1 **Title:** An almost nontoxic tetrodotoxin analog, 5,6,11-trideoxytetrodotoxin,
2 as an odorant for the grass puffer

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18

Abstract

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 acts as an attractive pheromone for some toxic puffers. To elucidate the physiological mechanism of putative pheromonal action of TTX, we examined whether grass puffers *Takifugu alboplumbeus* can detect TTX. Electroolfactogram (EOG) results suggest that the olfactory epithelium (OE) of grass puffers responded 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 by immunohistochemistry against neural activity marker (phosphorylated extracellular signal regulated kinase; pERK) revealed that labeled olfactory sensory neurons were localized in the region surrounding “islets” where there was considered as nonsensory epithelium. 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 toxic puffers may positively use this TTX analog, which has been present in their body with TTX but whose function was unknown, as an odorant for chemical communication or effective TTX accumulation.

Introduction

Toxic puffers accumulate the potent neurotoxin, tetrodotoxin (TTX), which is a well-known voltage-gated sodium channel blocker [1]. 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 [2].

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, produced by certain aquatic bacteria [1,3]. TTX and its analogs were detected in 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 food organisms [2]. Additionally, juveniles of the cultivated tiger puffer *Takifugu rubripes* became nontoxic in aquaria or cages that were suspended above the seafloor with a TTX-free diet [3,4]. These results suggest that TTX in pufferfish is a result of its accumulation through the food chain. Such a phenomenon of a nontoxic organism becoming toxic by taking toxins into its body is called toxification.

Why do toxic puffers accumulate TTX in their bodies? Some toxic puffers have TTX-secreting glands on the skin [5], and electrical stimulation induced TTX secretion from the body surface of these puffers [6]. In addition, TTX induces repulsive or avoidance reactions in predatory fish [7]; predators that ingested *Takifugu* larvae spat them out promptly [8,9]. 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 crude TTX that was extracted from other pufferfish [10]. Artificially raised nontoxic tiger puffer juveniles were also attracted to crude extract of TTX [11–13]. 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 tiger puffer juveniles disappeared after nose ablation [11].

In this study, we found that an almost nontoxic TTX analog, 5,6,11-trideoxyTTX (**Fig. 1**), that has also been known to accumulate in toxic puffers, is an odorant for the grass puffer. We demonstrated the presence of the olfactory sensing ability, specific olfactory sensory neurons (OSNs), and the chemotactic 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 TTX analog, which has been present in their body with TTX but whose function was unknown, as an odorant.

Results

EOG response of grass puffers to TTX

We first performed EOG recordings of adult grass puffers in response to commercially available TTX. The EOG response to a 10-s stimulus with an amino acid solution (10^{-5} M L-Arginine (L-Arg)) was a slow monophasic potential with a steep rising phase followed by an exponential decay toward the baseline (**Fig.2a**, bottom trace). However, TTX (10^{-7} and 10^{-5} M) did not induce significant changes in the EOG (**Fig. 2a** upper and middle traces, respectively). The EOG amplitude of 10^{-7} M TTX (0.027 ± 0.007 mV, n = 4; **Fig. 2b**○) and 10^{-5} M TTX (0.026 ± 0.007 mV, n = 7; **Fig. 2b**●) were significantly lower than that of 10^{-5} M L-Arg (0.074 ± 0.008 mV, n = 11; **Fig. 2b**■).

83

84 EOG response to TTX analogs

85 We then examined the EOG responses of the grass puffer to the two synthesized TTX analogs
86 that have been reported to be simultaneously detected with TTX in toxic puffers or their prey's
87 bodies. A TTX analog, 4,9-anhydroTTX, did not induce significant changes in EOG traces (10^{-5}
88 M; **Fig. 3a**, second trace) when the same concentration of L-Arg showed clear EOG responses
89 (**Fig. 3a**, bottom trace). However, the same concentration of another TTX analog, 5,6,11-
90 trideoxyTTX, induced a clear increase in EOG response (**Fig. 3a**, third trace). Vehicle control
91 (2.5×10^{-5} N acetic acid solution in artificial seawater (ASW)) did not induce any changes in
92 EOG traces (**Fig. 3a**, top trace). EOG amplitudes of 4,9-anhydroTTX, 5,6,11-trideoxyTTX, and
93 L-Arg (all 10^{-5} M) were 0.027 ± 0.004 mV [n = 4], 0.197 ± 0.029 mV [n = 7], and 0.128 ± 0.017
94 mV [n = 7], respectively. EOG amplitudes of 5,6,11-trideoxyTTX and L-Arg were significantly
95 larger than those of the vehicle (0.032 ± 0.007 mV [n = 5]; **Fig.3b**), and EOG amplitude of
96 5,6,11-trideoxyTTX showed a dose-dependent relationship (**Fig. 3c and d**). These results
97 suggest that the OE of the grass puffers can detect 5,6,11-trideoxyTTX.

98

99 Behavioral experiments

100 We next examined the behavioral response of adult grass puffers to 5,6,11-trideoxyTTX. One
101 mature grass puffer was put in a test aquarium filled with 5 L of fresh ASW (50 × 20 × 20 cm),
102 covered with a dark curtain, and acclimated to the test environment (>1 h). Then, 5 mL of
103 vehicle or 5,6,11-trideoxyTTX (10^{-5} M) was administered to one side of the aquarium (the final
104 estimated concentration of 5,6,11-trideoxy TTX was 10^{-8} M). Before administration, the fish

105 intermittently swam left and right and occasionally stopped in one place in the aquarium.
106 Administration of 5,6,11-trideoxyTTX caused the fish to approach and frequently stop on the
107 administered side (**Movie S1**). Such change in the swimming pattern was reflected in the time
108 vs. x-position plot of the fish (**Fig. 4a**). Similar changes in swimming patterns were observed
109 with food extract administration (Supplementary **Fig. S1**), but not with vehicle administration
110 (**Fig. 4d**).

