1 **Title:** A nontoxic tetrodotoxin analog, 5,6,11-trideoxytetrodotoxin,

2 as an olfactory chemoattractant for the grass puffer

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19

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 unknown function TTX analog as an olfactory chemoattractant.

38 **Introduction**

39 Toxic puffers accumulate the potent neurotoxin, tetrodotoxin (TTX), which is a well-known
40 voltage-gated sodium channel blocker [1]. TTX was first believed to be present only in pufferfish
41 from the family Tetraodontidae. However, it was later detected in taxonomically diverse animals
42 (e.g., teleosts, amphibians, echinoderms, crustaceans, mollusks, and worms) inhabiting
43 terrestrial, marine, freshwater, and brackish water environments [2].

44 Regardless of the abundant studies, the biological origin of TTX and its ecological
45 functions remain unclear. However, the presence of TTX in such a variety of organisms suggests
46 that the ultimate origin of TTX is exogenous or that it is symbiotically produced by certain
47 aquatic bacteria [1,3]. TTX and its analogs were detected in pufferfish prey, including starfish,
48 gastropods, crustaceans, flatworms, and ribbonworms, in addition to several species of bacteria
49 that are free-living or symbiotic with pufferfish or their food organisms [2]. Additionally, juveniles
50 of the cultivated tiger puffer *Takifugu. rubripes* became nontoxic in aquaria or cages that were
51 suspended above the seafloor with a TTX-free diet [4,5]. These results suggest that TTX in
52 pufferfish is a result of its accumulation through the food chain.

53 Why do toxic puffers accumulate TTX in their bodies? Some toxic puffers have TTX-
54 secreting glands on the skin [6], and electrical stimulation induced TTX secretion from the body
55 surface of these puffers [7]. TTX induces repulsive or avoidance reactions in predatory fish [8];
56 predators that ingested *Takifugu* larvae spat them out promptly [9,10]. Thus, in toxic puffers, TTX
57 functions as a biological defense substance against predators.

58 Although the main function of TTX in pufferfish appears to be defensive, several studies
59 have reported that pufferfish can exploit TTX as a source of communication. Sexually active
60 male grass puffers (*T. alboplumbeus*) were attracted to crude TTX that was extracted from other

61 pufferfish [11]. Artificially raised nontoxic tiger puffer juveniles were also attracted to crude
62 extract of TTX [12–14]. These studies suggest that pufferfish may sense TTX. However, the TTX-
63 sensing mechanism in these puffers remains unclear, although the attractive effect of TTX on
64 tiger puffer juveniles disappeared after nose ablation [12].

65 In this study, we found that a nontoxic TTX analog, 5,6,11-trideoxyTTX (**Fig. 1**), that
66 has also been known to accumulate in toxic puffers, is an olfactory chemoattractant for the grass
67 puffer. We demonstrated the presence of the olfactory sensing ability, specific olfactory sensory
68 neurons (OSNs), and the attraction behavior of grass puffers in response to 5,6,11-trideoxyTTX.
69 To the best of our knowledge, this is the first report that pufferfish positively use functionally
70 unknown TTX analogs for chemical communication.

71

72 **Results**

73 <u>EOG response of grass puffers to TTX</u>

74 We first performed EOG recordings of adult grass puffers in response to a commercially
75 available TTX. The EOG response to a 10-s stimulus with an amino acid solution (10^{-5} M L-
76 Arginine (L-Arg)) was a slow monophasic potential with a steep rising phase followed by an
77 exponential decay toward the baseline (**Fig.2a**, bottom trace). However, TTX (10^{-7} and 10^{-5} M)
78 did not induce significant changes in the EOG (**Fig. 2a** upper and middle traces, respectively).
79 The EOG amplitude of 10^{-7} M TTX (0.027 ± 0.007 mV, n = 4; **Fig. 2b**○) and 10^{-5} M TTX (0.026
80 ± 0.007 mV, n = 7; **Fig. 2b**●) were significantly lower than that of 10^{-5} M L-Arg (0.074 ± 0.008
81 mV, n = 11; **Fig. 2b**■).

82

83 EOG response to TTX analogs

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

96

97 Behavioral experiments

98 We next examined the behavioral response of adult grass puffers to 5,6,11-trideoxyTTX. One
99 mature grass puffer was put in a test aquarium filled with 5 L of fresh ASW (50 × 20 × 20 cm)
100 and acclimated to the fish for the test environment in the dark (>1 h). Then, 5 mL of Vehicle or
101 5,6,11-trideoxyTTX (10^{-5} M) was administered to one side of the aquarium (the final estimated
102 concentration of 5,6,11-trideoxy TTX was 10^{-8} M). Before administration, the fish intermittently
103 swam left and right and occasionally stopped in one place in the aquarium. Administration of
104 5,6,11-trideoxyTTX caused the fish to approach and frequently stop on the administered side
105 (**Movie S1**). Such change in the swimming pattern was reflected in the time vs. x-position

106 velocity plot of the fish (**Fig. 4a**). Similar changes in swimming patterns were observed with
107 food extract administration (Supplementary **Fig. S1**), but not with vehicle administration (**Fig.**
108 **4d**).

