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## **First evidence that emerging pinnatoxin-G, a contaminant of shellfish, reaches the brain and crosses the placental barrier**

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21 **ABSTRACT**

22 Massive proliferation of some toxic marine dinoflagellates is responsible for the occurrence of  
23 harmful algal blooms and the contamination of fish and shellfish worldwide. Pinnatoxins (PnTx) (A-  
24 H) comprise an emerging phycotoxin family belonging to the cyclic imine toxin group. Interest has  
25 been focused on these lipophilic, fast-acting and highly potent toxins because they are widely found in  
26 contaminated shellfish, and can represent a risk for seafood consumers. PnTx display a potent  
27 antagonist effect on nicotinic acetylcholine receptors (nAChR), and in this study we assessed *in vivo*  
28 the ability of PnTx-G to cross physiological barriers to reach its molecular target. Radiolabeled [<sup>3</sup>H]-  
29 PnTx-G synthesized with good radiochemical purity and yield retained the high affinity of the natural  
30 toxin. Oral gavage or intravenous administration to adult rats and digital autoradiographic analyses  
31 revealed the biodistribution and toxicokinetics of [<sup>3</sup>H]-PnTx-G, which is rapidly cleared from blood,  
32 and accumulates in the liver and small intestine. The labeling of peripheral and brain adult/embryo rat  
33 tissues highlights its ability to cross the intestinal, blood-brain and placental barriers. High-resolution  
34 3D-imaging and *in vitro* competition studies on rat embryo sections revealed the specificity of [<sup>3</sup>H]-  
35 PnTx-G binding and its selectivity for muscle and neuronal nAChR subtypes (such as  $\alpha 7$  subtype).  
36 The use of a human perfused cotyledon model and mass spectrometry analyses disclosed that PnTx-G  
37 crosses the human placental barrier. The increasing worldwide occurrence of both the dinoflagellate  
38 *Vulcanodinium rugosum* and PnTx-contaminated shellfish, due to climate warming, raises concerns  
39 about the potential adverse impact that exposure to pinnatoxins may have for human health.

40

41

42 **Keywords:** Pinnatoxins; Cyclic imine toxins; Nicotinic acetylcholine receptors; Harmful algal bloom;  
43 Toxicokinetics; Digital autoradiography

44

45 **Abbreviations:** CI, cyclic imine; PnTx, pinnatoxins; nAChR, nicotinic acetylcholine receptor; MS,  
46 mass spectrometry; FTR, fetal transfer rate; MLA, methyllycaconitine; SUG, superficial gray layer of  
47 the superior colliculus

## 49 **1. Introduction**

50 Cyclic imine (CI) toxins produced by various species of marine dinoflagellates constitute a widely  
51 distributed group of phycotoxins, comprising the prorocontrolides, spiro-prorocentrimine,  
52 gymnodimines, spirolides, pinnatoxins, pteriatoxins, and portimines, with increasing prevalence in the  
53 global environment (Molgó et al., 2017; Stivala et al., 2015). The warming of ocean temperatures and  
54 increases in nutrients have driven an intensification of harmful algal bloom events (Anderson et al.,  
55 2012; Farabegoli et al., 2018; Griffith and Gobler, 2020; Hallegraeff, 2010; Van Dolah, 2000). During  
56 these active dinoflagellate blooms, phycotoxins can accumulate in shellfish tissues and be transferred  
57 into crabs, fish, seabirds, marine mammals and ultimately to humans (Molgó et al., 2014).

