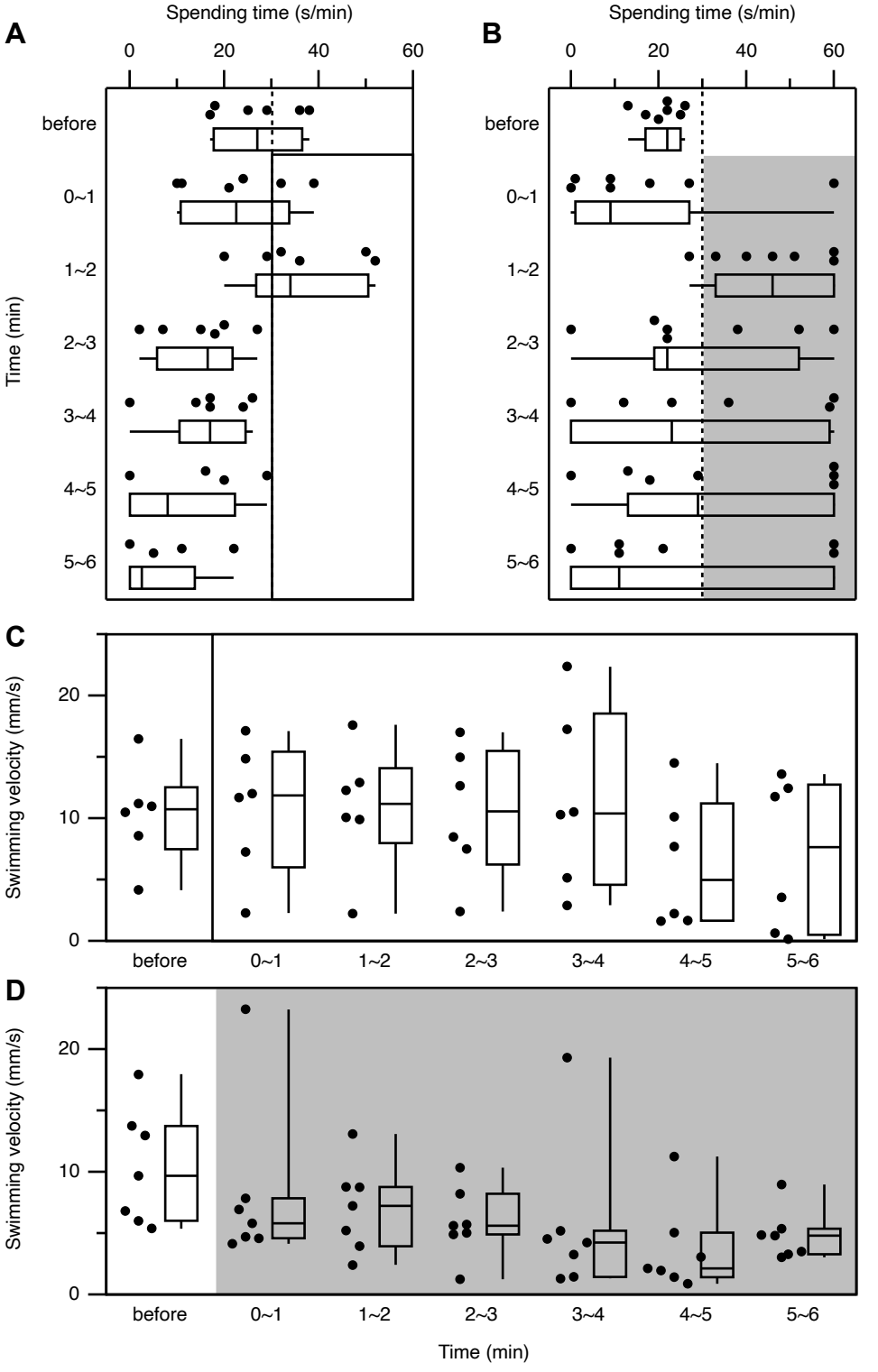

*
*
EOG amplitude (mV)
0.0
0.1
0.2
0.3
Vehicle
AnhydroTTX
(10-5M)
TrideoxyTTX
(10-5M)
Arg
(10-5M)