109 To quantify the degree of attraction, we calculated the time spent on the administered
110 sides and the mean swimming velocity of the individual grass puffers for every 1 min period
111 (**Fig. 4b and e**). The fish at first tended to move away from the administered side because of
112 the vibration of water surface caused by odorant administrations. The fish subsequently preferred
113 to stay on the administered side when 5,6,11-trideoxyTTX was administered, but tended to avoid
114 the administered side when Vehicle was administered. Time spent by the grass puffers on the
115 administered side was longer with the 5,6,11-trideoxyTTX treatment than with the vehicle
116 treatment ($p<0.01$, two-way ANOVA; **Fig. 4b and e**), and the swimming velocity of grass
117 puffers was decreased by 5,6,11-trideoxyTTX compared to the vehicle treatment ($p<0.01$, two-
118 way ANOVA; **Fig. 4c and f**). Non TTX-bearing fish (medaka) did not prefer to stay on the
119 administered side of 5,6,11-trideoxyTTX (Supplementary **Fig. S2**). These results suggest that
120 5,6,11-trideoxyTTX worked as a chemoattractant for grass puffers.

121

122 <u>Activity-dependent labeling of 5,6,11-trideoxyTTX sensitive OSNs</u>

123 To identify OSN that responded to 5,6,11-trideoxyTTX, we first observed the gross morphology
124 of the OE from the grass puffer. Differential interference microscopy images of live olfactory
125 lamellae showed that the surface of olfactory lamella had repeated structures in which the
126 elliptical "islets" of sensory epithelium (**se** in **Fig. 5a)** were bordered by oval cells and exposed
127 on the surface (arrowheads in **Fig. 5a**). The cross-sectional structure of the olfactory lamellae

showed that oval cells (arrowheads in **Fig. 5b**) surrounded small islets where there were some cilia on the surface (arrow in **Fig. 5b**).

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

Discussions

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

A previous behavioral study reported that TTX attracts male grass puffers during the spawning period [11]. TTX also attracted juvenile tiger puffers regardless of sex [12–14], and this attraction effect was blocked by nose ablation [12]. However, TTX did not induce any EOG responses in our study. Previous studies used crude TTX that was extracted from the wild-caught

150 tiger puffer ovary or liver [12]. Original TTX extraction study reported that their crude TTX from
151 pear puffer (*T. vermicularis*) ovaries contained both TTX and TTX analogs, including 5,6,11-
152 trideoxyTTX, 4-epi TTX, and 4,9-anhydroTTX [15]. We used a commercially available TTX in
153 this study. The manufacturer's datasheet indicated that this TTX was also extracted from
154 pufferfish ovary and liver, but the species were not specified. However, our electrospray
155 ionization mass spectrometry analysis indicated that the commercially available TTX that we
156 used did not contain detectable levels of these TTX analogs (except 4,9-anhydroTTX, a
157 chemically equilibrium analog of TTX; data not shown). Thus, we hypothesized that the crude
158 TTX extract from previous studies might contain TTX analogs, and some of these TTX analogs
159 may act as olfactory chemoattractants for the pufferfish.

160 To investigate this hypothesis, we examined the effects of two types of synthesized TTX
161 analogs (4,9-anhydroTTX and 5,6,11-trideoxyTTX) using EOG and a behavioral assay. Our
162 EOG recordings showed that 5,6,11-trideoxyTTX induced an EOG response in grass puffers. In
163 our recordings, the EOG amplitude of 10^{-5} M 5,6,11-trideoxyTTX was significantly greater than
164 that at the same concentration of L-Arg, which is a well-known odorant for many teleosts
165 (Figure 3a). Furthermore, 5,6,11-trideoxyTTX induced EOG responses even at 10^{-8}M (Figure 3
166 c and d). Because the stimulation threshold of amino acids on the fish olfactory system is 10^{-9}M
167 to 10^{-7}M [16], 5,6,11-trideoxyTTX is a potent, powerful odorant for grass puffers.

168 5,6,11-trideoxyTTX attracts grass puffers. In response to 5,6,11-trideoxyTTX
169 administration to one side of the aquarium, grass puffers were attracted to the administered side
170 of the aquarium and showed the behavior of slowing down and swimming in that area. We
171 estimated from our preliminary dye diffusion test that the grass puffers could recognize 5,6,11-
172 trideoxyTTX ranging 2×10^{-8} to 1×10^{-5} M within 2 minutes after administration (see Materials

173 and Methods). These estimated 5,6,11-trideoxyTTX concentrations correspond to the threshold
174 concentration of the EOG response, although the concentration is much higher than those of the
175 previous behavioral studies. Mature male grass puffers were attracted to 1.5×10^{-11} M TTX [11].
176 Both male and female juvenile tiger puffers also could recognize 3.7×10^{-9} [14] and 2.6×10^{-8} M [12]
177 of TTX, respectively. In these studies, they started their "choice" experiments after diffusion of
178 TTX from agar or gelatin on one side of the Y-maze aquarium. These differences in
179 experimental paradigms seem to contribute to the difference in the effective TTX concentration.
180 Alternatively, TTX analogs other than 5,6,11-trideoxyTTX may also involve chemoreception,
181 and multiple forms of TTX analogs other than TTX and 4,9-anhydroTTX may act
182 synergistically. In olfaction, a combination of several compounds can be perceived as a specific
183 odor, or a series of structurally similar compounds can be detected by a single olfactory receptor.
184 Most pheromone specificity is achieved using a combination of compounds [17]. For example,
185 female sex pheromones in moths usually consist of multicomponent blends of five to six
186 hydrocarbons including unbranched fatty acids, alcohols, acetates, or aldehydes in particular
187 combinations and ratios [18]. In goldfish and carp, a combination of species-specific metabolites of
188 prostaglandin F2α (PGF2α), a well-known releaser pheromone that induces sexual behavior,
189 strongly induces species-specific sexual behavioral responses [19]. Thus, it is possible that other
190 metabolites in pufferfish may play a synergistic action with 5,6,11-trideoxyTTX as a chemical
191 signal to attract the opposite sex.

