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

Authors: Yasuhisa Noguchi[1], Takehisa Suzuki[1], Keigo Matsutani[1], Ryo Sakakibara[2], Ryota Nakahigashi[2], Masaatsu Adachi[2†], Toshio Nishikawa[2], and Hideki Abe[1*]

Affiliations

1. Laboratory of Fish Biology, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-Cho, Chikusa-Ku, Nagoya, Aichi 464-8601, Japan
2. Laboratory of Organic Chemistry, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-Cho, Chikusa-Ku, Nagoya, Aichi 464-8601, Japan

[†] Present address: Laboratory of Heterocyclic Chemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan

Corresponding author's email address

habe@agr.nagoya-u.ac.jp (Hideki Abe)

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 (Magarlamov et al., 2017; 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

82 attractive effect of TTX on *T. rubripes* juveniles disappeared after nose ablation (Okita et al.,
83 2013).

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

90

91 **Materials and Methods**

92 Animals

93 One hundred fifty-nine grass puffers (body length, 5–12 cm; bodyweight, 6–32 g) were
94 collected from the coastal waters of Ise-bay in Minamichita-Cho on the Pacific coast of
95 central Japan by line-fishing. Grass puffers were fed commercial diets (Tetra Krill-E;
96 Spectrum Brands, Madison, WI, USA) in aerated 300 L tanks (10–25°C, approximately 50
97 fish per tank) filled with artificial seawater (ASW; Instant Oceans® Sea Salt, Cl^- 543 mM,
98 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
99 mM, I^- 0.002 mM, CO_3^{2-}/HCO_3^- 3.3 mM, pH 8.2; Instant Ocean, Blacksburg, VA, USA)
100 under a 12-h light/12-h dark photoperiod. In this study, both males and females were used
101 without distinction. All animals were maintained and used in accordance with the Nagoya
102 university regulations on animal care and use in research.

103

104 Odorant chemicals

105 TTX (for biochemistry, citrate buffered, cat. No. 206–11071, FUJIFILM Wako Pure
106 Chemical Corporation, Osaka, Japan) was dissolved in ultra-pure water at 1.5×10^{-3} M. Two
107 types of TTX analogs, 4,9-anhydroTTX and 5,6,11-trideoxyTTX, were synthesized and
108 purified according to the methods described by Adachi et al. (2014a) and Adachi et al.
109 (2014b), respectively. Synthesized TTX analogs were dissolved in 2.5×10^{-2} N acetic acid at
110 10^{-3} M and stored at −30°C until use. L-Arginine (Arg; a food odor cue for teleosts;
111 FUJIFILM Wako) was prepared as a 10^{-2} M stock in artificial cerebrospinal fluid (ACSF;
112 NaCl 231 mM, KCl 8.1 mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 2.4 mM, D-glucose 10 mM, 4-(2-

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

120

121 Electroolfactogram recordings

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

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

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

149

150 Behavioral experiments

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

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

173

174 Activity-dependent labeling of ORNs

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

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

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

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

210 5 min with 0.2% NGS in PBST. Sections were then incubated with Alexa Fluor® 488-
211 conjugated secondary antibody (goat anti-rabbit IgG (H+L); A-11008, Thermo Fisher
212 Scientific, 1:500) that was diluted with 0.2% NGS in PBST for 1 day at 4°C. The sections
213 were washed three times for 5 min in PBST. Finally, sections were counterstained with DAPI
214 (1:1000) for 5 min and mounted using mounting medium.

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

222

223 Data analysis

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

231

232 **Results**

233 EOG response of grass puffers to TTX

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

240 ± 0.007 mV, n = 7; Fig. 1B●) were significantly lower than that of 10^{-5} M L-Arg (0.074 ±
241 0.008 mV, n = 11; Fig. 1B■).

242

243 EOG response to TTX analogs

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

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

260

261 Behavioral experiments

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

272 stop on the administered side (Fig. 5D and E, Movie S1). Although the time vs. x-axis plot
273 did not show an apparent change in the swimming pattern (Fig. 5G), 5,6,11-trideoxyTTX
274 administration decreased the swimming velocity of the fish (Fig. 5H).

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

282

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

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

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

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

304 OE (Fig. 8A, Movie S2), whereas L-Arg administration labeled elongated cells with strongly
305 labeled cilia whose somata were in the middle layer of the OEs (close to the basal membrane;
306 Fig. 8B, Movie S3). Additionally, 5,6,11-trideoxyTTX-sensitive OSNs were localized in the
307 NSE area of Fig. 7A. However, Vehicle administration did not activate any type of OSNs
308 (Fig. 8C).

309

310 **Discussions**

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

316

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

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

To investigate this hypothesis, we examined the effects of two types of synthesized 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,

368 alcohols, acetates, or aldehydes in particular combinations and ratios (de Bruyne and Baker,
369 2008). In goldfish and carp, a combination of species-specific metabolites of prostaglandin
370 F2α (PGF2α), a well-known releaser pheromone that induces sexual behavior, strongly
371 induces species-specific sexual behavioral responses (Lim and Sorensen, 2011). Thus, it is
372 possible that other metabolites in pufferfish may play a synergistic action with 5,6,11-
373 trideoxyTTX as a chemical signal to attract the opposite sex.

374

375 5,6,11-trideoxyTTX sensitive OSNs on the olfactory epithelium of grass puffers

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

385 It is widely known that teleost fish are equipped with three types of OSNs that are
386 intermingled in the OE but show distinct morphology (Bazáes et al., 2013). First, there are
387 the ciliated cells with long dendrites and a few cilia, and second, there are microvillous cells
388 with shorter dendrites than the ciliated cells, which have microvilli extending from their
389 apical surface. The third type is the crypt cells. Crypt cells have an oval shape, are located
390 close to the epithelial surface, and have few cilia and microvilli (Hansen and Eckart, 1998).
391 Cells that were labeled with fluorescent dextran by 5,6,11-trideoxyTTX co-administration
392 had no cilia or microvilli and were located on the epithelial surface around the islets of
393 ciliated epithelium. Based on these morphological characteristics, the 5,6,11-trideoxyTTX-
394 sensitive OSNs are presumed to be crypt or another unknown type of OSN.