58 In recent years, there is increasing evidence for the occurrence of emerging pinnatoxins (PnTx) in  
59 various wild or commercial shellfish species, collected at different periods of the year, and in different  
60 marine waters. Among the eight known PnTxs (A to H), PnTx-G is the most commonly found  
61 contaminating shellfish in European waters (Aráoz et al., 2020; Kvirgić et al., 2021; McCarthy et al.,  
62 2015; Moreiras et al., 2019; Otero et al., 2019; Otero et al., 2020; Rambla-Alegre et al., 2018; Varriale  
63 et al., 2021). In addition, PnTx-G, as well as PnTx-E and PnTx-F, have been detected in Japan,  
64 Australia, New Zealand and Canada (reviewed in (Molgó et al., 2014)), and in commercial mussels  
65 from Chile (Otero et al., 2020). The cosmopolitan dinoflagellate *Vulcanodinium rugosum* is the  
66 producer of PnTx-G (Nézan and Chomérat, 2011; Rhodes et al., 2011), and its growth rate and PnTx-  
67 G production are reported to be highest at temperatures ranging between 25 and 30°C (Abadie et al.,  
68 2016). PnTx-G levels in contaminated mussels at the Mediterranean Ingril lagoon (France) ranged  
69 between 261 and 1,244 µg/kg (Anses, 2019; Hess et al., 2013), values that are much higher than those  
70 reported in contaminated shellfish from other locations: 115 µg/kg in Norway (Rundberget et al.,  
71 2011), 59 µg/kg in Spain (Garcia-Altare et al., 2014) and 83 µg/kg in Canada (McCarron et al.,  
72 2012). In 2019, the French Agency for Food, Environmental and Occupational Health and Safety  
73 (Anses, 2019; Arnich et al., 2020), established a detailed view regarding the occurrence of PnTxs in  
74 shellfish, establishing a contamination value of 23 µg PnTx-G/kg of total meat not to be exceeded.

75 Consequently, a risk for human consumers may exist (Arnich et al., 2020; Delcourt et al., 2019) when  
76 PnTx shellfish accumulation reaches higher levels.

77 PnTx were identified as potent antagonists of embryonic and mature muscle ( $\alpha_1\beta\gamma\delta$  and  $\alpha_1\beta\epsilon\delta$ ),  
78 and neuronal ( $\alpha_7$ ,  $\alpha_3\beta_2$ ,  $\alpha_4\beta_2$ ) nicotinic acetylcholine receptor (nAChR) subtypes (Aráoz et al., 2011;  
79 Benoit et al., 2019; Hellyer et al., 2015). At the structural level, their seven-membered CI ring fits  
80 perfectly with the receptor subunit interface of nAChRs, while the unique substructures of the  
81 macrocyclic framework of the PnTx extend radially from the binding pocket to engage in distinctive  
82 interactions with the complementary and variable face F of the nAChRs (Bourne et al., 2015). The  
83 interaction of PnTx with nAChRs was shown in mice to induce exceptionally rapid lethality associated  
84 with central neurological symptoms and flaccid paralysis (Munday et al., 2012; Selwood et al., 2010).  
85 In addition, due to their high chemical stability (Jackson et al., 2012; Selwood et al., 2014), PnTx  
86 exhibit high oral acute toxicity in mice (Arnich et al., 2020; Delcourt et al., 2019; Munday et al., 2012;  
87 Sosa et al., 2020), suggesting that they cross the intestinal barrier.

88 Interestingly, a recent publication reported that a *V. rugosum* bloom in Cuba induced acute skin  
89 irritation in swimmers. The bloom contained CI toxins such as pinnatoxins and portimine, which could  
90 have caused dermatitis (Moreira-González et al., 2021). Even though no information has been reported  
91 yet linking CI toxins to neurotoxic events in humans, the potent interaction of PnTx with central and  
92 peripheral nAChRs raises concerns about the harmful downstream effects of PnTx exposure.

93 In the present study, we chose to study the ability of PnTx-G to cross physiological barriers,  
94 because: (i) it is a chemically stable molecule and is considered as the precursor of other pinnatoxins  
95 (A-C) in mollusks, (ii) it is a common shellfish contaminant distributed worldwide, and (iii) it can be  
96 chemically synthesized and radiolabeled with tritium allowing its detection using high-resolution  
97 digital autoradiographic analyses. As demonstrated herein, the toxicokinetic profile of [ $^3\text{H}$ ]-PnTx-G in  
98 rats reveals its rapid blood clearance, large tissue distribution, and its ability to cross the intestinal,  
99 blood-brain and placental barriers. Finally, using a human perfused placenta cotyledon model and  
100 mass spectrometry analysis, the ability of PnTx-G to pass through the human placental barrier is  
101 confirmed.

102

## 103 **2. Materials and Methods**

### 104 *2.1. Synthesis and spectral analysis of radiolabeled [<sup>3</sup>H]-PnTx-G*

105 Among the PnTx family, PnTx-G is the only one that has proven amenable to tritium labeling so far  
106 by partial hydrogenation reaction (Fig. S1, Supplementary materials). Based on a previously reported  
107 total synthesis (Aráoz et al., 2011), the protocol afforded [<sup>3</sup>H]-PnTx-G with a radiochemical purity  
108 greater than 97%, and a specific activity of 40 Ci/mmol.