192 5,6,11-trideoxyTTX activates oval OSNs that were distributed in the border area around
193 the ciliary "islets" on the olfactory lamella of grass puffers. Comparative histological studies
194 reported that the sensory epithelium of grass puffers was dispersed in many discrete islets on the
195 surface of the olfactory lamella, and the border area of the sensory islet was classified as

196 undifferentiated epithelium [20]. However, our immunohistochemistry with pERK antibody, a
197 neuronal activity marker, suggested that oval cells that were activated by 5,6,11-trideoxyTTX
198 were present in the border area around the sensory islets. These oval cells were also labeled with
199 simultaneous administration of fluorescent dextran with 5,6,11-trideoxyTTX.

200 It is widely known that teleost fish are equipped with three types of OSNs that are
201 intermingled in the OE but show distinct morphology [21]. First, there are the ciliated cells with
202 long dendrites and a few cilia, and second, there are microvillous cells with shorter dendrites
203 than the ciliated cells, which have microvilli extending from their apical surface. The third type
204 is the crypt cells. Crypt cells have an oval shape, are located close to the epithelial surface, and
205 have few cilia and microvilli [22]. Cells that were labeled by 5,6,11-trideoxyTTX co-administration
206 had no cilia or microvilli and were located on the epithelial surface around the islets of ciliated
207 epithelium. Based on these morphological characteristics, the 5,6,11-trideoxyTTX-sensitive
208 OSNs are presumed to be crypt or another unknown type of OSN. Our recent study using the
209 green spotted puffers suggested that cells that were immunopositive for S-100, a marker protein
210 of crypt-type OSNs, were also labeled by another neuronal activity marker, phosphorylated
211 ribosomal protein S6, antibody that is formed after 5,6,11-trideoxyTTX administration[23]. This
212 result further supports that the 5,6,11-trideoxyTTX sensitive OSNs are crypt-type olfactory
213 sensory neurons.

214 Based on these experimental data, we concluded that adult grass puffers can detect
215 5,6,11-trideoxyTTX by olfaction. 5,6,11-trideoxyTTX has been reported to be present in the
216 body of toxic pufferfish. The distribution of TTX and its analogs throughout the body of grass
217 puffers was reported, and 5,6,11-trideoxyTTX accumulated mainly in the ovary [24]. Remarkably,
218 5,6,11-trideoxyTTX accumulates more in the ovary than TTX. A receptor binding assay study of

219 voltage-gated sodium channels in rat brain suspensions reported that the dissociation equilibrium
220 constant of 5,6,11-trideoxyTTX (> 5000 nM) was extremely high compared with that of TTX
221 (1.8 ± 0.1 nM) [25]. These studies suggest that 5,6,11-trideoxyTTX does not act as a toxin and that
222 it does not act as a defense substance against predators. Intramuscular injection study reported
223 that the transformation from TTX to its deoxy analogs or oxidized analogs does not occur in the
224 body of grass puffers [26]. This result suggests that 5,6,11-trideoxy-TTX is a precursor to TTX, but
225 it remains unclear whether 5,6,11-trideoxy-TTX is converted to TTX inside the toxic puffers or
226 other organisms [27,28]. Therefore, the biological significance of why toxic puffers accumulate such
227 nontoxic TTX analogs remains unclear.

228 In the previous study, TTX was treated as an attractive sex pheromone for toxic puffers.
229 Pheromones are defined as follows: "an odor or mixture of odorous substances, released by an
230 individual (the sender) and evoking in conspecifics (the receivers) adaptive, specific, and
231 species-typical response(s), the expression of which needs not require prior experience or
232 learning" [29] or "molecules that are evolved signals, in defined ratios in the case of multiple
233 component pheromones, which are emitted by an individual and received by a second individual
234 of the same species, in which they cause a specific reaction, for example, a stereotyped behavior
235 or a developmental process" [30]. In fish, 17,20β-dihydroxy-4-pregnen-3-one and prostaglandin
236 F2α (goldfish), a type of sex steroid that is found in ovarian cavity fluid and urine, and a bile acid
237 that is released from gills, urine, and feces (jawless sea lamprey, rainbow trout, and lake char)
238 have been reported to act as attractive sex pheromones [29]. TTX is secreted from the secretory
239 glands on the skin of *T. pardalis, T. poecilonotus, T. vermicularis*, and grass puffers [7] and is
240 released from ovulated oocytes in the grass puffers [11]. Because 5,6,11-trideoxyTTX is

241 particularly abundant in pufferfish ovaries [31], 5,6,11-trideoxyTTX may also be released from the
242 ovarian luminal fluid during spawning periods.

243 These chemicals, known as fish pheromones, are synthesized by conspecific fish.
244 However, current circumstantial evidence suggests that 5,6,11-trideoxyTTX, unlike other
245 pheromones, is acquired from TTX-bearing organisms by eating them [32,33]. In some animals,
246 molecules made by other organisms are harvested and used as pheromones. For example, male
247 neotropical orchid bees can unsuccessfully display and attract females without a species-specific
248 pheromone mix, such as limonenes, from the right kinds of orchid flowers [34]. Animals may also
249 use molecules that are produced by bacteria as pheromones. Phase-change pheromones of the
250 locust are guaiacol (2-methoxyphenol) and phenol, and they are produced by locust gut bacteria
251 [35].