395

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

397 On the basis of these experimental data, we concluded that adult grass puffers can detect
398 5,6,11-trideoxyTTX by olfaction. 5,6,11-trideoxyTTX has been reported to be present in the
399 body of toxic pufferfish. The distribution of TTX and its analogs throughout the body of

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

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

429 These chemicals, known as fish pheromones, are synthesized by conspecific fish.
430 However, current circumstantial evidence suggests that 5,6,11-trideoxyTTX, unlike other
431 pheromones, is acquired from TTX-bearing organisms by eating them (Lago et al., 2015;
432 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.

Acknowledgment

We would like to express our gratitude to Prof. Naoyuki Yamamoto for his advice during experimentation.

465 **Competing interests**

466 The authors declare no competing or financial interests.

467

468 **Author contributions**

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

474

475 **Funding**

476 This work was supported by Grant-in-Aids for Scientific Research (16K07435 and 19K06762
477 to H.A., 17K19195 to T.N. and H.A., and 17H06406 to T.N.).

478

479 **References**

480 **Adachi, M., Imazu, T., Sakakibara, R., Satake, Y., Isobe, M., and Nishikawa, T.** (2014a).
481 Total synthesis of Chiriquitoxin, an analogue of tetrodotoxin isolated from the skin of
482 a dart frog. *Chem. – Eur. J.* **20**, 1247–1251. doi: 10.1002/chem.201304110

483 **Adachi, M., Sakakibara, R., Satake, Y., Isobe, M., and Nishikawa, T.** (2014b). Synthesis
484 of 5,6,11-trideoxytetrodotoxin. *Chem. Lett.* **43**, 1719–1721. doi: 10.1246/cl.140684

485 **Bazáes, A., Olivares, J., and Schmachtenberg, O.** (2013). Properties, projections, and
486 tuning of teleost olfactory receptor neurons. *J. Chem. Ecol.* **39**, 451–464.
487 doi: 10.1007/s10886-013-0268-1

488 **Biechl, D., Tietje, K., Gerlach, G., and Wullimann, M.F.** (2016) Crypt cells are involved in
489 kin recognition in larval zebrafish. *Sci. Rep.* **6**, 24590. doi: 10.1038/srep24590.

490 **de Bruyne, M. and Baker, T. C.** (2008) Odor detection in insects: volatile codes. *J. Chem.*
491 *Ecol.* 34, 882–897. doi: 10.1007/s10886-008-9485-4

492 **Døving, K., Hansson, K.-A., Backström, T., and Hamdani, H.** (2011). Visualizing a set of
493 olfactory sensory neurons responding to a bile salt. *J. Exp. Biol.* **214**, 80–87.

doi: 10.1242/jeb.046607

Hansen, A. and Eckart, Z. (1998). The peripheral olfactory organ of the zebrafish, *Danio rerio*: An Ultrastructural Study. *Chem. Senses* **23**, 39–48. doi: 10.1093/chemse/23.1.39

Hara, T. (1994). Olfaction and gustation in fish: an overview. *Acta Physiol Scand* **152**, 207–217. doi: 10.1111/j.1748-1716.1994.tb09800.x

Honda, S., Arakawa, O., Takatani, T., Tachibana, K., Yagi, M., Tanigawa, A., and Noguchi, T. (2005). Toxification of cultured puffer fish *Takifugu rubripes* by feeding on tetrodotoxin-containing diet. *Nippon Suisan Gakkaishi* **71**, 815–820. doi:10.2331/suisan.71.815

Hwang, P.-A., Noguchi, T., and Hwang, D.-F. (2004). Neurotoxin tetrodotoxin as attractant for toxic snails. *Fish. Sci.* **70**, 1106–1112. doi: 10.1055/s-2005-917664

Ikeda, K., Murakami, Y., Emoto, Y., Ngy, L., Taniyama, S., Yagi, M., Takatani, T., and Arakawa, O. (2009). Transfer profile of intramuscularly administered tetrodotoxin to nontoxic cultured specimens of the pufferfish *Takifugu rubripes*. *Toxicon* **53**, 99–103. doi: 10.1016/j.toxicon.2008.10.018

Itoi, S., Kozaki, A., Komori, K., Tsunashima, T., Noguchi, S., Kawane, M., and Sugita, H. (2015). Toxic *Takifugu pardalis* eggs found in *Takifugu niphobles* gut: Implications for TTX accumulation in the pufferfish. *Toxicon* **108**, 141–146. doi: 10.1016/j.toxicon.2015.10.009

Itoi, S., Sato, T., Takei, M., Yamada, R., Ogata, R., Oyama, H., Teranishi, S., Kishiki, A., Wada, T., Noguchi, K., et al. (2020). The planocerid flatworm is a main supplier of toxin to tetrodotoxin-bearing fish juveniles. *Chemosphere* **249**, 126217. doi: 10.1016/j.chemosphere.2020.126217

Itoi, S., Suzuki, M., Asahina, K., Sawayama, E., Nishikubo, J., Oyama, H., Takei, M., Shiibashi, N., Takatani, T., Arakawa, O., et al. (2018a). Role of maternal tetrodotoxin in survival of larval pufferfish. *Toxicon* **148**, 95–100. doi: 10.1016/j.toxicon.2018.04.014

Itoi, S., Ueda, H., Yamada, R., Takei, M., Sato, T., Oshikiri, S., Wajima, Y., Ogata, R., Oyama, H., Shitto, T. et al. (2018b). Including planocerid flatworms in the diet effectively toxifies the pufferfish, *Takifugu niphobles*, *Sci. Rep.* **8**, 12302.