111 To analyze odorant preference, we calculated the time spent on the administered side and
112 the mean swimming velocity of the individual grass puffer during the three-minute periods from
113 the second to the fifth minute before and after the administration, when the odorant were
114 considered to have sufficiently diffused to the administered side in the aquarium. This period
115 was determined based on the preliminary dye diffusion test (see Materials and Methods;
116 excluded the data within 2 minutes before and after the start of odorant administration from the
117 analysis to exclude the effects associated with the administration procedure). The time spent by
118 the grass puffers on the administered side increased (62.1 ± 5.1 to 102.9 ± 14.6 s, $p<0.05$; **Fig.**
119 **4b**), and the swimming velocity of grass puffers decreased after the 5,6,11-trideoxyTTX
120 administration (10.4 ± 1.8 to 6.2 ± 1.5 mm/s, n=7, $p<0.05$; **Fig. 4c**). In contrast, there are no
121 significant changes in time spent (81.5 ± 10.8 to 67.7 ± 5.1 s; **Fig. 4d**) and swimming velocity
122 (10.3 ± 1.6 to 10.9 ± 2.3 mm/s; **Fig. 4e**) during the three-minute periods before and after the
123 vehicle administration (n= 6 fish). Furthermore, we examined the behavioral response of non-
124 TTX bearing fish to 5,6,11-trideoxyTTX using medaka (*Oryzias latipes*; inhabiting freshwater
125 without any TTX-bearing prey organisms; Supplementary **Fig. S2**). Medaka did not prefer to
126 stay on the administered side of 5,6,11-trideoxyTTX. These results suggest that grass puffers
127 prefer 5,6,11-trideoxyTTX.

128

129 Activity-dependent labeling of 5,6,11-trideoxyTTX sensitive OSNs

130 To identify OSN that responded to 5,6,11-trideoxyTTX, we first observed the gross morphology
131 of the OE from the grass puffer. Differential interference microscopy images of live olfactory
132 lamellae showed that the surface of olfactory lamella had repeated structures in which the
133 elliptical "islets" of the sensory epithelium (**se** in **Fig. 5a)** were bordered by oval cells and
134 exposed on the surface (arrowheads in **Fig. 5a**). The cross-sectional structure of the olfactory
135 lamellae showed that oval cells (arrowheads in **Fig. 5b**) surrounded small islets where there
136 were some cilia on the surface (arrow in **Fig. 5b**).

137 We performed immunohistochemistry of excised OE from grass puffers with the neural
138 activity marker (phosphorylated extracellular signal regulated kinase; pERK) antibody as an
139 activity indicator. Administration of 5,6,11-trideoxyTTX (10^{-6} M for 1min) labeled oval cells
140 clustering on the surface of the OE (**Fig. 5c**), whereas L-Arg administration (10^{-6} M for 1min)
141 labeled elongated cells with strongly labeled cilia whose somata were in the middle layer of the
142 OEs (close to the basal membrane; **Fig. 5d**). In addition, administration of 5,6,11-trideoxyTTX
143 also induced an activity-dependent uptake of Alexa Fluor 555 -conjugated dextran (10 kDa) to
144 oval cells in the surrounding region of the islets (Supplementary **Fig. S3**). However, vehicle
145 administration did not label any OSNs (**Fig. 5e**).

146

147 **Discussions**

148 In this paper, we electrophysiologically, morphologically, and behaviorally demonstrated that
149 one of the TTX analogs, 5,6,11-trideoxyTTX, is an odorant for grass puffers, *T. alboplumbeus*. A

150 previous behavioral study reported that TTX attracts male grass puffers during the spawning
151 period [10]. TTX also attracted juvenile tiger puffers regardless of sex [11–13], and this attraction
152 effect was blocked by nose ablation [11]. However, TTX did not induce any EOG responses in our
153 study. Previous studies used crude TTX extracted from the wild-caught tiger puffer ovary or
154 liver [11]. The original TTX extraction method used in previous studies reported that their crude
155 TTX from pear puffer (*T. vermicularis*) ovaries contained both TTX and TTX analogs, including
156 5,6,11-trideoxyTTX, 4-*epi* TTX, and 4,9-anhydroTTX [14]. However, our electrospray ionization
157 mass spectrometry analysis indicated that the commercially available TTX that we used in our
158 study did not contain detectable levels of these TTX analogs (data not shown; except 4,9-
159 anhydroTTX because both 4,9-anhydroTTX and TTX are epimerized each other in aqueous
160 solution and are in chemical equilibrium.). Therefore, since the crude TTX extracts obtained in
161 previous studies contain TTX analogs, we hypothesized that some of these TTX analogs might
162 act as odorants for grass puffers and examined the effects of two types of synthesized TTX
163 analogs (4,9-anhydroTTX and 5,6,11-trideoxyTTX). In our recordings, 5,6,11-trideoxyTTX
164 induced an EOG response in grass puffers, but 4,9-anhydroTTX and TTX did not. In addition,
165 the EOG amplitude of 10^{-5} M 5,6,11-trideoxyTTX was significantly greater than that at the same
166 concentration of L-Arg, which is a well-known odorant for many teleosts (Figure 3a).
167 Furthermore, 5,6,11-trideoxyTTX induced EOG responses even at 10^{-8} M (Figure 3 c and d).
168 Because the stimulation threshold of amino acids on the fish olfactory system is 10^{-9} M to 10^{-7} M
169 [15], 5,6,11-trideoxyTTX is considered to be fully capable of being an odorant for grass puffers.

170 5,6,11-trideoxyTTX elicits chemotaxis of grass puffers. In response to 5,6,11-
171 trideoxyTTX administration to one side of the aquarium, grass puffers were attracted to the
172 administered side and showed the behavior of slowing down and swimming in that area. Our

173 preliminary dye diffusion test estimated that the grass puffers could recognize 5,6,11-
174 trideoxyTTX ranging 2×10^{-8} to 1×10^{-5} M within 2 minutes after administration (see Materials
175 and Methods). These estimated 5,6,11-trideoxyTTX concentrations correspond to the threshold
176 concentration of the EOG response, although the concentration is much higher than those of the
177 previous behavioral studies. Mature male grass puffers were attracted to 1.5×10^{-11} M crude
178 TTX extract [10]. Male and female juvenile tiger puffers could also recognize 3.7×10^{-9} [13] and 2.6
179 $\times 10^{-8}$ M [11] of crude TTX extract, respectively. In these studies, they started their "choice"
180 experiments after diffusion of TTX from agar or gelatin on one side of the Y-maze aquarium.
181 They then examined which side of the Y-maze aquarium the pufferfish swam in. Alternatively,
182 TTX analogs other than 5,6,11-trideoxyTTX may also be involved in chemoreception
183 synergistically. For example, female sex pheromones in moths usually consist of
184 multicomponent blends of five to six hydrocarbons, including unbranched fatty acids, alcohols,
185 acetates, or aldehydes in particular combinations and ratios [16]. These differences seem to
186 contribute to effective TTX concentrations in previous studies and that of 5,6,11-trideoxyTTX in
187 our study.