252 Wild toxic puffers tend to feed on benthic organisms, such as flatworms, starfish, small
253 gastropods, and skeleton shrimp that are known to possess TTX [3]. A recent study reported that a
254 TTX-bearing planocerid flatworm was found in the gut content of grass puffers [36] and *Chelondon*
255 *patoca* [37]. Furthermore, some carnivorous gastropods have 5,6,11-trideoxyTTX in addition to
256 TTX (*Charonia lampas lampas*[38]; *Nassarius spp.* [39]), and toxic snails (*Polinices didyma, Natica*
257 *lineata, Natica vitellus, Zeuxis sufflatus, Niotha clathrata, Oliva miniacea, Oliva mustelin*a, and
258 *Oliva hirasei*) have been reported to be attracted to crude TTX extracts [40]. Additionally, grass
259 puffers ingested toxic eggs of another pufferfish, *T. pardalis,* which contained 5,6,11-
260 trideoxyTTX [41]. All these studies support that the pufferfish seems to be attracted by 5,6,11-
261 trideoxyTTX and that it ingests TTX-bearing organisms as food, resulting in accumulating TTX
262 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 unknown function 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.

Materials and Methods

Animals

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

286

287 Odorant chemicals

288 TTX (for biochemistry, citrate buffered, FUJIFILM Wako Pure Chemical Corporation, Osaka,
289 Japan) was dissolved in ultra-pure water at 1.5×10^{-3} M. Two types of TTX analogs, 4,9-
290 anhydroTTX and 5,6,11-trideoxyTTX, were chemically synthesized and purified by HPLC on
291 ion-exchange column (Hitachi gel 3013C). These synthesized analogs do not contain other
292 derivatives and reagents before use, judging by their ^{1}H and ^{13}C-NMR spectra. These
293 purification procedures and the spectra were described in the papers and the supporting
294 information cited in reference [42] and [43], respectively. Synthesized TTX analogs were dissolved in
295 2.5×10^{-2} N acetic acid at 10^{-3} M and stored at −30°C until use. L-Arg (FUJIFILM Wako) was
296 prepared as a 10^{-2} M stock in artificial cerebro-spinal fluid (ACSF; NaCl 231 mM, KCl 8.1 mM,
297 $MgCl_2$ 1.3 mM, $CaCl_2$ 2.4 mM, D-glucose 10 mM, 4-(2-hydroxyethyl)-1-
298 piperazineethanesulfonic acid (HEPES) 10 mM; pH adjusted 7.4 with NaOH) that was modified
299 from the Ringer's solution for marine fish [44]. For testing, test odorant solutions were prepared
300 fresh as required by diluting aliquots of concentrated stock in ACSF or in ASW. L-Arg was
301 always tested at 10^{-5} M. When testing a series of solutions with different concentrations, the
302 lowest dose was tested first, followed by solutions with concentrations that increased by a factor
303 of 10 to 10^{-4} M. A vehicle solution was prepared in an identical manner by diluting 2.5×10^{-2} N
304 acetic acid in ACSF or ASW (final concentration, 2.5×10^{-4} N). The food extract solution was
305 prepared by obtaining the supernatant of ground commercial diet (0.5 g) suspended in artificial
306 seawater (50 mL).

307

308 Electroolfactogram recordings

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

320 EOG responses were recorded using a saline–agar (0.6%)-filled glass capillary (tip
321 diameter, 100 μm) that was bridged with an Ag–AgCl wire filled with ACSF. The indifference
322 electrode was placed lightly on the head skin surface near the perfused olfactory cavity, and the
323 recording electrode was positioned over the olfactory rosette using micromanipulators. The
324 electrodes were insulated with a paraffin–Vaseline paste that was placed on the surface of the
325 fish's head. Electrical signals were differentially amplified (gain 1,000×; Band-pass 0.8–100 Hz)
326 using a DC preamplifier (AVH-11 in VC-11 digital oscilloscope; Nihon-Kohden, Tokyo, Japan).
327 In this study, recordings were not made in full DC mode to minimize the slow drift of the
328 baseline caused by the changing contact area between the odorant solutions constantly dripped
329 into the nose and the artificial seawater being perfused the gill for respiration. The amplified
330 EOG signal was digitized (10 kHz) and stored on a laptop computer using a USB-6251 data

331 acquisition device (National Instruments, Austin, TX, USA) and WinEDR software (ver. 3.2.7;
332 http://spider.science.strath.ac.uk/sipbs/software_ses.htm; Strathclyde University, Glasgow, UK).
333

334 Behavioral experiments

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

344 Time-lapse image acquisitions from the top of the test aquarium were started (1 s
345 intervals). The time-lapse images were acquired on a laptop computer using image acquisition
346 software (Micro-Manager ver. 1.4.22; https://micro-manager.org)[45] and a USB-video capture
347 unit (PCA-DAV, Princeton, Tokyo, Japan) at a resolution of 640 × 480 pixels. After 6 min of
348 image acquisition, 5 mL of 10^{-5} M 5,6,11-trideoxyTTX or vehicle (5 mL of 2.5×10^{-4} N acetic
349 acid) was gently applied to one side of the test aquarium through a silicone tube attached to a 10
350 mL syringe. We randomly altered the administered side of odor solutions. Then, image
351 acquisitions were continued for up to 6 min. We excluded the data that fish stopped swimming
352 longer than 5 seconds before the odorant administrations (20% of the experiments). A
353 preliminary test indicated that diffusion indicator dye reached half of the experimental aquarium

354 where a grass puffer was swimming within 2 min after administration and had spread throughout
355 the aquarium at the end of the observation period. The numeric x–y coordinates representing the
356 movement of the fish over time (one coordinate in a frame) were extracted from each video using
357 the UMA tracker software (Release-15; http://ymnk13.github.io/UMATracker/)[46].