525 doi: 10.1038/s41598-018-30696-z

526 **Itoi, S., Yoshikawa, S., Asahina, K., Suzuki, M., Ishizuka, K., Takimoto, N., Mitsuoka,**
527 **R., Yokoyama, N., Detake, A., Takayanagi, C., et al.** (2014). Larval pufferfish
528 protected by maternal tetrodotoxin. *Toxicon* **78**, 35–40.
529 doi: 10.1016/j.toxicon.2013.11.003

530 **Jang, J.-H., Lee, J.-S., and Yotsu-Yamashita, M.** (2010). LC/MS Analysis of Tetrodotoxin
531 and Its Deoxy Analogs in the Marine Puffer Fish *Fugu niphobles* from the Southern
532 Coast of Korea, and in the Brackishwater Puffer Fishes *Tetraodon nigroviridis* and
533 *Tetraodon biocellatus* from Southeast Asia. *Mar. Drugs* **8**, 1049–1058.
534 doi: 10.3390/md8041049

535 **Kodama, M., Ogata, T., and Sato, S.** (1985). External secretion of tetrodotoxin from puffer
536 fishes stimulated by electric shock. *Mar. Biol.* **87**, 199–202.
537 doi: 10.1007/BF00539429

538 **Kodama, M., Sato, S., Ogata, T., Suzuki, Y., Kaneko, T., and Aida, K.** (1986).
539 Tetrodotoxin secreting glands in the skin of puffer fishes. *Toxicon* **24**, 819–829.
540 doi: 10.1016/0041-0101(86)90107-8

541 **Kono, M., Matsui, T., Furukawa, K., Takase, T., Yamamori, K., Kaneda, H., Aoki, D.,**
542 **Jang, J.-H., and Yotsu-Yamashita, M.** (2008). Examination of transformation
543 among tetrodotoxin and its analogs in the living cultured juvenile puffer fish,
544 kusafugu, *Fugu niphobles* by intramuscular administration. *Toxicon* **52**, 714–720.
545 doi: 10.1016/j.toxicon.2008.08.002

546 **Lago, J., Rodríguez, L., Blanco, L., Vieites, J., and Cabado, A.** (2015). Tetrodotoxin, an
547 extremely potent marine neurotoxin: Distribution, toxicity, origin and therapeutical
548 uses. *Mar. Drugs* **13**, 6384–6406. doi: 10.3390/md13106384

549 **Lim, H. and Sorensen, P.** (2011). Polar metabolites synergize the activity of prostaglandin
550 F2α in a species-specific hormonal sex pheromone released by ovulated common
551 carp. *J. Chem. Ecol.* **37**, 695–704. doi: 10.1007/s10886-011-9976-6

552 **Luo, X., Yu, R., Wang, X.-J., and Zhou, M.-J.** (2012). Toxin composition and toxicity
553 dynamics of marine gastropod *Nassarius spp.* collected from Lianyungang, China.
554 *Food Addit. Contam. Part Chem. Anal. Control Expo. Risk Assess.* **29**, 117–27.
555 doi: 10.1080/19440049.2011.61506

Magarlamov, T. Y., Melnikova, D. I., and Chernyshev, A. V. (2017). Tetrodotoxin-producing bacteria: detection, Distribution and migration of the toxin in aquatic systems. *Toxins* **9**, 166. doi: 10.3390/toxins9050166

Matsumura, K. (1995). Tetrodotoxin as a pheromone. *Nature* **378**, 563–564. doi: 10.1038/378563b0

Narahashi, T. (2001). Pharmacology of tetrodotoxin. *J Toxicol* **20**, 67–84.

Noguchi, T. and Arakawa, O. (2008). Tetrodotoxin – Distribution and accumulation in aquatic organisms, and cases of human intoxication. *Mar. Drugs* **6**, 220–242. doi: 10.3390/md20080011

Noguchi, T., Arakawa, O., and Takatani, T. (2006a). TTX accumulation in pufferfish. *Comp. Biochem. Physiol. Part D Genomics Proteomics* **1**, 145–152. doi: 10.1016/j.cbd.2005.10.006

Noguchi, T., Arakawa, O., and Takatani, T. (2006b). Toxicity of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. *Comp. Biochem. Physiol. Part D Genomics Proteomics* **1**, 153–157. doi: 10.1016/j.cbd.2005.11.003

Noguchi, T., Onuki, K., and Arakawa, O. (2011). Tetrodotoxin poisoning due to pufferfish and gastropods, and their intoxication mechanism. *ISRN Toxicol.* **2011**, e276939. doi: 10.5402/2011/276939

Okita, K., Yamazaki, H., Sakiyama, K., Yamane, H., Niina, S., Takatani, T., Arakawa, O., and Sakakura, Y. (2013). Puffer smells tetrodotoxin. *Ichthyol. Res.* **60**, 386–389. doi: 10.1007/s10228-013-0353-z

Pener, M. P. and Simpson, S.J. (2009). Locust phase polyphenism: An update. *In Advances in Insect Physiology, edited by S. J. Simpson and M. P. Pener*, **36**, 1–272. Academic Press. doi: 10.1016/S0065-2806(08)36001-9.

Puilingi, C., Kudo, Y., Cho, Y., Konoki, K., and Yotsu-Yamashita, M. (2015). Tetrodotoxin and its analogues in the pufferfish *Arothron hispidus* and *A. nigropunctatus* from the Solomon Islands: A comparison of their toxin profiles with the same species from Okinawa, Japan. *Toxins* **7**, 3436–3454. doi: 10.3390/toxins7093436

Rodriguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., and Botana, L. (2008). First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet

shell *Charonia lampas lampas* in Europe. *Anal. Chem.* **80**, 5622–5629. doi: 10.1021/ac800769e

Saito, T., Kageyu, K., Goto, H., Murakami, K., and Noguchi, T. (1997). Tetrodotoxin attracts pufferfish ('torafugu', *Takifugu rubripes*). *Toxicon* **35**, 489. doi: 10.1016/S0041-0101(97)84745-9

Saito, T., Kageyu, K., Goto, H., Murakami, K., and Noguchi, T. (2000). Tetrodotoxin attracts pufferfish ("torafugu" *Takifugu rubripes*). *Bull. Inst. Ocean. Res. Dev. Tokai Univ.* **21**, 93–96.

Sakakura, Y., Takatani, T., Nakayasu, J., Yamazaki, H., and Sakiyama, K. (2017). Administration of tetrodotoxin protects artificially raised juvenile tiger puffer *Takifugu rubripes* from predators. *Fish. Sci.* **83**, 191–197. doi: 10.1007/s12562-016-1046-0

Sorensen, P. and Stacey, N. (2004). Brief review of fish pheromones and discussion of their possible uses in the control of non-indigenous teleost fishes. *N. Z. J. Mar. Freshw. Res.* **38**, 399–417. doi: 10.1080/00288330.2004.9517248

Ueyama, N., Sugimoto, K., Kudo, Onodera, K., Cho, Y., Konoki, K., Nishikawa, T., and Yotsu-Yamashita, M. (2018). Spiro bicyclic guanidino compounds from pufferfish: possible biosynthetic intermediates of tetrodotoxin in marine environments. *Chem. – Eur. J.* **24**, 7250–7258. doi: 10.1002/chem.201801006

Wan, Y., Otsuna, H., Chien, C.-B., and Hansen, C. (2012). FluoRender: An application of 2D image space methods for 3D and 4D confocal microscopy data visualization in neurobiology research. In *2012 IEEE Pacific Visualization Symposium*, pp. 201–208. doi: 10.1109/pacificvis.2012.6183592

Wyatt, T. D. (2014). Animals in a chemical world *In: Wyatt, T. D., Pheromones and Animal Behavior: Chemical Signals and Signatures 2nd ed.*, Cambridge University Press, New York, pp.1–48.