188 Immunohistochemistry with pERK antibody suggested that 5,6,11-trideoxyTTX activates
189 oval cells distributed in the border area around the ciliary "islets" on the olfactory lamella, which
190 were classified as undifferentiated epithelium in previous study [17]. These oval cells were also
191 labeled with simultaneous administration of fluorescent dextran with 5,6,11-trideoxyTTX. It is
192 widely known that teleost fish are equipped with three types (ciliated, microvillous, and crypt) of
193 OSNs intermingled in the OE but show distinct morphology [18]. Among them, crypt OSNs have
194 an oval shape, are located close to the epithelial surface, and have few cilia and microvilli [19].
195 Cells labeled by 5,6,11-trideoxyTTX co-administration had no cilia or microvilli and were

196 located on the epithelial surface around the islets of ciliated epithelium. Based on these
197 morphological characteristics, the 5,6,11-trideoxyTTX-sensitive OSNs are presumed to be crypt
198 or another unknown type of OSN. Our recent study using the green spotted puffers suggested
199 that cells immunopositive for S-100, a marker protein of crypt-type OSNs, were also labeled by
200 another neuronal activity marker, phosphorylated ribosomal protein S6, antibody that is formed
201 after 5,6,11-trideoxyTTX administration [20]. This result further supports that the 5,6,11-
202 trideoxyTTX sensitive OSNs are crypt-type OSNs.

203 Here, let us consider the physiological significance of 5,6,11-trideoxyTTX as an odorant
204 for the grass puffers. 5,6,11-trideoxyTTX has been reported to be present in the body of toxic
205 pufferfish. The distribution of TTX and its analogs throughout the body of grass puffers was
206 reported, and 5,6,11-trideoxyTTX accumulated mainly in the ovary [21]. A receptor binding assay
207 study of voltage-gated sodium channels in rat brain suspensions reported that the dissociation
208 equilibrium constant of 5,6,11-trideoxyTTX (> 5000 nM) was extremely high compared with
209 that of TTX (1.8 ± 0.1 nM) [22]. With such a low affinity for voltage-gated sodium channels,
210 5,6,11-trideoxyTTX can hardly act as a toxin, suggesting that it cannot function as a defense
211 substance against predators. Therefore, the biological significance of why toxic pufferfish
212 accumulate such almost nontoxic TTX analog remains unclear.

213 One possibility is that 5,6,11-trideoxyTTX functions as an attractive sex pheromone for
214 toxic pufferfish. It has been reported that TTX is secreted from the secretory glands on the skin
215 of *T. pardalis, T. flavipterus, T. vermicularis*, and grass puffers [6] and is released from ovulated
216 oocytes in the grass puffers [10]. Interestingly, grass puffer's whole body TTX content was higher
217 during the spawning period [23]. Because 5,6,11-trideoxyTTX is particularly abundant in pufferfish

218 ovaries [24], 5,6,11-trideoxyTTX may also be leaked from the ovarian luminal fluid with TTX
219 during spawning periods, which attracts the males.

220 Another possibility is that grass puffers use 5,6,11-trideoxyTTX as an olfactory cue to
221 locate and feed on TTX-bearing organisms. Wild toxic puffers tend to feed on benthic
222 organisms, such as flatworms, starfish, small gastropods, and skeleton shrimp that are known to
223 possess TTX [2]. For example, a recent study reported that a TTX-bearing planocerid flatworm
224 was found in the gut content of grass puffers [25] and *Chelondon patoca* [26]. Furthermore, some
225 carnivorous gastropods have 5,6,11-trideoxyTTX in addition to TTX (*Charonia lampas*
226 *lampas*[27]; *Nassarius spp.* [28]), and toxic snails have been reported to be attracted to crude TTX
227 extracts [29]. Additionally, grass puffers ingested toxic eggs of another pufferfish, *T. pardalis,*
228 which contained 5,6,11-trideoxyTTX [30]. All these studies support the idea that the pufferfish
229 seems to be attracted by 5,6,11-trideoxyTTX and feed on TTX-bearing organisms, resulting in
230 TTX accumulation.

231 In conclusion, the grass puffers detect 5,6,11-trideoxyTTX as an odorant. Our findings
232 suggest that pufferfish sense a TTX analog, which is present in their body with TTX but whose
233 function was unknown, as an odorant. Further studies are needed to determine whether other
234 TTX analogs can also be detected by olfaction and whether multiple TTX analogs synergistically
235 induce such effects. However, we believe that our results provide a new perspective on the use of
236 TTX and its analogs by toxic puffers.

237

238 **Materials and Methods**

239 <u>Animals</u>

240 One hundred seventy-four grass puffers (body length, 5–12 cm; bodyweight, 6–32 g) were
241 collected from the coastal waters of Ise-bay in Minamichita-Cho on the Pacific coast of central
242 Japan by line-fishing. Grass puffers were fed commercial diets (Tetra Krill-E; Spectrum Brands,
243 Madison, WI, USA) in aerated 300 L breeding tanks (10–25°C, approximately 50 fish per tank)
244 filled with ASW (Instant Oceans Sea Salt; Instant Ocean, Blacksburg, VA, USA) under a 12-h
245 light/12-h dark photoperiod. In this study, we used randomly selected fish from the breeding tank
246 in the experiment. Thus, both males and females were used without distinction. Animal Use and
247 Care Protocols were approved by the Center for Animal Research and Education of the Nagoya
248 University (Approved number: A210871-004). All animals were maintained and used in
249 accordance with ARRIVE guidelines and the Tokai National Higher Education and Research
250 System Regulations on Animal Care and Use in Research (Regulation No.74). No statistical
251 methods were used to predetermine the sample size because many of the outcomes were
252 unknown.