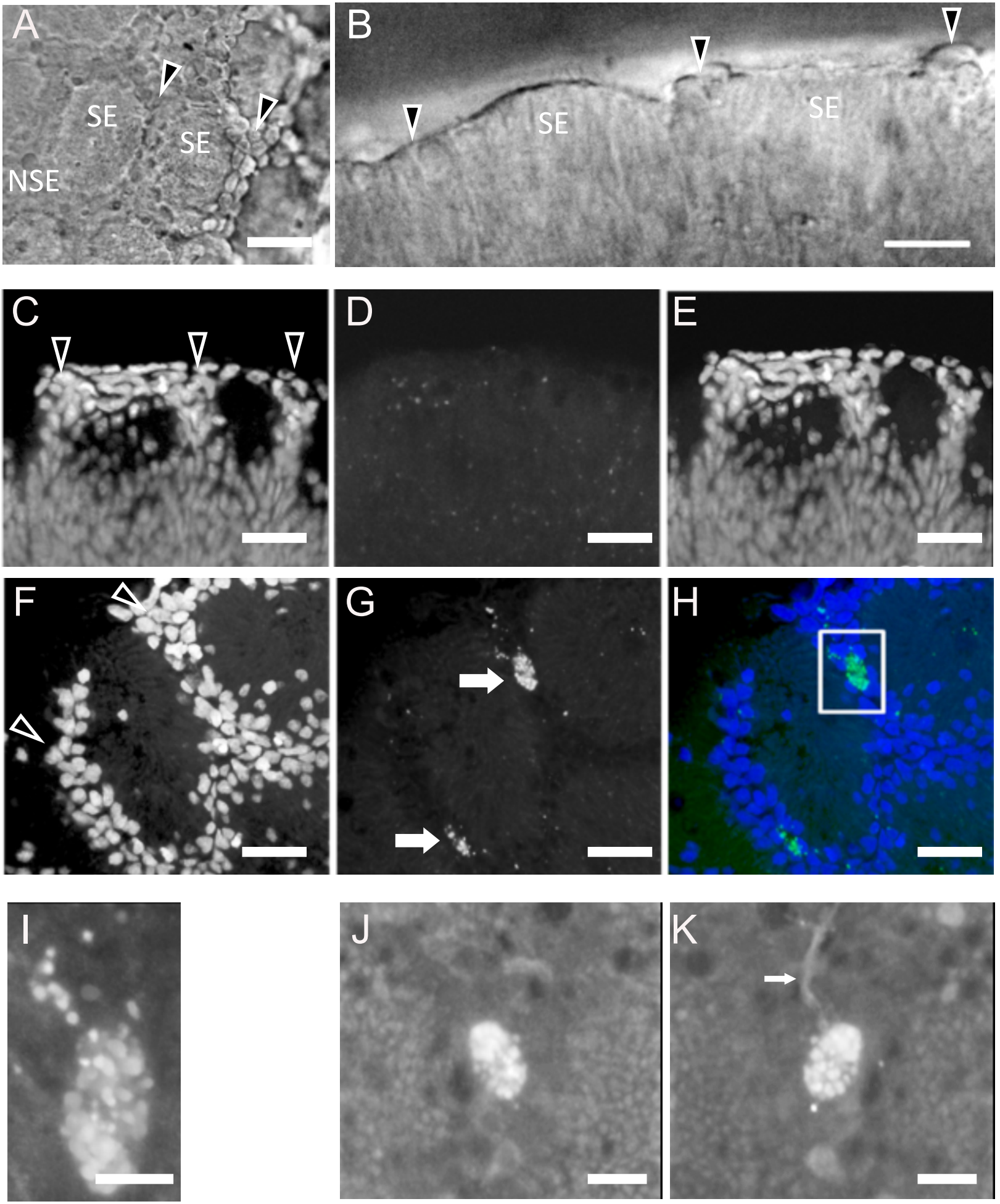

A
5,6,11-TrideoxyTTX
Concentration (M)
10-4
10-5
10-6
10-7
10-8
0.2 mV
2 s

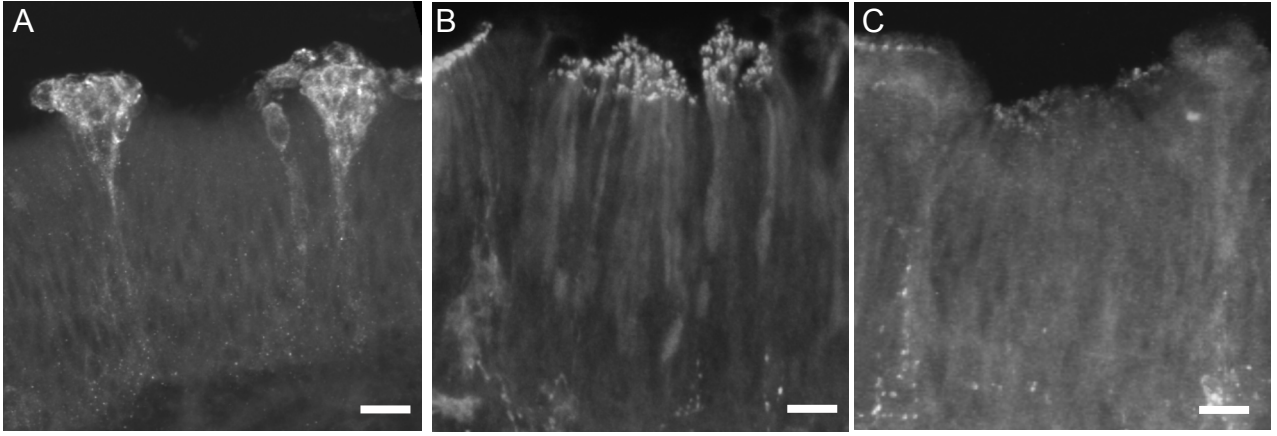

B
EOG amplitude (mV)
0.0
0.1
0.2
0.3
0.4
0.5
10-8
10-7
10-6
10-5
10-4
5,6,11-TrideoxyTTX (logM)


A
-360~-240s
B
-240~-120s
C
-120~0s
D
0~120s
+Vehicle
E
120~240s
F
240~360s
G
X Position
-360
Time (s)
0
+Vehicle
360
H
Swimming Velocity (mm/s)
0
20
40
60
80
+Vehicle


A
-360~-240s
B
-240~-120s
C
-120~0s
D
0~120s
+ TrideoxyTTX
E
120~240s
F
240~360s
G
X Position
-360
Time (s)
0
+ TrideoxyTTX
360 s
H
Swimming Velocity (mm/s)
0
20
40
60
80
-360
Time (s)
0
+ TrideoxyTTX
360 s


A
Spending time (s/min)
0
20
40
60
Time (min)
before
0~1
1~2
2~3
3~4
4~5
5~6
B
Spending time (s/min)
0
20
40
60
before
0~1
1~2
2~3
3~4
4~5
5~6
C
Swimming velocity (mm/s)
0
10
20
before
0~1
1~2
2~3
3~4
4~5
5~6
D
Swimming velocity (mm/s)
0
10
20
before
0~1
1~2
2~3
3~4
4~5
5~6
Time (min)


A
SE
SE
NSE
B
SE
SE
C
D
E
F
G
H
I
J
K


A
B
C