Yamamoto, M. and Ueda, K. (1979). Comparative morphology of fish olfactory epithelium, IX. Tetraodontiformes. *Zool. Mag.* **88**, 210–218.

Yamanaka, O. and Takeuchi, R. (2018). UMATracker: an intuitive image-based tracking platform. *J. Exp. Biol.* **221**, jeb182469. doi: 10.1242/jeb.182469

Yotsu-Yamashita, M., Sugimoto, A., Takai, A., and Yasumoto, T. (1999). Effects of

specific modifications of several hydroxyls of tetrodotoxin on its affinity to rat brain membrane. *J. Pharmacol. Exp. Ther.* **289**, 1688–1696.

Yotsu-Yamashita, M., Abe, Y., Kudo, Y., Ritson-Williams, R., Paul, V., Konoki, K., Cho, Y., Adachi, M., Imazu, T., Nishikawa, T., et al. (2013). First identification of 5,11-dideoxytetrodotoxin in marine animals, and characterization of major fragment ions of tetrodotoxin and its analogs by high resolution ESI-MS/MS. *Mar. Drugs* **11**, 2799–2813. doi: 10.3390/md11082799

Zimmermann, Y., Ramírez, S., and Eltz, T. (2009). Chemical niche differentiation among sympatric species of orchid bees. *Ecology* **90**, 2994–3008. doi: 10.1890/08-1858.1

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

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

633 B: EOG amplitudes of TTX (10^{-7} M, n = 6, open circle; 10^{-5} M, n = 7, filled circle) and L-
634 Arg (10^{-5} M, n = 11, filled square). Box plots show the median, quartiles (boxes), and
635 10%–90% range (whiskers), and dot plots show raw EOG amplitudes. Steel's multiple
636 comparison test: ** $p<0.01$.

637

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

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

642 B: EOG amplitudes of Vehicle (n = 5, open circles), 4,9-anhydroTTX (n = 4, filled circles),
643 5,6,11-trideoxyTTX (n = 7, filled triangles), and L-Arg (n = 7, filled square). Box plots
644 show the median, quartiles (boxes), and 10%–90% range (whiskers) of EOG
645 amplitudes, and dot plots show the raw value of EOG amplitudes. Steel's multiple
646 comparison test: * $p < 0.05$.

647

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

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

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

658

659

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

661 Tracked location of a grass puffer (2-min periods) before (A–C) and after Vehicle
662 administration (D–F). Glid: 100 mm.

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

666

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

669 Tracked location of a grass puffer (2-min periods) before (A–C) and after 5,6,11-
670 trideoxyTTX application (D–F). Glid: 100 mm.

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

675

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

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

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

685

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

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

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

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

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

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

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

708

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

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

713

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

716 Vehicle or 5,6,11-trideoxyTTX solutions were added from the right side of the test aquarium
717 6 minutes after the start of image acquisitions.

718

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

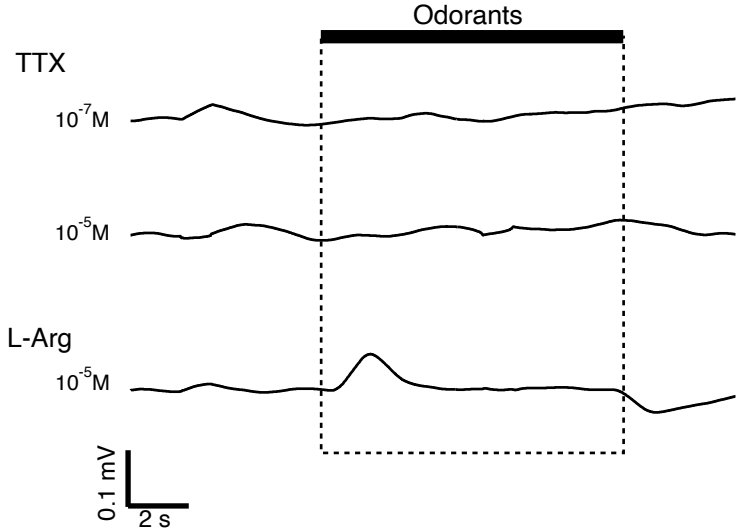
721 pERK-immunopositive OSNs after exposure to 5,6,11-trideoxyTTX. The same confocal
722 microcopy data as in Fig.8A was used. Scale bar: 10 μm.

723

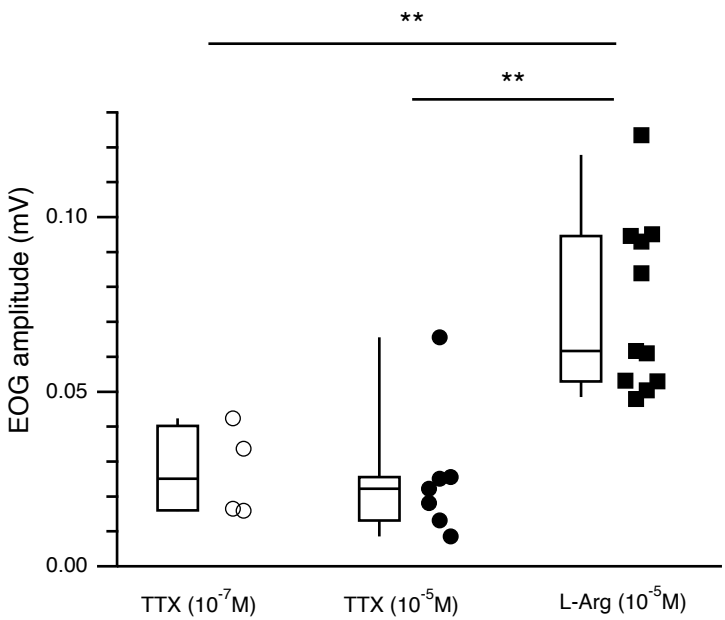
724 Movie S3: **Reconstructed 3D view of the L-Arg-sensitive OSNs in the middle layer of**
725 **olfactory epithelium shown in Fig. 8B.**