253

254 Odorant chemicals

255 TTX (for biochemistry, citrate buffered, FUJIFILM Wako Pure Chemical Corporation, Osaka,
256 Japan) was dissolved in ultra-pure water at 1.5×10^{-3} M. Two types of TTX analogs, 4,9-
257 anhydroTTX and 5,6,11-trideoxyTTX, were chemically synthesized and purified by HPLC on
258 ion-exchange column (Hitachi gel 3013C). These synthesized analogs do not contain other
259 derivatives and reagents before use, judging by their ^{1}H and ^{13}C-NMR spectra. These
260 purification procedures and the spectra were described in the papers and the supporting
261 information cited in references [31] and [32], respectively. Synthesized TTX analogs were dissolved
262 in 2.5×10^{-2} N acetic acid in artificial cerebrospinal fluid (ACSF; NaCl 231 mM, KCl 8.1 mM,

263 $MgCl_2$ 1.3 mM, $CaCl_2$ 2.4 mM, D-glucose 10 mM, 4-(2-hydroxyethyl)-1-
264 piperazineethanesulfonic acid (HEPES) 10 mM; pH adjusted 7.4 with NaOH), that was modified
265 from the Ringer's solution for marine fish[33], at 10^{-3} M and stored at −30°C until use. L-Arg
266 (FUJIFILM Wako) was prepared as a 10^{-2} M stock in ACSF. For testing, test odorant solutions
267 were prepared fresh as required by diluting aliquots of concentrated stock in ASW. L-Arg was
268 always tested at 10^{-5} M. When testing a series of solutions with different concentrations, the
269 lowest dose was tested first, followed by solutions with concentrations that increased by a factor
270 of 10 to 10^{-4} M. A vehicle solution was prepared in an identical manner by diluting 2.5×10^{-2} N
271 acetic acid in ASW (final concentration, 2.5×10^{-4} N). The food extract solution was prepared by
272 obtaining the supernatant of ground commercial diet (0.5 g) suspended in ASW (50 mL).

273

274 Electroolfactogram recordings

275 EOG recordings were performed on secured grass puffers on a holding apparatus that was placed
276 into a plastic experimental trough. The tricaine methanesulfonate (MS–222; 0.02%) anesthetized
277 grass puffer was mounted on the center groove of a hand-made plastic holder by wrapping it in a
278 paper towel and aluminum foil after attaching an Ag–AgCl plate (1 × 1 cm) with the body wall
279 as a reference electrode. The gills were perfused with ASW containing 0.01% MS–222 through a
280 tube inserted into the mouth throughout the experiments. The skin and cartilage covering the
281 right olfactory cavity were opened, and the exposed olfactory cavity was continuously perfused
282 with ASW using an 18 G stainless needle (NN–1838S; Terumo, Tokyo, Japan). The perfusion
283 rate of ASW was 1.0 mL/min. A computer-controlled solenoid valve (UMG1–T1; CKD,
284 Komaki, Japan) delivered 10-s pulses of test solutions by switching between the ASW and test
285 solutions (diluted appropriately in ASW) at 3-min intervals.

286 EOG responses were recorded using a saline–agar (0.6%)-filled glass capillary (tip
287 diameter, 100 μm) that was bridged with an Ag–AgCl wire filled with ASW. The indifference
288 electrode was placed lightly on the head skin surface near the perfused olfactory cavity, and the
289 recording electrode was positioned over the olfactory rosette using micromanipulators. The
290 electrodes were insulated with paraffin–Vaseline paste that was placed on the surface of the
291 fish's head. Electrical signals were differentially amplified (gain 1,000×; Band-pass 0.08–100
292 Hz) using a DC preamplifier (AVH-11 in VC-11 digital oscilloscope; Nihon-Kohden, Tokyo,
293 Japan). In this study, recordings were not made in full DC mode to minimize the slow drift of the
294 baseline caused by the changing contact area between the odorant solutions constantly dripped
295 into the nose and the artificial seawater being perfused on the gill for respiration. The amplified
296 EOG signal was digitized (10 kHz) and stored on a laptop computer using a USB-6251 data
297 acquisition device (National Instruments, Austin, TX, USA) and WinEDR software (ver. 3.2.7;
298 http://spider.science.strath.ac.uk/sipbs/software_ses.htm; Strathclyde University, Glasgow, UK).

299

300 Behavioral experiments

301 Behavioral experiments were performed using adult male and female grass puffers. For the
302 behavioral assay, a grass puffer was transferred to a test aquarium (50 × 20 × 20 cm) filled with
303 5 L of fresh ASW. The test aquarium was covered with a black curtain and illuminated by an
304 infrared LED-array illuminator (940 nm, ca. 2 W; AE-LED56V2, Akizuki Denshi Tsusho Co.,
305 Tokyo, Japan) so that the experimenter was invisible to the fish. A video camera (XC-ST50,
306 Sony, Tokyo, Japan) equipped with a low distortion C-mount lens (f = 4.4 mm; LM4NC1M,
307 Kowa, Nagoya, Japan) was mounted vertically above the aquarium, and the camera system was

308 adjusted so that the aquarium filled the camera frame. The fish was allowed to acclimate to a
309 dark environment for 90–180 min.