109

### 110 *2.2. Characterization and validation of [<sup>3</sup>H]-PnTx-G*

111 The  $\alpha 7/5HT_3$  chimera was transiently transfected into tsA-201 cells using 15  $\mu$ g cDNA for ca.  $10^7$   
112 cells per Petri dish. All binding experiments were performed in 96-well GF/C plates pre-soaked in  
113 0.5% polyethylenimine, and using PBS buffer complemented with 0.1% BSA. The reaction was  
114 stopped by simultaneous filtration of the 96 wells with a FilterMate harvester (PerkinElmer, France).  
115 The filters were washed twice with ice-cold buffer and dried, 25  $\mu$ l of MicroScint was added, and the  
116 bound radioactivity was counted by scintillation spectrometry on a TopCount beta counter  
117 (PerkinElmer, France).

118 Equilibrium competition with the  $\alpha 7/5HT_3$  chimera used a cell density adjusted to bind specifically  
119 no more than 10% of [<sup>3</sup>H]-PnTx-G (1 nM). IC<sub>50</sub> values were determined by fitting the competition  
120 data with a Hill equation and conversion to K<sub>i</sub> constants using the equation:  $K_i = IC_{50}/(1+L^*/K_d)$ .  
121 Association kinetic experiments were performed with 3 different concentrations of [<sup>3</sup>H]-PnTx-G (1,  
122 1.9 and 3.6 nM) and the apparent association constants obtained from these experiments were plotted  
123 against the tracer concentrations. The slope of this line corresponds to the association kinetic constant  
124 K<sub>on</sub>. Dissociation experiments were performed at 1.9 nM of [<sup>3</sup>H]-PnTx-G and the dissociation was  
125 measured for 3 h after the addition of 1  $\mu$ M of PnTx-G. Each experiment was performed two times in  
126 duplicate.

127

### 128 *2.3. In vivo experiments*

129           2.3.1 Ethical compliance

130           Animal care and surgical procedures were performed according to the Directive 2010/63/EU of the  
131 European Parliament, which had been approved by the Ministry of Agriculture, France. The project  
132 was submitted to the French Ethics Committee CEEA (Comité d’Ethique en Expérimentation  
133 Animale) and obtained the authorization APAFIS#4111-2016021613253432 v5. All experiments were  
134 performed in accordance with relevant named guidelines and regulations. Experiments were conducted  
135 on adult Wistar rats from Janvier Labs (Le Genest St Isle, France).

136

137           2.3.2 Toxicokinetics, organ distribution, and elimination

138           Wistar male rats were anesthetized with 2% isoflurane (Iso-vet®, Piramal, UK). A 22G Surflo i.v.  
139 catheter (Terumo, Japan) was inserted into the tail vein for intravenous (i.v.) injection of [<sup>3</sup>H]-PnTx-G  
140 at 3 µg/kg. This dose was selected because was not lethal to rats, as determined by a 24 h period of  
141 observation. Blood samples were collected between 2 and 15 min after i.v. injection. Animals were  
142 euthanized 15 min after [<sup>3</sup>H]-PnTx-G administration, and perfused with 0.9% NaCl (30 mL/rat, 300  
143 mL/h) for collection of major organs. Urine and feces were collected 4 h after administration.  
144 Quantification of β-radioactivity contained in blood and organs was performed using a Liquid  
145 Scintillation Analyzer (Tri-Carb 2100TR, Packard BioScience Company, USA). Toxicokinetic  
146 parameters were calculated with the non-compartmental analysis Linear up/Log down method, using  
147 the PKSolver add-in program (Excel).

148           2.3.3 Investigation of biological barriers crossing

149           Fasted rats were exposed by oral gavage to 100 µg/kg of [<sup>3</sup>H]-PnTx-G in PBS. The dose was  
150 chosen on the basis of our own preliminary studies, given that it is not lethal to rats, and because it  
151 corresponds to about 4 times the proposed maximum tolerable concentration of PnTX-G  
152 contaminating shellfish (23 µg PnTX G/kg of total meat) (see Introduction). We used oral gavage,  
153 instead of intraperitoneal administration, since the oral route of administration is the one that may be  
154 related to consumption of contaminated shellfish in humans, and because it assures less variability,  
155 pain, and discomfort for the animals. The administration by gavage followed the OECD

156 recommendations for the testing of chemicals. Rats were euthanized 6 h after exposure to the  
157 radiolabeled toxin.