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

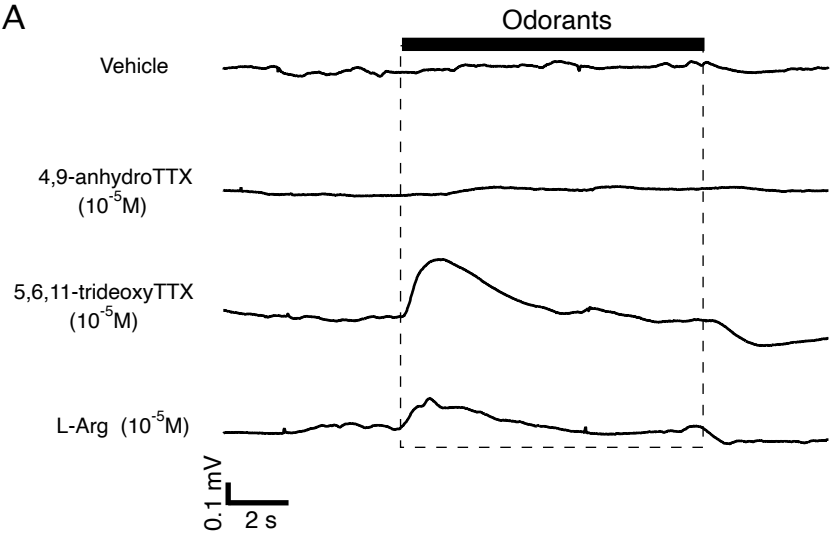
A


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

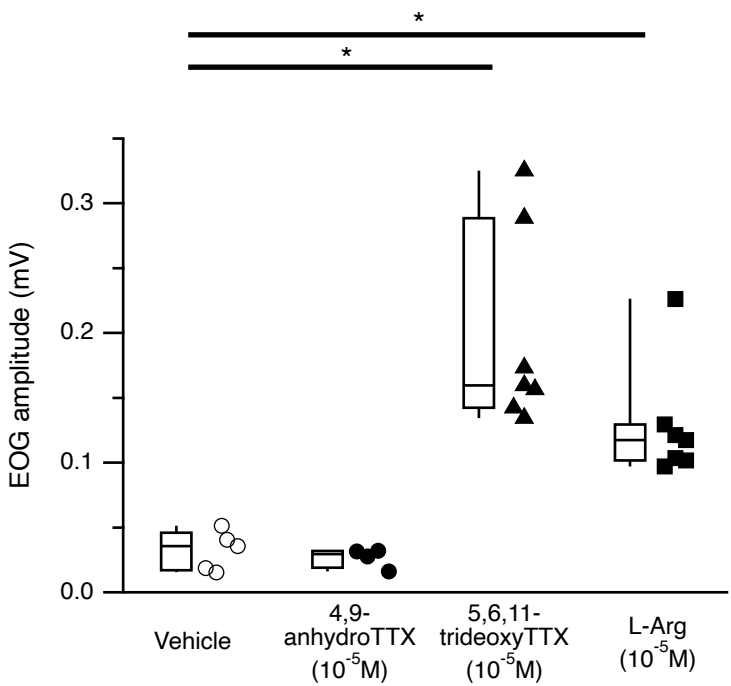
B

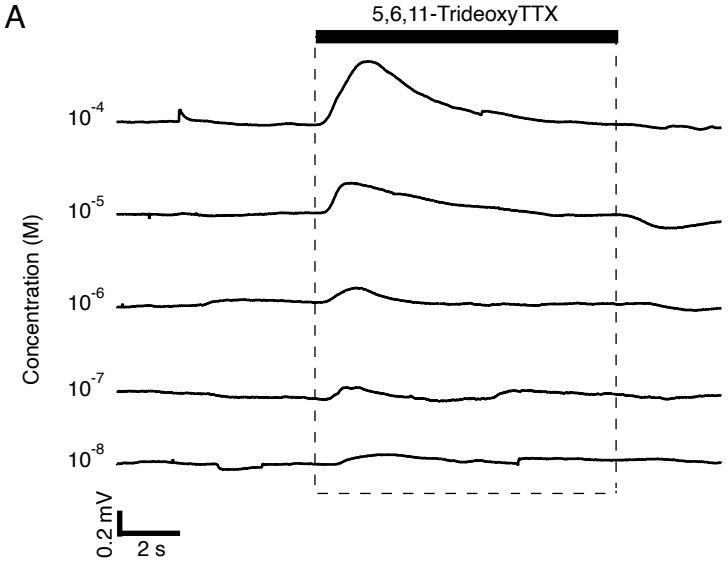