310 Time-lapse image acquisitions from the top of the test aquarium were started (1 s
311 intervals). The time-lapse images were acquired on a laptop computer using image acquisition
312 software (Micro-Manager ver. 1.4.22; https://micro-manager.org)[34] and a USB-video capture
313 unit (PCA-DAV, Princeton, Tokyo, Japan) at a resolution of 640 × 480 pixels. After 6 min of
314 image acquisition, 5 mL of 10^{-5} M 5,6,11-trideoxyTTX or vehicle (5 mL of 2.5×10^{-4} N acetic
315 acid) was gently applied to one side of the test aquarium through a silicone tube attached to a 10
316 mL syringe. We randomly altered the administered side of odor solutions. Then, image
317 acquisitions were continued for up to 6 min. We excluded the data that fish stopped swimming
318 longer than 5 seconds before the odorant administration (20% of the experiments). A preliminary
319 test indicated that diffusion indicator dye (1% methylene blue in ASW) reached half of the
320 experimental aquarium where a grass puffer was swimming within 2 min after administration
321 and had spread throughout the aquarium at the end of the observation period. The numeric x–y
322 coordinates representing the movement of the fish over time (one coordinate in a frame) were
323 extracted from each video using the UMA tracker software (Release-15;
324 http://ymnk13.github.io/UMATracker/)[35].

325

326 Activity-dependent labeling of OSNs

327 To study the ligand specificity of OSNs, we excised the olfactory lamellae from anesthetized
328 fish. The excised olfactory lamella was washed three times for 5 min in ASW and stimulated
329 with odorant solutions (vehicle, L-Arg, TTX, or 5,6,11-trideoxyTTX; 10^{-6} M, 1 mL, diluted with
330 ASW) for 5 min in a 1.5 mL tube. After odor stimulation, OE was fixed in 4% paraformaldehyde

331 and 15% picric acid in 0.1 M PBS overnight at 4°C, rinsed in PBS, and cryoprotected in 30%
332 sucrose in PBS overnight at 4ºC. Cross cryosections (14–20 µm) of OE mounted onto gelatin-
333 coated slides and dried. After rehydration in PBS, the sections were blocked in 2% normal goat
334 serum (NGS) in PBS with 0.05% Triton-X100 (PBST) for 3 h at 28°C. The sections were then
335 incubated with anti-phosphorylated-p44/42 mitogen-activated protein kinase (MAPK;
336 extracellular regulated kinase, ERK1/2) rabbit monoclonal antibody (#4370, Cell Signaling
337 Technology, Danvers, MA, USA; 1:500), which was used in zebrafish OE[36], as a neural activity
338 marker that was diluted with 0.2% NGS in PBST for 2 days at 4°C. The sections were washed
339 three times for 5 min with 0.2% NGS in PBST. Sections were then incubated with Alexa Fluor
340 488-conjugated secondary antibody (goat anti-rabbit IgG (H+L); A-11008, Thermo Fisher
341 Scientific, 1:500) diluted with 0.2% NGS in PBST for 1 day at 4°C.

342 We also used odor-induced uptake of fluorescent dye-conjugated dextran [48]. The fish was
343 anesthetized using 0.02% MS-222 and secured on the holding apparatus used in EOG
344 experiments. The gills were continuously perfused with ASW containing 0.01% MS-222 through
345 a tube inserted into the mouth throughout the procedure. The skin and cartilage covering the
346 olfactory cavity were opened, and the exposed olfactory cavity was continuously perfused with
347 ASW using an 18G stainless needle. The olfactory cavity of anesthetized fish was perfused with
348 L-Arg (10^{-5} M) or 5,6,11-trideoxyTTX (10^{-5} M) together with a dextran conjugated with Alexa
349 Fluor 555 (M.W. 10,000, 10^{-6} M; D34679, Thermo Fisher Scientific, MA, USA) for 5 min. In the
350 control experiments, the olfactory cavity of anesthetized fish was exposed to only Alexa Fluor
351 555-dextran (10^{-6} M) for 5 min. The flow rate was approximately 1.0 mL/min. After the odorant
352 application, the olfactory cavity was rinsed with a continuous flow of ASW for at least 5 min.
353 Fish were kept alive for 3 to 4 h in aerated ASW at room temperature. Then, the fish was re-

354 anesthetized using 0.1% MS-222 and decapitated. The decapitated head was immersed in 4%
355 paraformaldehyde in 0.1 M PBS (pH 7.2, 4°C, 1 h). After fixation, the olfactory lamellae were
356 dissected and rinsed with PBS. The olfactory lamellae were then counterstained with 4′,6-
357 diamidino-2-phenylindole (DAPI; 1 ng/mL, 5 min) and mounted on a hole glass slide with
358 mounting medium (10% Mowiol 4-88, 25% glycerol, 0.2 M Tris-HCl (pH 9.0), and 2.5%-3.0%
359 1,4-diazabicyclo [2,2,2] octane; diluted with ultrapure water).

360 A confocal microscope (FluoVIEW 1000D, IX81, Olympus, Tokyo, Japan) was used to
361 observe the details of olfactory lamellae. Pictures were taken in planes and were separated by
362 0.45 μm z-axis steps. Confocal microscopy images were analyzed using FluoRender (ver. 2.26.3;
363 http://www.sci.utah.edu/software/fluorender.html) for three-dimensional (3D) reconstruction and
364 visualization of dextran-labeled cells[38]. Pre-processing of images before 3D reconstruction
365 (median filtering [radius: 1 pixel] and trimming) was performed by Fiji (https://fiji.sc/)[39].

366

367 Data analysis

368 All animal experiments were performed on fish of both sexes. Statistical analyses and graph
369 preparations in this study were performed using R (ver. 4.0.4; https://www.r-project.org) and
370 Igor Pro (ver. 9.00; WaveMetrics Inc., Lake Oswego, OR, USA). Data are presented as a dot plot
371 or a box–whisker plot (box: median and quartiles; whiskers: 10%–90% range) or mean ±
372 standard error of the mean (SEM).

373

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.N. and H.A. conceived the study, designed experiments, and wrote the manuscript. R.S., R.N., M.A., and T.N. synthesized and purified TTX analogs. Y.N. performed electrophysiology, behavioral experiments, and activity labeling of olfactory sensory neurons by fluorescent dextran. T.S. and K.M. performed immunohistochemistry of olfactory sensory neurons.

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Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

396

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485

486 **Figure legends**

487 **Figure 1: Molecular structures of tetrodotoxin and its analogs used in this study**

488

489 **Figure 2: Grass puffer did not respond to TTX.**

490 **a**: EOG responses of the olfactory epithelium of *T. alboplumbeus* to TTX (10^{-7} M, upper trace;
491 10^{-5} M, middle trace) and L-Arg (10^{-5} M, under trace).