158 Tissue sections were prepared on a microtome and analyzed by digital autoradiography.

159 Pregnant female rats were i.v. injected 19 days after fertilization with 10 µg/kg of [<sup>3</sup>H]-PnTx-G to  
160 explore the ability of PnTx-G to cross the placental barrier. The dose was chosen for the reason that is  
161 not lethal to pregnant rats, according to our preliminary studies. Rats were euthanized 30 min later and  
162 perfused with 0.9% NaCl. Embryos and placentas were rapidly collected and snap-frozen to prepare  
163 whole-body sagittal tissue sections, for digital autoradiographic analyses and to measure radioactivity  
164 content.

165

#### 166 *2.4. Digital autoradiography*

167 The analyzed organs were maintained in mounting medium, and tissue sections (20 µm thick) were  
168 cut at -20 °C with a slicing microtome (Leica Microsystems, France). The microtome was carefully  
169 cleaned with water-ethanol solution between treatment of each sample to remove any trace of  
170 radioactivity and avoid contamination between different organs. The absence of radioactive  
171 contaminants in the rinsing solution was checked by radioimaging of drop samples. The tissue sections  
172 were placed on glass slides and were either stored in a freezer for microscopy analyses or kept at room  
173 temperature for 1 day in the presence of silica gel to ensure complete drying for proper radioimaging.  
174 A high-performance autoradiography imager (β-imager 2000, Biospace Lab, Paris, France) allowing  
175 real-time radioactive imaging through direct β-particle counting and absolute radioactivity  
176 quantification (detection threshold of 0.001 cpm/mm<sup>2</sup> for <sup>3</sup>H) was used to measure radioactivity in  
177 dried tissue sections. These sections were analyzed by radioimaging at high resolution and sensitivity  
178 (125 µm resolution and 0.5 pCi detection threshold) using digital autoradiography, to determine the  
179 count/per tissue section volume, which was converted to total radioactivity per organ on the basis of  
180 the total volume of each organ (determined by organ weighing)

181 The time of acquisition on the β-Imager was 1 h for peripheral organ preparations and 24 h for  
182 brain sections.

183 *2.5. HPLC analysis of organ extracts*

184 Soluble extracts of spleen, liver and whole brain from rats sacrificed 30 min post-injection were  
185 prepared as follows: organs were resuspended in 3 mL TFA (trifluoroacetic acid) and homogenized  
186 using an Ultra Turrax® system (IKA-Werke, Gmbh & Co Kg, Staufen, Germany). The homogenate  
187 was rinsed with 15 mL of MilliQ H<sub>2</sub>O and was kept on ice for 5 min before being centrifuged (8,000  
188 rpm, 15 min at 4°C). Supernatants were collected, analyzed with radio-HPLC (Kromasyl HPLC C18  
189 column 4.6 x 150 mm) and compared to control analysis of [<sup>3</sup>H]-PnTx-G.

190

191 *2.6. Reconstruction 3D images*

192 Six embryos from 2 pregnant rats were recovered for histological and autoradiographic analyses.  
193 After euthanasia, embryos were immediately frozen at -40 °C in isopentane. They were cut into 20- $\mu$ m  
194 thick sections (sagittal embryo sections, coronal brain sections) with a CM3050S cryostat (Leica,  
195 Rueil-Malmaison, France). Every fourth section was mounted on Superfrost glass slides, and rapidly  
196 dried in a desiccator. After autoradiography imaging, the same sections were processed for Nissl  
197 staining, to obtain additional information about brain anatomy. Images from the embryo surface,  
198 corresponding to sections subsequently processed, were recorded before sectioning using a digital  
199 camera (Oscar F-810C, 8 megapixels, Allied Vision Technologies, Stadroda, Germany) with an in-  
200 plane resolution of 27  $\times$  27  $\mu$ m<sup>2</sup>. Block-face photographs were stacked and tissue was automatically  
201 segmented using a histogram analysis method. As these images were taken prior to sectioning at the  
202 exact same position section after section when the imaged tissue was still attached to the block, so the  
203 resulting stack of photographs was therefore intrinsically 3D spatially coherent.