358

359 Activity-dependent labeling of OSNs

360 To study the ligand specificity of OSNs, we excised the olfactory lamellae from
361 anesthetized fish. The excised olfactory lamella was washed three times for 5 min in ACSF and
362 stimulated with odorant solutions (vehicle, L-Arg, TTX, or 5,6,11-trideoxyTTX; 10^{-6} M, 1 mL,
363 diluted with ACSF) for 5 min in a 1.5 mL tube. After odor stimulation, OE was fixed in 4%
364 paraformaldehyde and 15% picric acid in 0.1 M PBS overnight at 4°C, rinsed in PBS, and
365 cryoprotected in 30% sucrose in PBS overnight at 4ºC. Cross cryosections (14–20 μm) of OE
366 mount onto gelatin-coated slides and dried. After rehydration in PBS, the sections were blocked
367 in 2% normal goat serum (NGS) in PBS with 0.05% Triton-X100 (PBST) for 3 h at 28°C. The
368 sections were then incubated with anti-phosphorylated-p44/42 mitogen-activated protein kinase
369 (MAPK; extracellular regulated kinase, ERK1/2) rabbit monoclonal antibody (#4370, Cell
370 Signaling Technology, Danvers, MA, USA; 1:500), which was used in zebrafish OE[47], as a
371 neural activity marker that was diluted with 0.2% NGS in PBST for 2 days at 4°C. The sections
372 were washed three times for 5 min with 0.2% NGS in PBST. Sections were then incubated with
373 Alexa Fluor 488-conjugated secondary antibody (goat anti-rabbit IgG (H+L); A-11008, Thermo
374 Fisher Scientific, 1:500) that was diluted with 0.2% NGS in PBST for 1 day at 4°C.

375 We also used odor-induced uptake of fluorescent dye-conjugated dextran [48]. The fish was
376 anesthetized using 0.02% MS-222 and secured on the holding apparatus used in EOG

377 experiments. The gills were continuously perfused with ASW containing 0.01% MS-222 through
378 a tube inserted into the mouth throughout the procedure. The skin and cartilage covering the
379 olfactory cavity were opened, and the exposed olfactory cavity was continuously perfused with
380 ACSF using an 18G stainless needle. The olfactory cavity of anesthetized fish was perfused with
381 L-Arg (10^{-5} M) or 5,6,11-TrideoxyTTX (10^{-5} M) together with a dextran conjugated with Alexa
382 Fluor 555 (M.W. 10,000, 10^{-6} M; D34679, Thermo Fisher Scientific, MA, USA) for 5 min. In the
383 control experiments, the olfactory cavity of anesthetized fish was exposed to only Alexa Fluor
384 555-dextran (10^{-6} M) for 5 min. The flow rate was approximately 1.0 mL/min. After the odorant
385 application, the olfactory cavity was rinsed with a continuous flow of ACSF for at least 5 min.
386 Fish were kept alive for 3 to 4 h in aerated ASW at room temperature. Then, the fish was re-
387 anesthetized using 0.1% MS-222 and decapitated. The decapitated head was immersed in 4%
388 paraformaldehyde in 0.1 M PBS (pH 7.2, 4°C, 1 h). After fixation, the olfactory lamellae were
389 dissected and rinsed with PBS. The olfactory lamellae were then counterstained with 4′,6-
390 diamidino-2-phenylindole (DAPI; 1 ng/mL, 5 min) and mounted on a hole glass slide with
391 mounting medium (10% Mowiol 4-88, 25% glycerol, 0.2 M Tris-HCl (pH 9.0), and 2.5%-3.0%
392 1,4-diazabicyclo [2,2,2] octane; diluted with ultrapure water).

393 A confocal microscope (FluoVIEW 1000D, IX81, Olympus, Tokyo, Japan) was used to
394 observe the details of olfactory lamellae. Pictures were taken in planes and were separated by
395 0.45 μm z-axis steps. Confocal microscopy images were analyzed using FluoRender (ver. 2.26.3;
396 http://www.sci.utah.edu/software/fluorender.html) for three-dimensional (3D) reconstruction and
397 visualization of dextran-labeled cells[49]. Pre-processing of images before 3D reconstruction
398 (median filtering [radius: 1 pixel] and trimming) was performed by Fiji (https://fiji.sc/)[50].

399

400 Data analysis

401 All animal experiments were performed on fish of both sexes. Statistical analyses were
402 performed using Kyplot (ver. 5; KyensLab Inc., Tokyo, Japan) and Igor Pro (ver. 9.00;
403 WaveMetrics Inc., Lake Oswego, OR, USA). Data are presented as a dot plot or a box–whisker
404 plot (box: median and quartiles; whiskers: 10%–90% range) or mean ± standard error of the
405 mean (SEM).

406

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410

411 **Competing interests**

412 The authors declare no competing or financial interests.

413

414 **Author contributions**

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

420

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541

542 **Figure legends**

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

544

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

546 **a**: EOG responses of the olfactory epithelium of *T. alboplumbeus* to TTX (10^{-7} M, upper trace;
547 10^{-5} M, middle trace) and L-Arg (10^{-5} M, under trace).

548 **b**: EOG amplitudes of TTX (10^{-7} M, n = 6, open circle; 10^{-5} M, n = 7, filled circle) and L-Arg
549 (10^{-5} M, n = 11, filled square). Box plots show the median, quartiles (boxes), and 10%–
550 90% range (whiskers), and dot plots show raw EOG amplitudes. Steel's multiple
551 comparison test compared with L-Arg (TTX 10^{-7}M, P = 0.009 ; TTX 10^{-5}M, P = 0.011: *
552 $p<0.05$, ** $p<0.01$.

553

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

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

558 **b**: EOG amplitudes of Vehicle (n = 5, open circles), 4,9-anhydroTTX (n = 4, filled circles),
559 5,6,11-trideoxyTTX (n = 7, filled triangles), and L-Arg (n = 7, filled square). Box plots
560 show the median, quartiles (boxes), and 10%–90% range (whiskers) of EOG amplitudes,
561 and dot plots show the raw value of EOG amplitudes. Steel's multiple comparison test
562 compared with Vehicle (5,6,11-trideoxyTTX, P = 0.012; L-Arg, P = 0.012): * $p < 0.05$.