492 **b**: EOG amplitudes of TTX (10^{-7} M, n = 6, open circle; 10^{-5} M, n = 7, filled circle) and L-Arg
493 (10^{-5} M, n = 11, filled square). Box plots show the median, quartiles (boxes), and 10%–
494 90% range (whiskers), and dot plots show raw EOG amplitudes. Steel's multiple
495 comparison test compared with L-Arg (TTX 10^{-7}M, P = 0.009 ; TTX 10^{-5}M, P = 0.011: *
496 $p<0.05$, ** $p<0.01$.

497

498 **Figure 3: 5,6,11-trideoxyTTX induced EOG response of the grass puffer.**

499 **a**: EOG responses to Vehicle (top trace), 4,9-anhydroTTX (10^{-5} M, second trace), 5,6,11-
500 trideoxyTTX (10^{-5} M, third trace), and L-Arg (10^{-5} M, bottom trace) from same grass
501 puffer.

502 **b**: EOG amplitudes of Vehicle (n = 5, open circles), 4,9-anhydroTTX (n = 4, filled circles),
503 5,6,11-trideoxyTTX (n = 7, filled triangles), and L-Arg (n = 7, filled square). Box plots
504 show the median, quartiles (boxes), and 10%–90% range (whiskers) of EOG amplitudes,
505 and dot plots show the raw value of EOG amplitudes. Steel's multiple comparison test
506 compared with Vehicle (5,6,11-trideoxyTTX, P = 0.012; L-Arg, P = 0.012): * $p < 0.05$.

507 **c:** Representative EOG responses by a grass puffer were evoked by increasing the
508 concentration of 5,6,11-trideoxyTTX. The period of 5,6,11-trideoxyTTX administration is
509 shown by the black bar.

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

515

516 **Figure 4: 5,6,11-trideoxyTTX attracts grass puffers.**

517 Tracked location of a grass puffer before and after 5,6,11-trideoxyTTX administration (5 mL
518 of 10^{-5} M dissolved in 2.5×10^{-4} N acetic acid solution, Glid: 100 mm) and positions of the
519 fish are plotted along the horizontal axis of the aquarium every 1 s. 5,6,11-trideoxyTTX was
520 applied at time 0 and remained in the aquarium thereafter (**a**). Time spent on the administered
521 side (**b**) and mean swimming velocity (**c**) during the 3-minute periods before and after the
522 5,6,11-trideoxyTTX administration (n=7). Paired *t*-test compared before and after the
523 administrations. (Time spent, P = 0.037). *p < 0.05.

524 Tracked location of a grass puffer before and after vehicle administration (5 mL of 2.5
525 $\times 10^{-4}$ N acetic acid solution, Glid: 100 mm) and positions of the fish are plotted along the
526 horizontal axis of the aquarium every 1 s. Vehicle was applied at time 0 and remained in the
527 aquarium thereafter (**d**). No significant changes in the time spent (3-min period) (**e**) or
528 swimming velocity (mm/s; **f**) in response to the vehicle administered on one side of the

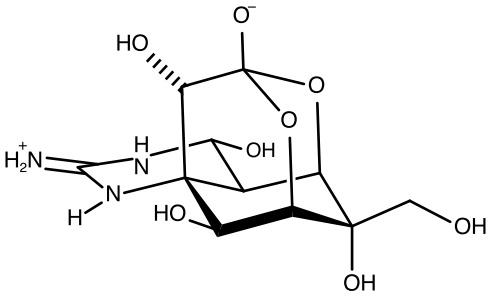
experimental aquarium (n=6). Paired *t*-test compared before and after the administrations. (Time spent, P = 0.202; Swimming velocity, P=0.728).

Open or colored dots and boxes in the plot represent the data before and after odorant administrations. The line and scatter plots show the value from individual fish, and box plots show the median, quartiles (boxes), and 10%–90% range (whiskers).

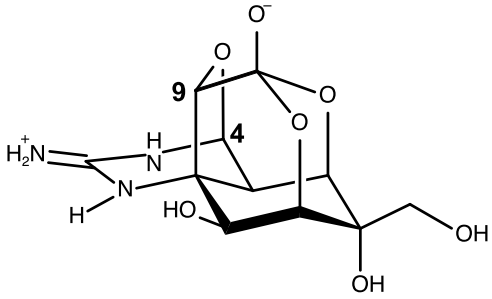
Figure 5: Activity-dependent labeling by pERK antibody labeled oval-shaped cells on the surface of the olfactory epithelium.

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 surrounding the sensory epithelium "islets" (SE). Arrow represents sensory cilia on the surface of the sensory epithelium. Scale bar = 20 μm.

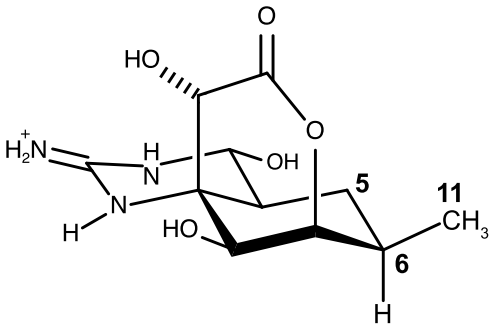
pERK-immunopositive OSNs after exposure to 5,6,11-trideoxyTTX (**c**) or L-Arg (**d**). No labeled cells were observed with the Vehicle control (**e**). Scale bar: 5 μm. Six fish examined for each stimulus showed similar results.



tetrodotoxin (TTX)