**
**
EOG amplitude (mV)
0.10
0.05
0.00
TTX (10-7M)
TTX (10-5M)
L-Arg (10-5M)

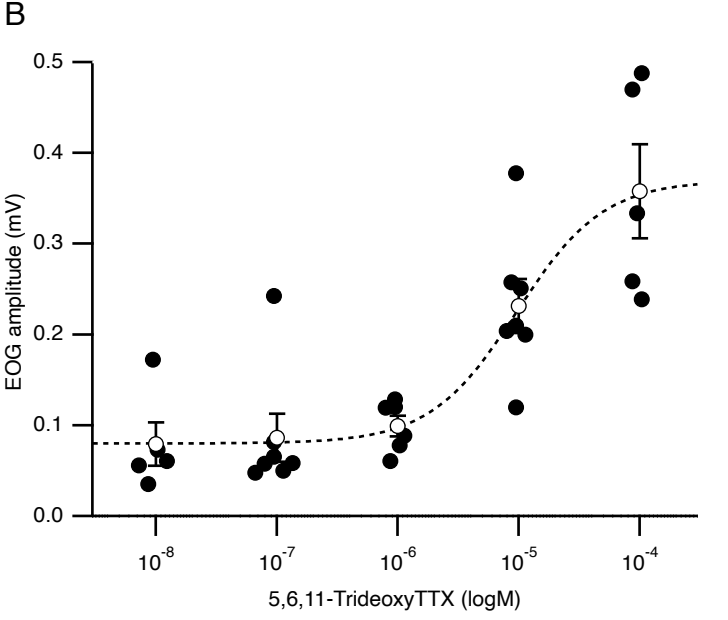
A

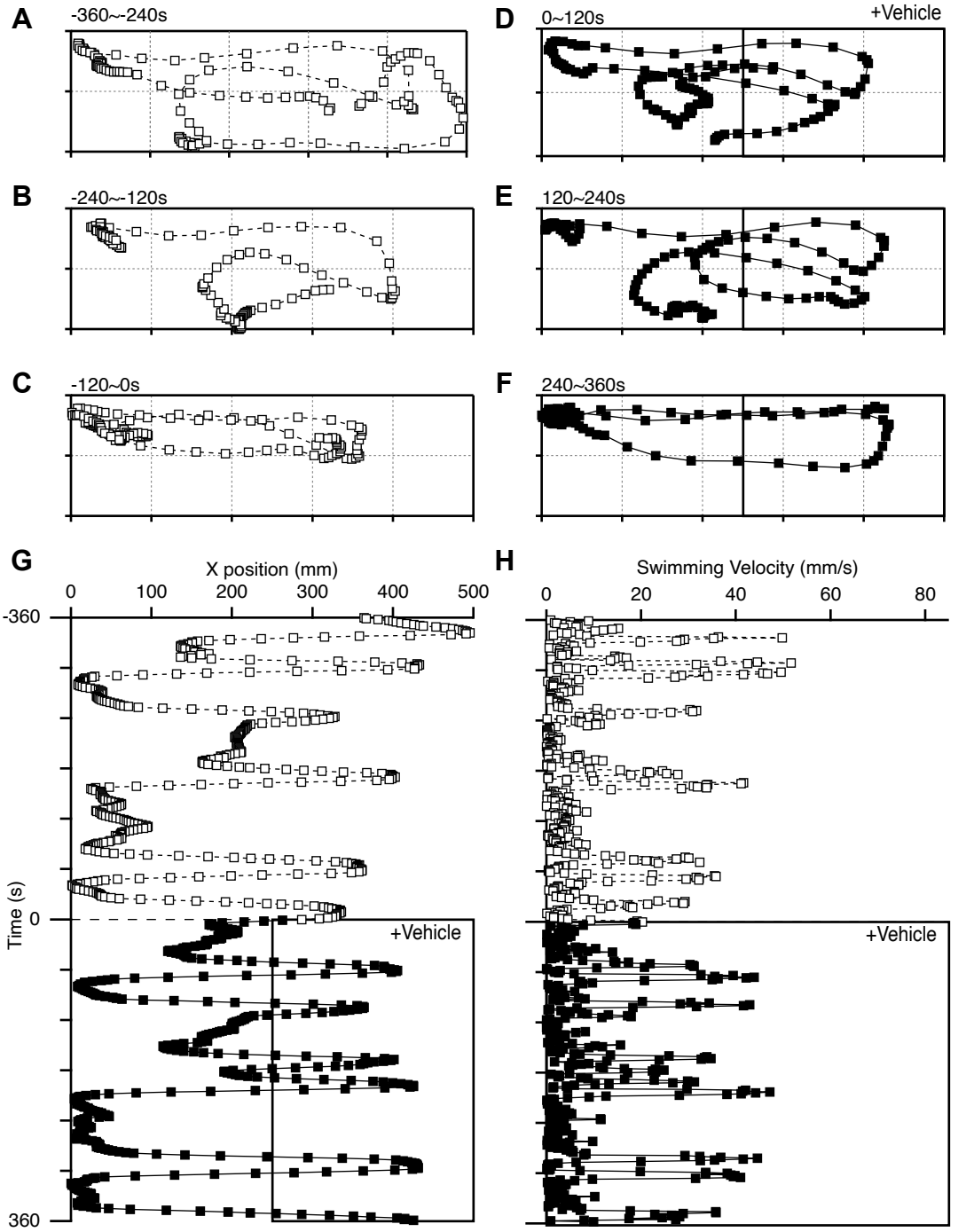

Odorants
Vehicle
4,9-anhydroTTX
(10-5M)
5,6,11-trideoxyTTX
(10-5M)
L-Arg (10-5M)
0.1 mV
2 s