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

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

571

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

573 Tracked location of a grass puffer before and after 5,6,11-trideoxyTTX administration (5 mL
574 of 10^{-5} M dissolved in 2.5×10^{-4} N acetic acid solution, Glid: 100 mm) and positions of the
575 fish are plotted along the horizontal axis of the aquarium every 1 s. 5,6,11-trideoxyTTX was
576 applied at time 0 and remained in the aquarium thereafter (**a**). Changes in the time (1-min
577 period) spent by grass puffers (**b**) or changes in the swimming speed (mm/s; **c**) in response to
578 5,6,11-trideoxyTTX administered on one side of the experimental aquarium (n=7). One
579 sample t-test for significant deviation from the mean time spent before 5,6,11-trideoxyTTX
580 administration (20.7 s; 1~2 min, P = 0.002). *$p < 0.05$.

581 Tracked location of a grass puffer before and after vehicle administration (5 mL of 2.5
582 $\times 10^{-4}$ N acetic acid solution, Glid: 100 mm) and positions of the fish are plotted along the
583 horizontal axis of the aquarium every 1 s. Vehicle was applied at time 0 and remained in the
584 aquarium thereafter (**d**). No changes in the time (1-min period) spent by grass puffers (**e**) or
585 swimming speed (mm/s; **f**) in response to vehicle administered on one side of the
586 experimental aquarium (n=6). One sample t-test for significant deviation from the mean time

587 spent before Vehicle administration (27.2 s; 2~3 min, P = 0.021; 3~4 min, P = 0.034; 4~5
588 min, P = 0.024; 5~6 min, P = 0.002). *p < 0.05.

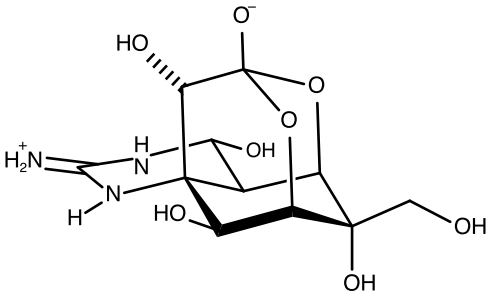
589 The vertical dotted line in **b** and **e** represents the chance level (= No odorant
590 preference). Open or colored dots and boxes in the plot represent the data before and after
591 odorant administrations. Dot plots show the value from individual fish, and box plots show
592 the median, quartiles (boxes), and 10%–90% range (whiskers). The results of the statistical
593 analysis between the odorant administrations are shown in the text.

594

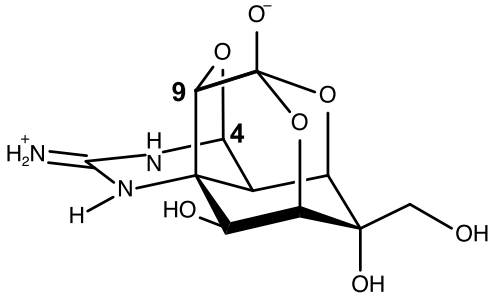
595 **Figure 5: Activity-dependent labeling by pERK antibody labeled oval-shaped cells**
596 **on the surface of the olfactory epithelium.**

597 Differential interference contrast photomicrographs of the surface (**a**) and the edge (**b**) of the
598 olfactory lamella of grass puffer. Arrowheads represent the non-ciliated epithelium that
599 surrounds the "islets" of the sensory epithelium (SE). Arrow represents sensory cilia on the
600 surface of the sensory epithelium. Scale bar = 20 μm.

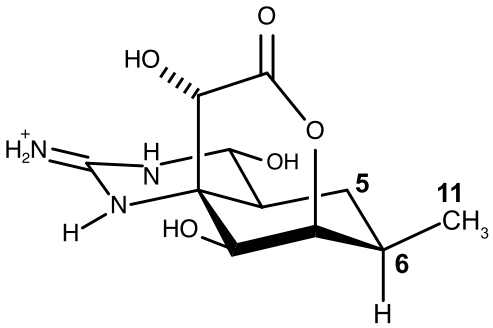
601 pERK-immunopositive OSNs after exposure to 5,6,11-trideoxyTTX (**c**) or L-Arg (**d**). No
602 labeled cells were observed with the Vehicle control (**e**). Scale bar: 5 μm. Six fish examined
603 for each stimulus showed similar results.



tetrodotoxin (TTX)