204 To further refine the toxin biodistribution in the rat embryo, we applied a protocol previously  
205 developed to obtain 3D imaging of adult animal brain from tissue sections (Dauguet et al., 2007). As  
206 shown in supplementary material (Fig. S3, Supplementary information) 66 sagittal embryo section  
207 images were produced consecutively by photography, histological coloration (cresyl violet) and digital  
208 autoradiography, allowing 3D reconstruction of the volumes by co-alignment of the block face,  
209 histological and autoradiographic slices. The digitalization of the autoradiography and histological

210 sections and the 3D reconstruction of the images are described in detail in the supplementary  
211 materials.

212

## 213 *2.7. Passage through the human placental barrier*

### 214 2.7.1 Mass calibration and analysis

215 The mass spectrometry analysis of PnTx-G was described in supplementary materials section.

216

### 217 2.7.2 Human placental perfused cotyledon model

218 Term placentas (37-40 weeks of gestation) from uneventful pregnancies were immediately  
219 collected after vaginal delivery or cesarean section from the Port-Royal Hospital Obstetrics  
220 Department (Paris, France). The enrolled patients did not receive any medication, except for epidural  
221 analgesia or oxytocin during labor, and did not present any vascular disease such as diabetes mellitus,  
222 preeclampsia, or intra-uterine growth restriction. These placentas were obtained following informed  
223 patient written consent and approval from our local ethics committee (CPP Paris Ile-de-France 3, N-  
224 18-05, Paris, France). Collected placentas (n = 3) were perfused in a double circuit according to a  
225 validated method as previously described (Ceccaldi et al., 2009). Maternal and fetal solutions were  
226 prepared with Earle's medium containing 25 g/L bovine serum albumin (Euromedex,  
227 Souffelweyersheim, France). The pH of the maternal and fetal compartments was adjusted to  $7.4 \pm 0.1$   
228 and  $7.2 \pm 0.1$ , respectively.

229 Perfusion experiments were started within 20 min after delivery. After a visual examination to  
230 confirm the vascular integrity of both maternal and fetal sides, a distal branch of a fetal artery and its  
231 associated vein that were supplying a selected cotyledon were cannulated. The fetal circulation was  
232 established at a flow rate of  $6 \text{ mL}\cdot\text{min}^{-1}$  (to ensure a balance between arterial and fetal venous flow).  
233 Placentas with evidence of vascular leakage were discarded. On the maternal side, the perfused area  
234 progressively whitened, which allowed visualization of the chosen cotyledon. After a wash step of 10  
235 min, the maternal perfusion was initiated by inserting two catheters into the intervillous space on the  
236 maternal side. The maternal circulation was established at a flow rate of  $12 \text{ mL}\cdot\text{min}^{-1}$ . Parameters such  
237 as perfusion pressure in the fetal vasculature and potential fluid leakage from the fetal circulation were

238 monitored to check the technical process. After an additional wash step of 5 min, a freely diffusing  
239 marker, antipyrine, was added to the maternal medium (final concentration 20 mg/L) and PnTx-G (100  
240 nM) was subsequently added to the same compartment. The perfusion duration was 90 min with  
241 maternal recirculation. Samples from the maternal circulation and venous fetal circulation were  
242 collected at 0, 15, 30, 45, 60, 75 and 90 min, in order to monitor toxin concentrations. At the end of  
243 perfusion, all the samples were stored at -80°C until toxin analysis. Standard parameters were  
244 calculated according to Challier's formulas (Challier, 1985). Placental transfer was estimated from two  
245 transport parameters: the fetal transfer rate (FTR) and the clearance index. Calculating the clearance  
246 index allowed comparison of results during different experiments and evaluation of the index of trans-  
247 placental transfer from one placental unit to another.

248 The two transport parameters were calculated as follows:

- 249 • The Fetal Transfer Rate (FTR) =  $(C_f / C_m) \times 100$ , where  $C_f$  is the venous fetal concentration  
250 of a molecule and  $C_m$  is the maternal concentration of the same molecule. The result is given  
251 as a percentage. FTR for antipyrine had to be over 20% as described by Challier (Challier,  
252 1985).
- 253 • The Clearance Index =  $FTR(\text{pinnatoxin}) / FTR(\text{antiypirine})$ .