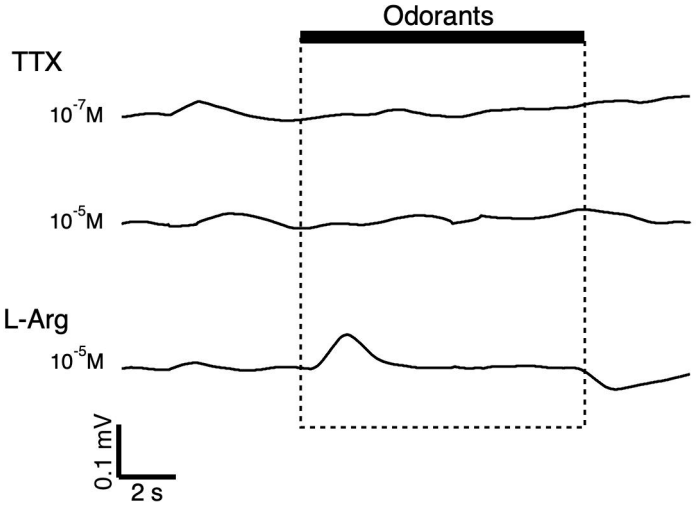


4,9-anhydroTTX

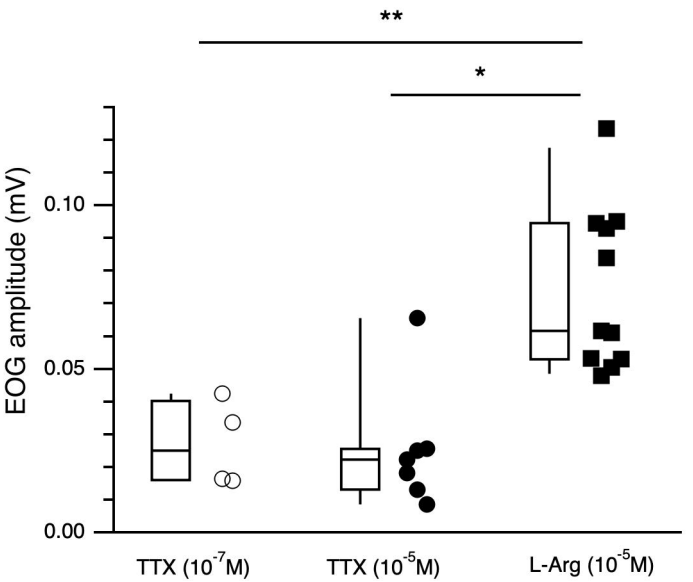


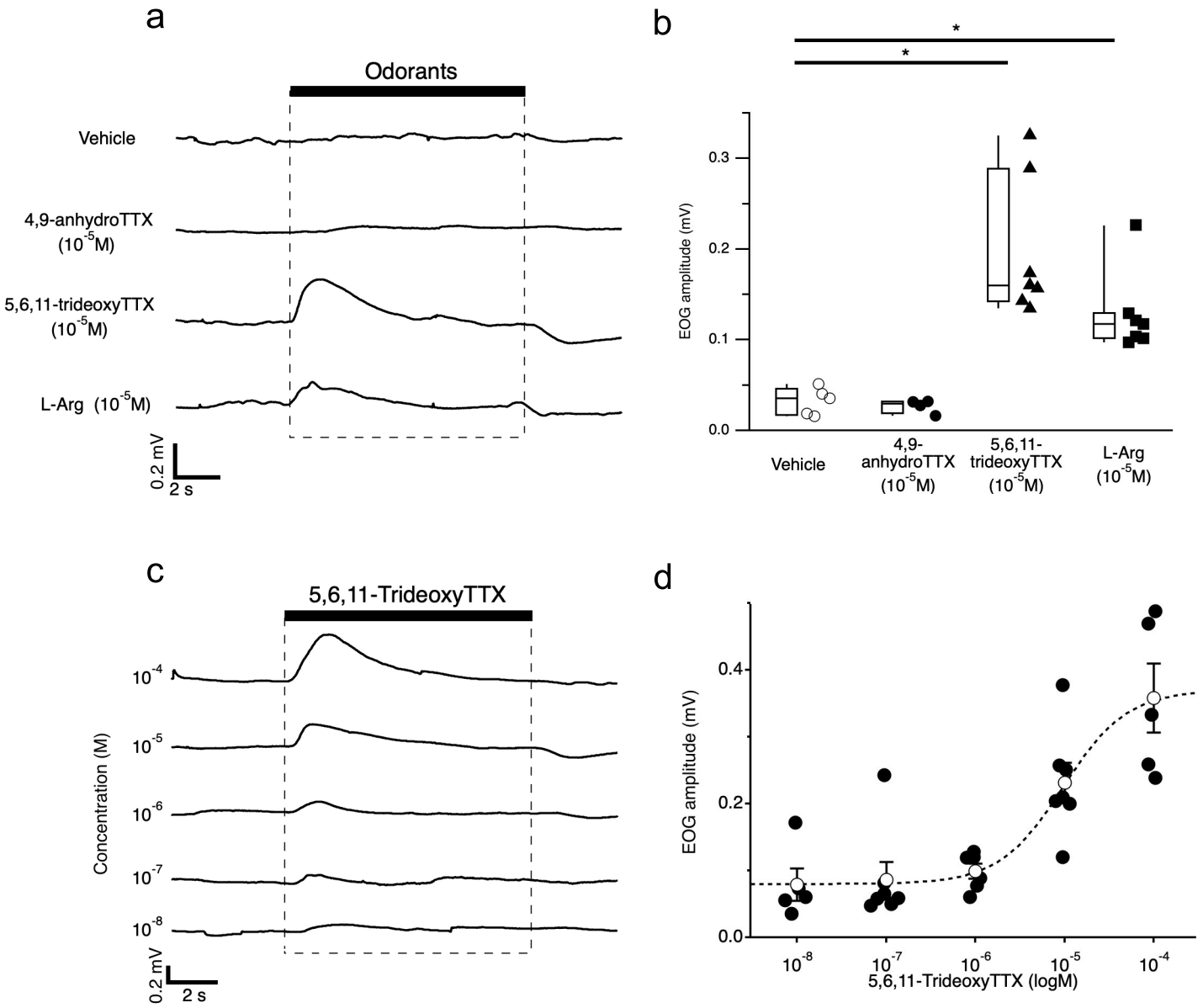
5,6,11-trideoxyTTX

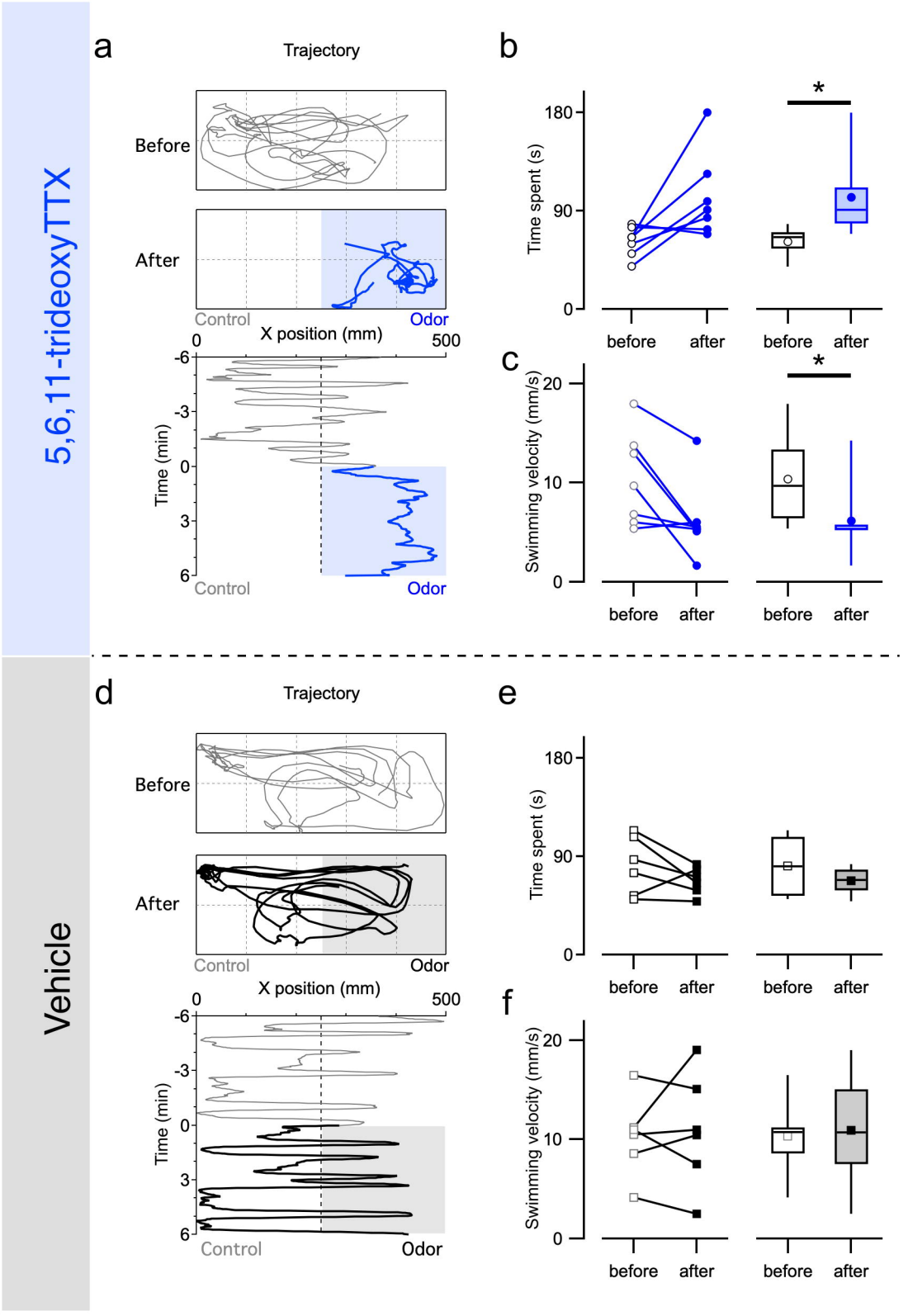
a

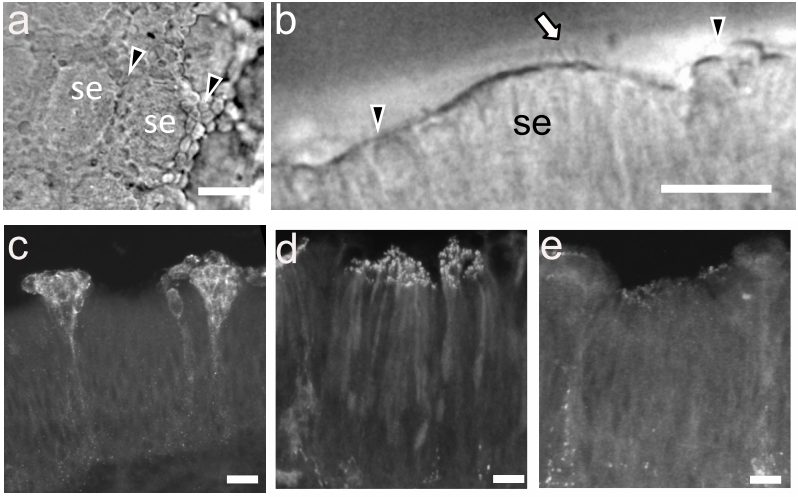

Odorants
TTX
10-7M
10-5M
L-Arg
10-5M
0.1 mV
2 s

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)
0.2 mV
2 s
b
*
*
EOG amplitude (mV)
0.0
0.1
0.2
0.3
Vehicle
4,9-
anhydroTTX
(10-5M)
5,6,11-
trideoxyTTX
(10-5M)
L-Arg
(10-5M)
c
5,6,11-TrideoxyTTX
Concentration (M)
10-4
10-5
10-6
10-7
10-8
0.2 mV
2 s
d
EOG amplitude (mV)
0.0
0.2
0.4
10-8
10-7
10-6
10-5
10-4
5,6,11-TrideoxyTTX (logM)


5,6,11-trideoxyTTX
a
Trajectory
Before
After
Control
Odor
X position (mm)
0
500
Time (min)
-6
-3
0
3
6
b
Time spent (s)
180
90
0
*
before
after
c
Swimming velocity (mm/s)
20
10
0
Vehicle
d
e
f


a
se
se
b
se
c
d
e