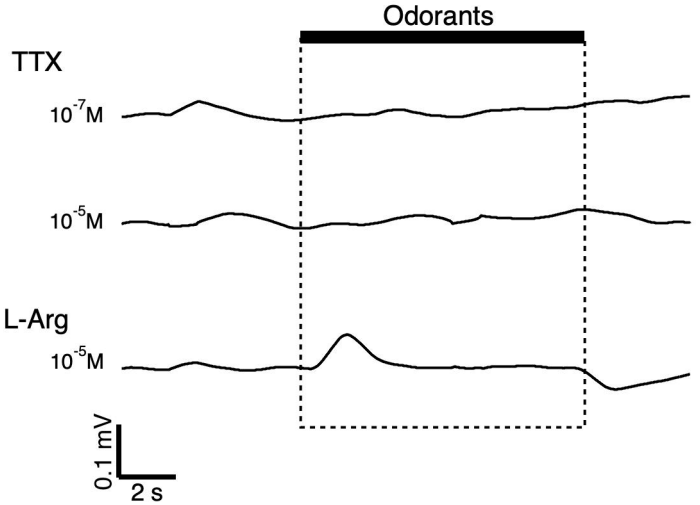


4,9-anhydroTTX

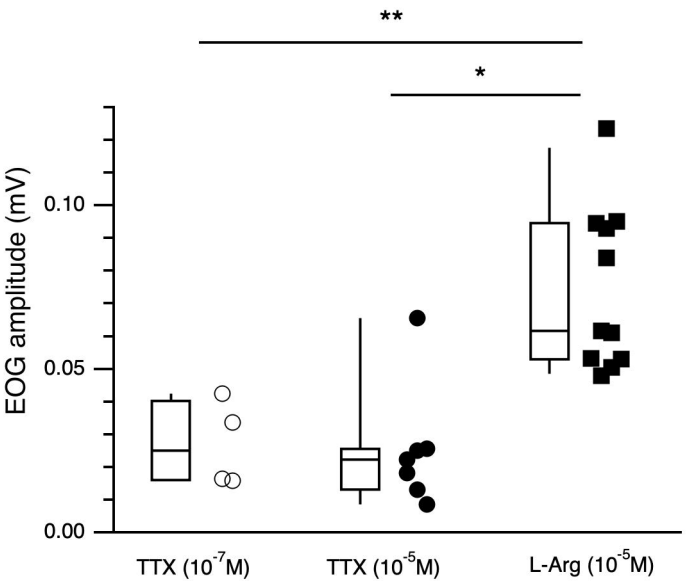


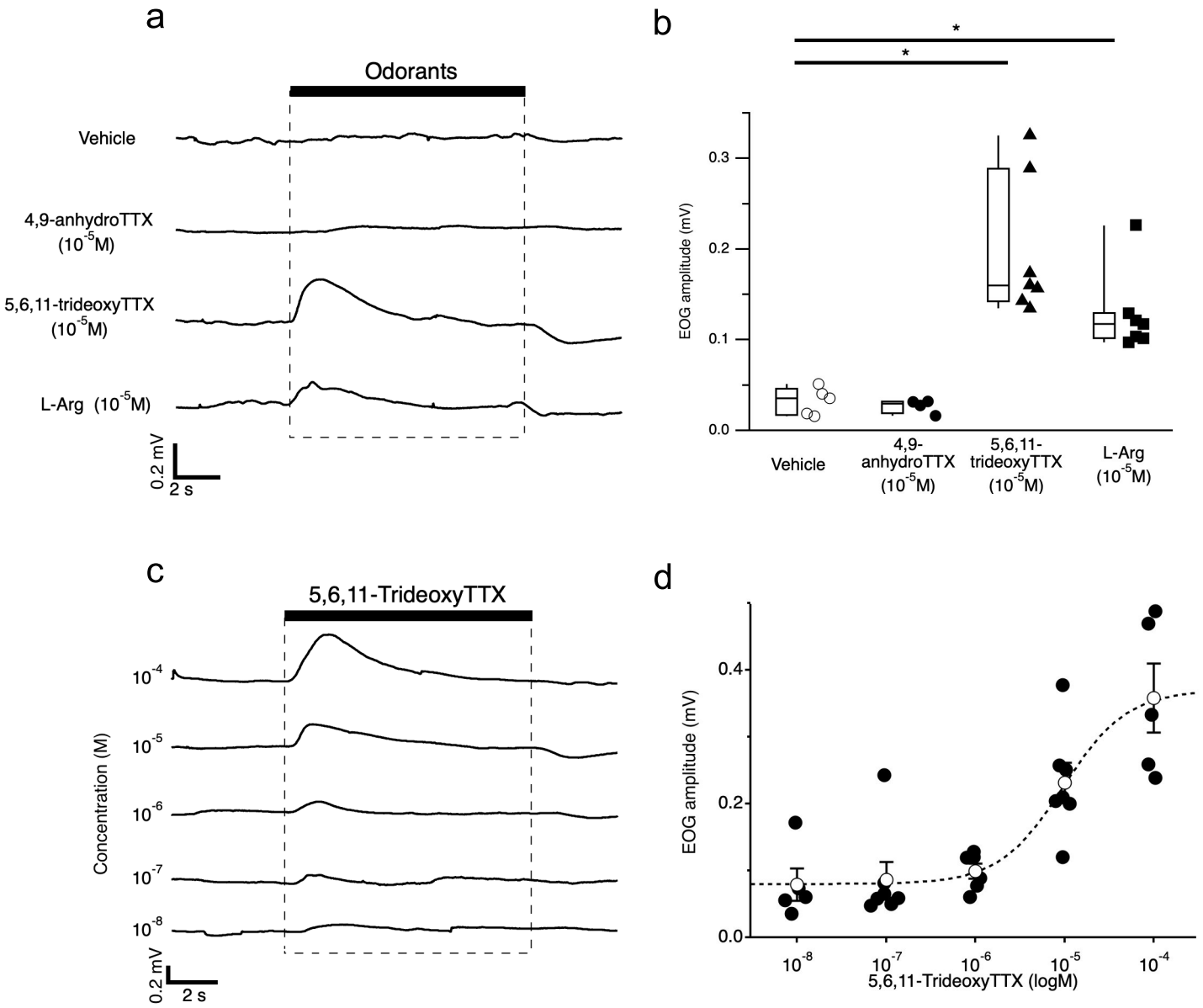
5,6,11-trideoxyTTX

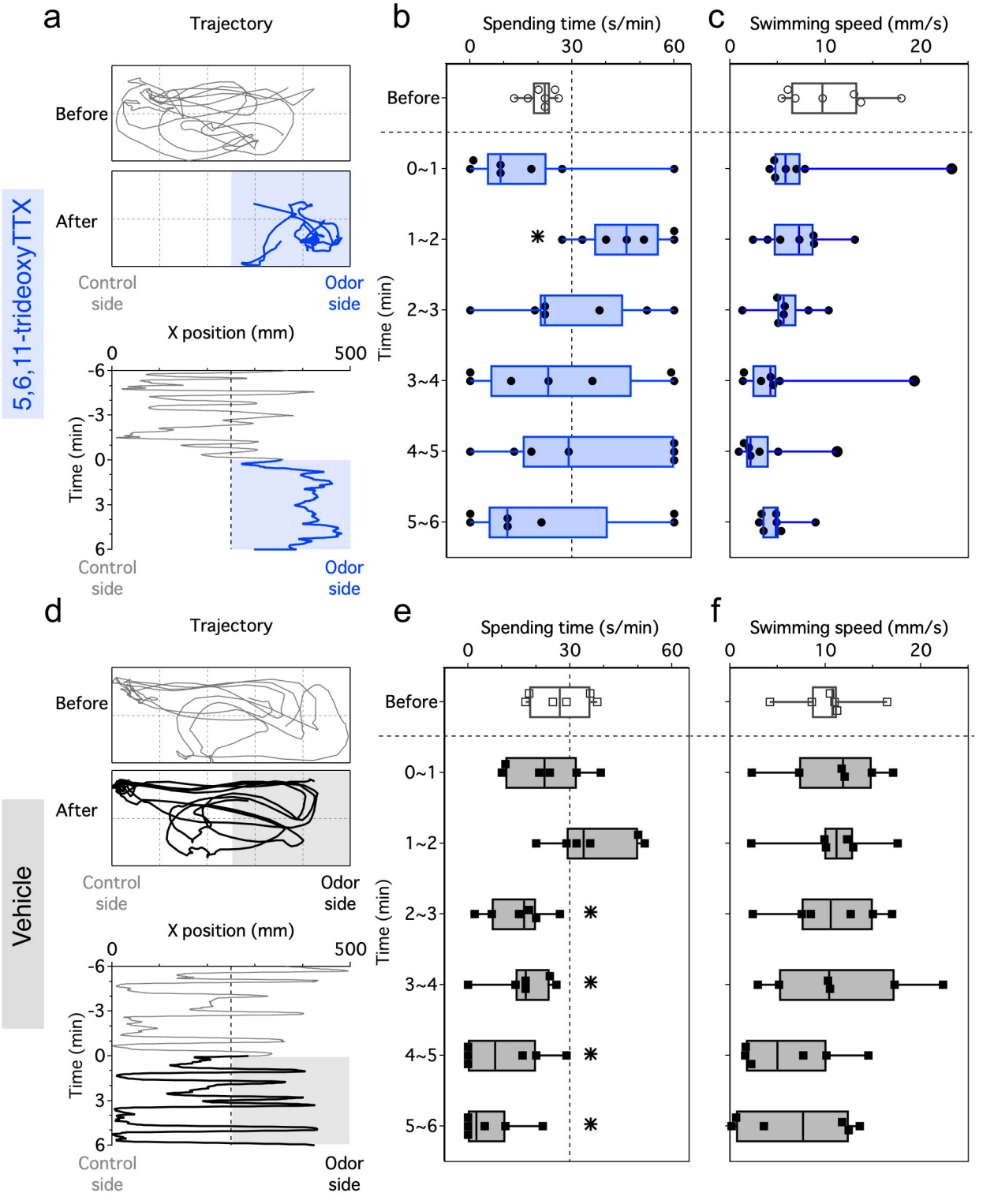
a

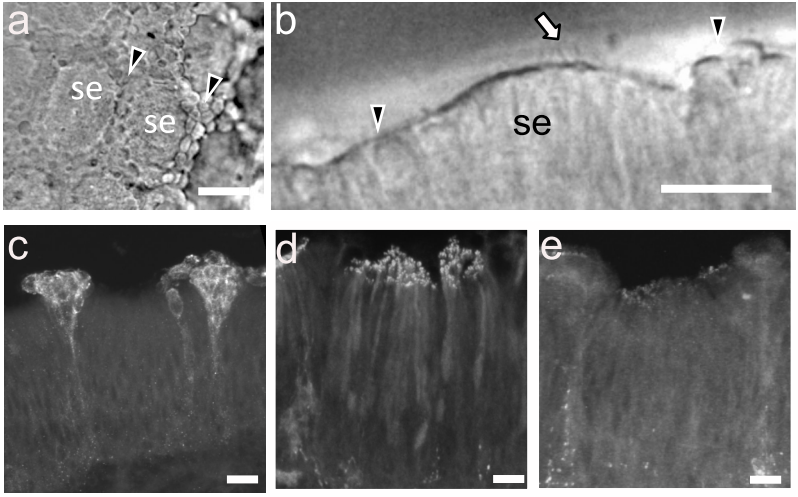

Odorants
TTX
10-7M
10-5M
L-Arg
10-5M
0.1 mV
2 s

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)


a
Trajectory
Before
After
Control side
Odor side
X position (mm)
0
500
Time (min)
-6
-3
0
3
6
Control side
Odor side
5,6,11-trideoxyTTX
b
Spending time (s/min)
0
30
60
Before
0~1
1~2
2~3
3~4
4~5
5~6
Time (min)
c
Swimming speed (mm/s)
0
10
20
d
Trajectory
Before
After
Control side
Odor side
X position (mm)
0
500
Time (min)
-6
-3
0
3
6
Control side
Odor side
Vehicle
e
Spending time (s/min)
0
30
60
Before
0~1
1~2
2~3
3~4
4~5
5~6
Time (min)
f
Swimming speed (mm/s)
0
10
20


a
se
se
b
se
c
d
e