254

## 255 2.8. *In vitro* competition experiments

256 Adult rats or rat embryos were euthanized and the whole embryos ( $n = 3$ ) or adult brains ( $n = 3$ )  
257 were immediately frozen at -80°C. In turn, 16 and 42 coronal sections (20  $\mu\text{m}$ ) of embryo and brain  
258 from each rat were cut and mounted onto chrome/gelatin coated slides and allowed to air dry overnight  
259 before being frozen again. All sections were preincubated in KRH buffer (20 mM HEPES, 118 mM  
260 NaCl, 4.8 mM KCl, 2.5 mM  $\text{CaCl}_2$  and 1.2 mM  $\text{MgSO}_4$ ) at 4°C for 30 min. Then, all sections were  
261 incubated in cold KRH buffer for 90 min with 0.3 nM [ $^3\text{H}$ ]-PnTx-G with or without the different  
262 competitors (cold 0.3  $\mu\text{M}$  PnTx-G, 0.25  $\mu\text{M}$   $\alpha\text{C}$ -conotoxin PrXA, 100 nM MLA for embryo slices and  
263 0.3  $\mu\text{M}$  PnTx-G, 100 nM MLA (methyllycaconitine) or 5 nM A-85380 for brain preparations). The  
264 sections were rinsed twice in cold KRH buffer then twice in cold 20 mM HEPES buffer, and finally

265 rinsed in distilled water. As previously described, these sections were analyzed by digital  
266 autoradiography for 24 h (embryo sections) or 64 h (brain sections).

267

### 268 2.9. Statistics and Reproducibility

269 All quantitative measures were performed as independent replicates. All attempts to replicate the data  
270 were consistent. Statistical analyses and sample sizes are described in the pertinent figure legends.  
271 Statistical analysis was performed using Prism 7 software (GraphPad Software, San Diego, CA, USA).  
272 Differences between values were tested using the unpaired Student's *t*-test, and samples were  
273 considered statistically significant when  $P < 0.05$ .

274

## 275 3. Results

276

### 277 3.1. Binding properties of [<sup>3</sup>H]-PnTx-G

278 *In vitro* and *in vivo* properties of PnTx-G were evaluated using its tritium labeled form ([<sup>3</sup>H]-PnTx-  
279 G), a labeling that fully preserves toxin bioactivity. The pharmacological properties of [<sup>3</sup>H]-PnTx-G  
280 were evaluated by kinetic binding experiments on the  $\alpha 7$  receptor subtype ( $\alpha 7$ -5HT<sub>3</sub> chimeric  
281 receptor) in order to measure its association and dissociation rate constants. These values, were  
282 respectively  $7.7 \cdot 10^5 \text{ M}^{-1} \cdot \text{sec}^{-1}$  and  $1.7 \cdot 10^{-4} \text{ sec}^{-1}$  (Fig. S2a, S2b, Supplementary information), leading to  
283 a dissociation constant ( $K_d = 0.22 \text{ nM}$ ), similar in value to that determined by competition binding  
284 experiments, or in previously described results using [<sup>125</sup>I]- $\alpha$ -bungarotoxin ([<sup>125</sup>I]- $\alpha$ -BgTx) as  
285 radiotracer (Fig. S2c, Table S1, Supplementary information).

286

### 287 3.2. *In vivo* toxicokinetics, organ distribution and elimination of [<sup>3</sup>H]-PnTx-G

288 Maximum blood  $\beta$ -radioactivity concentration of [<sup>3</sup>H]-PnTx-G was observed 2 min after i.v.  
289 administration ( $T_{\max}$ ), representing  $16.3 \pm 3.2\%$  of the administered dose (Fig. 1). The blood  $\beta$ -  
290 radioactivity concentration of [<sup>3</sup>H]-PnTx-G decreased after  $T_{\max}$ , showing a value of  $6.9 \pm 0.4\%$  of the  
291 administered dose 15 min after administration (Fig. 1a). The kinetic profile of [<sup>3</sup>H]-PnTx-G presented