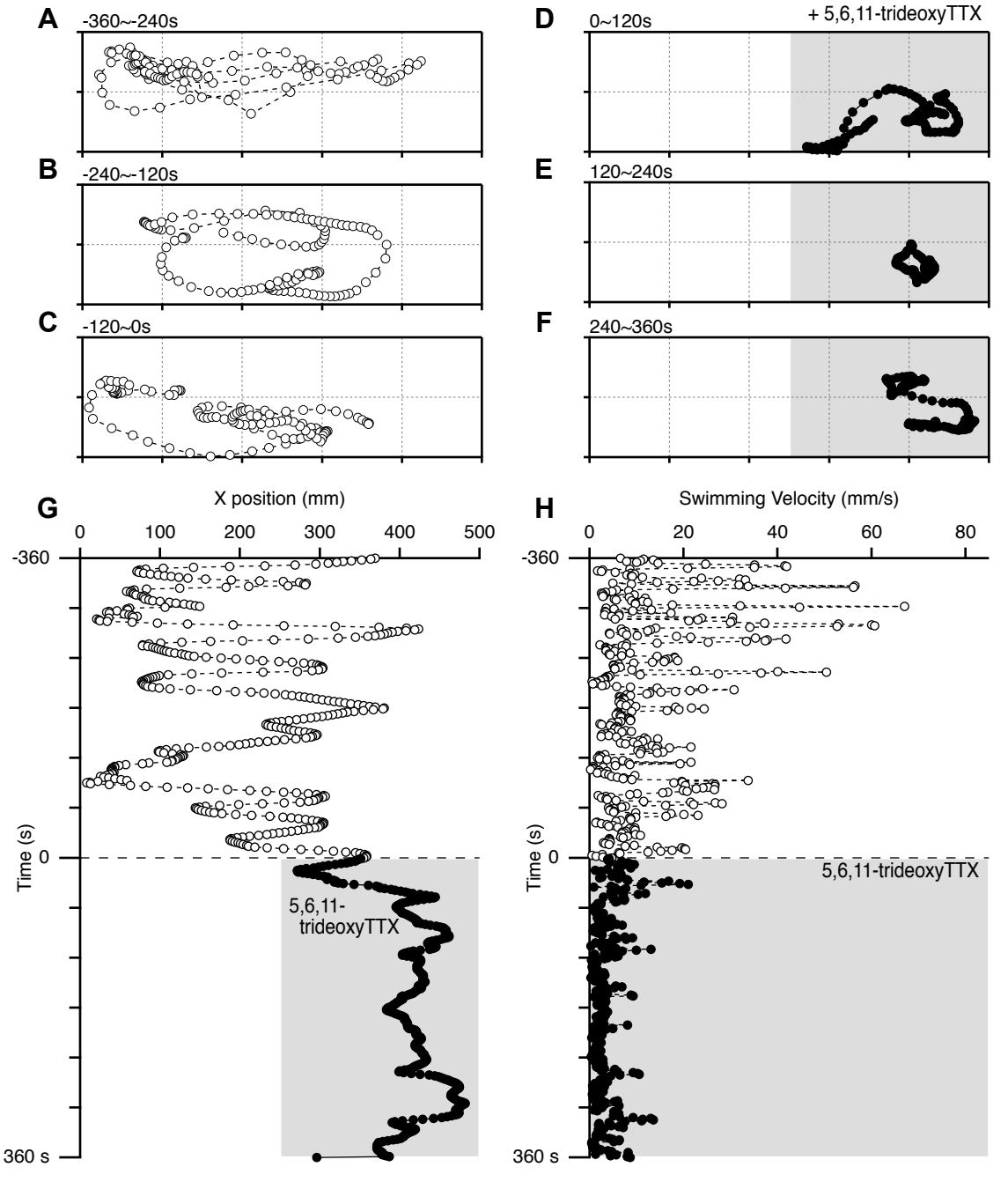
B

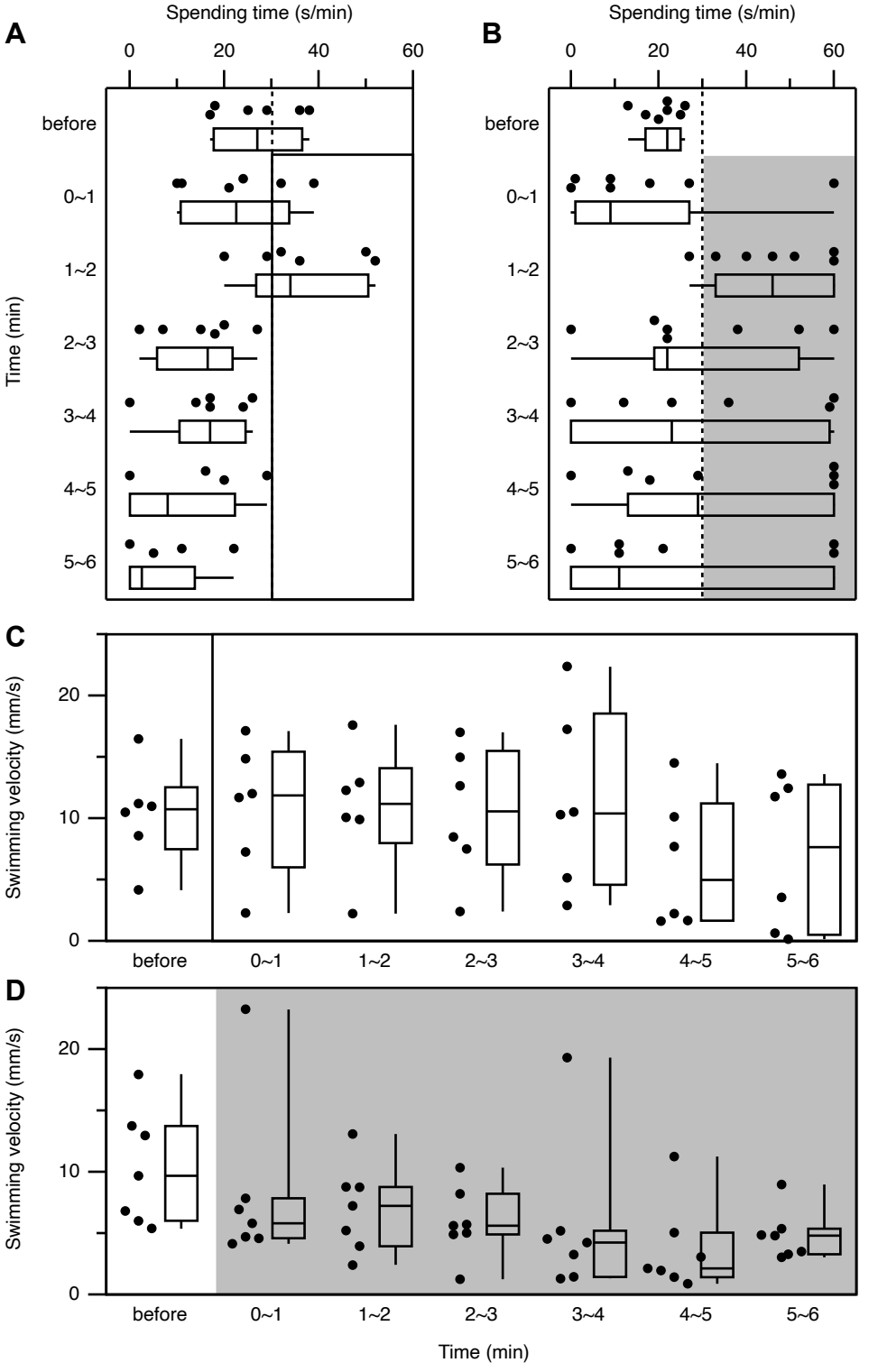

*
*
EOG amplitude (mV)
0.3
0.2
0.1
0.0
Vehicle
4,9-anhydroTTX
(10-5M)
5,6,11-trideoxyTTX
(10-5M)
L-Arg
(10-5M)