292 an exponential decrease in radioactivity with a final elimination characterized by a terminal half-life,  
293  $T_{1/2}$ , equal to  $13 \pm 4$  min and an elimination constant equal to  $0.06 \pm 0.02 \text{ min}^{-1}$ . The estimated  $AUC_{0-t}$   
294 was  $5.24 \pm 0.76 \text{ min} \cdot \mu\text{Ci/mL}$ . The distribution volume was  $117.4 \pm 34.4 \text{ mL}$  and the clearance  $6.3 \pm$   
295  $0.1 \text{ mL/min}$  (Table 1). Maximum organ radioactivity concentrations of [ $^3\text{H}$ ]-PnTx-G were observed in  
296 the liver and small intestine 15 min after administration, with  $14.6 \pm 4.4\%$  and  $17.5 \pm 6.0\%$  of the  
297 administered dose, respectively (Fig. 1b). Urine elimination represented  $3.6 \pm 0.6\%$  of the  
298 administered dose, 15 min after injection, whereas feces elimination after 4 h accounted for only  $0.04$   
299  $\pm 0.02\%$  of the administered dose (Fig. 1b).

300

### 301 3.3 *PnTx-G crosses the intestinal and blood-brain barriers of adult rats*

302 Previous studies have reported acute toxicity of PnTx-G in mice through respiratory failure after  
303 oral administration (Arnich et al., 2020; Munday et al., 2012; Sosa et al., 2020), suggesting the  
304 capacity of this toxin to cross the intestinal barrier.

305 As shown in Fig. 2a, when [ $^3\text{H}$ ]-PnTx-G was administered orally to rats the radioactivity was  
306 observed in peripheral organs such as liver, kidney and spleen, but also in various brain regions such  
307 as the hippocampus and superficial gray layer of the superior colliculus (SuG). Interestingly, the  
308 biodistribution of the toxin 15 min after i.v. injection in the brain was similar to that observed after  
309 oral gavage. Further systematic tissue analyses revealed the presence of radioactivity in skeletal  
310 muscles such as the *extensor digitorum longus* (EDL) and gastrocnemius muscles (Fig. 2b). In these  
311 muscles, the particular labeling of the radioactivity signal followed the distribution of the  $\alpha 1_2\beta 1\epsilon\delta$   
312 muscle-type nAChR at skeletal neuromuscular junctions, an observation in agreement with the  
313 interaction of PnTx-G with this receptor subtype. Similarly, the presence of radioactivity in the  
314 hippocampus, SuG and hypothalamus would correspond to the toxin binding to various neuronal  
315 nAChR subtypes known to be expressed in these brain structures.

316 Soluble extracts of spleen, whole brain and liver from rats euthanized 30 min post-injection were  
317 prepared and analyzed by HPLC coupled to a radioactivity detector. For the spleen, a single  
318 radioactivity peak eluting at the retention time of the native standard toxin was observed,

319 demonstrating the stability of pinnatoxin during its distribution phases in this organ (Fig. 3a). In brain  
320 extracts, in addition to the major native PnTx-G peak, minor metabolite peaks were also detected,  
321 corresponding to 20% of the injected toxin. This percentage rose to 45% in liver extracts (Fig. 3a),  
322 indicating a possible role of this organ in PnTx-G metabolization.

323

#### 324 3.4 *PnTx-G crosses the placental barrier*

325 The ability of PnTx-G to cross the placental barrier was evaluated by injecting i.v. [<sup>3</sup>H]-PnTx-G to  
326 pregnant rats (19 days after fertilization) and determining the presence of the radiolabeled toxin in the  
327 placenta and in embryos. Thirty minutes after the [<sup>3</sup>H]-PnTx-G injection, the mean radioactivity  
328 present in placentas corresponded to 0.73 % of the injected dose, while in embryos represented 0.45  
329 %.

330 As shown by the whole-body sagittal autoradiographic sections in Fig. 4a, different rat embryo  
331 tissues were labeled by the [<sup>3</sup>H]-PnTx-G, including the tongue, limb skeletal muscles, the spinal cord  
332 and various brain regions, demonstrating the toxin's ability to cross the placental barrier and to  
333 distribute into various tissues/organs. This labeling was highly reproducible in different sections from  
334 three different embryos (Fig. 4a), and the radioactivity was associated with the intact toxin, as revealed  
335 by radio-HPLC analyses of whole embryo extracts (Fig. 3b) (Dauguet et al., 2007). To further refine  
336 the [<sup>3</sup>H]-PnTx-G toxin biodistribution in the rat embryo, 3D reconstruction imaging was performed.  
337 As can be seen in Fig. 4b, the toxin labels several peripheral organs such as the tongue, the liver, the  
338 hindlimb and forelimb skeletal muscles, and several areas of the central nervous system, such as the  
339 medulla oblongata, the pons, the trigeminal nerve and the cervical and thoracic ganglia.