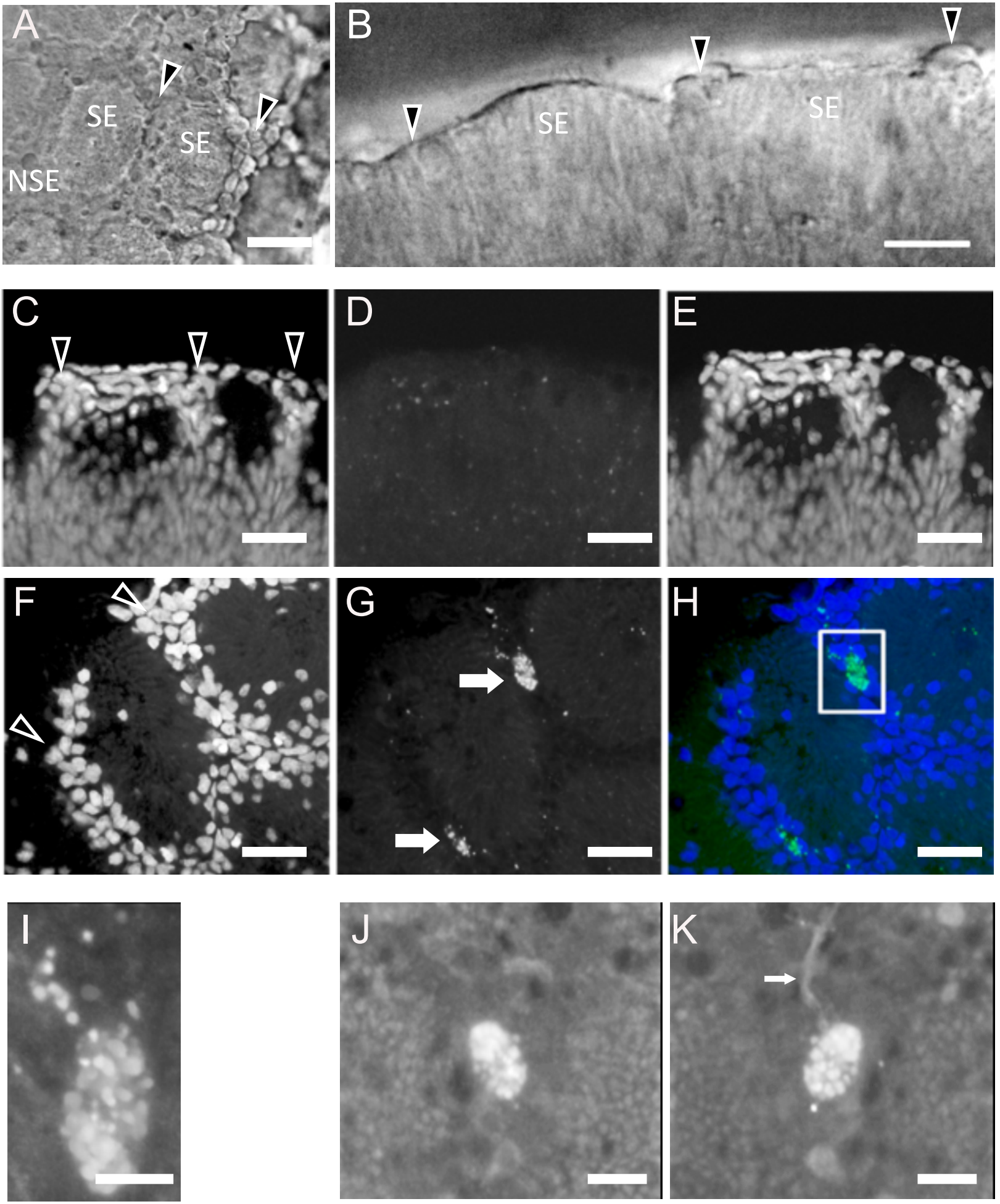

A
5,6,11-TrideoxyTTX
Concentration (M)
10-4
10-5
10-6
10-7
10-8
0.2 mV
2 s

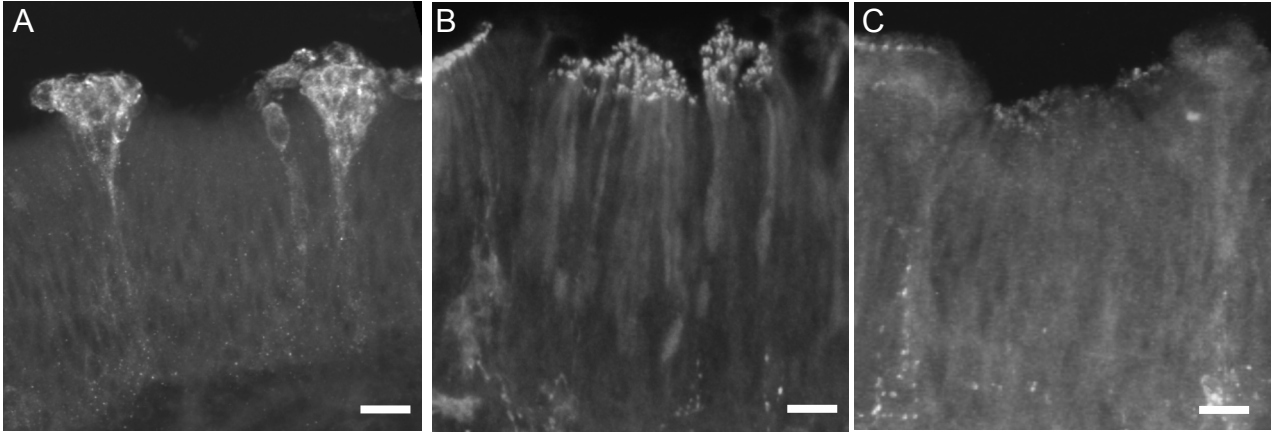

B
EOG amplitude (mV)
0.0
0.1
0.2
0.3
0.4
0.5
10-8
10-7
10-6
10-5
10-4
5,6,11-TrideoxyTTX (logM)


A
-360~-240s
B
-240~-120s
C
-120~0s
D
0~120s
+Vehicle
E
120~240s
F
240~360s
G
X position (mm)
0
100
200
300
400
500
-360
Time (s)
0
360
+Vehicle
H
Swimming Velocity (mm/s)
0
20
40
60
80
+Vehicle


A
-360~-240s
B
-240~-120s
C
-120~0s
D
0~120s
+ 5,6,11-trideoxyTTX
E
120~240s
F
240~360s
G
X position (mm)
0
100
200
300
400
500
-360
Time (s)
0
360 s
5,6,11-
trideoxyTTX
H
Swimming Velocity (mm/s)
0
20
40
60
80
-360
Time (s)
0
360 s
5,6,11-trideoxyTTX


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