340 The capacity of PnTx-G to cross the placental barrier was studied further, using a human perfused  
341 *ex vivo* cotyledon model, and quantitative detection of the intact toxin in maternal and fetal  
342 compartments (Fig. 5d) by mass spectrometry. For this, a mass spectrometry analysis of the PnTx-G  
343 standard was first performed (Fig. 5a), followed by the construction of a calibration curve for PnTx-G  
344 spiked at different known concentrations in naïve human placental perfusates (Fig. 5b) and submitted  
345 to an extraction protocol before analysis by MALDI-TOF spectrometry. The PnTx-G concentrations

346 detected in the maternal and fetal compartments, as a function of time (0-90 min), are shown in Fig.  
347 5c, and the results of three placentas that were successfully perfused and validated using the mean fetal  
348 transfer rate (FTR) of antipyrine are shown in Fig. 5e. These studies showed that a maximum value of  
349 FTR was reached after 15 min of perfusion, and decreased slightly due to maternal recirculation (Fig.  
350 5d-e). In comparison, the kinetics of the PnTx-G clearance index during the perfusion procedure  
351 increased up to 30 min, and then reached a plateau up to 90 min (Fig. 5e). The mean clearance index  
352 of PnTx-G was  $1.65 \pm 0.17$ , indicating that the FTR of the toxin is higher than the FTR of antipyrine,  
353 which undergoes only passive diffusion and is used to measure tissue integrity/membrane  
354 permeability.

355 Since antipyrine neither binds to proteins nor accumulates in placental tissue, its transfer rate  
356 depends only on fetal and maternal flows, which should be constant during the perfusion. Hence, a  
357 clearance index value close to 1 demonstrates a mechanism of placental transfer by passive diffusion  
358 without placental accumulation (Challier, 1985). In this case, PnTx-G transfer was significantly higher  
359 than the transfer rate of antipyrine, reaching a value close to 2, indicating a rapid significant facilitated  
360 transfer of the toxin ( $p = 0.032$ ).

361

### 362 3.5 *The nAChR receptor subtypes targeted by PnTx-G in rat embryos and adult rats*

363 In an attempt to identify the main nAChR subtypes targeted by PnTx-G in rat embryos, whole-body  
364 sagittal sections of rat embryos were prepared and incubated with [<sup>3</sup>H]-PnTx-G (0.3 nM) alone, or in  
365 the presence of three competitive ligands known to interact with different nAChR subtypes with  
366 various selectivity profiles (Table S2, Supplementary information). Digital autoradiographic analyses  
367 of these embryo sections (Fig. 6) highlighted first that the [<sup>3</sup>H]-PnTx-G labeling obtained in this *in*  
368 *vitro* experiment is impressively similar to that previously observed after *in vivo* toxin exposure (see  
369 Fig. 4). This observation validates the use of *in vitro* labeling to study the binding specificity of the  
370 toxin *in vivo*. Secondly, unlabeled PnTx-G (0.3 μM) largely displaced (up to 90%) [<sup>3</sup>H]-PnTx-G  
371 binding, emphasizing the specificity of the toxin labeling in these tissues (Fig. 6a). Interestingly, αC-  
372 conotoxin PrXA (0.25 μM), a ligand known to selectively interact with muscle-type nAChR (El-Aziz  
373 et al., 2017; Jimenez et al., 2007), induced a large decrease of the labeling, highlighting the crucial role

374 of this receptor subtype in the toxin interaction. This was particularly obvious in peripheral organs  
375 such as the tongue or forelimb skeletal muscles, while the spinal cord and brain regions remained  
376 largely labeled. In contrast, MLA (100 nM), a specific  $\alpha 7$  antagonist (Ward et al., 1990), displaced  
377 [ $^3\text{H}$ ]-PnTx-G labeling only weakly, but more efficiently in the CNS, such as the pineal gland and  
378 spinal cord regions (Fig. 6a). These observations suggest that  $\alpha 1_2\beta 1\gamma\delta$  and  $\alpha 7$  nAChRs are important  
379 targets for the toxin, in good agreement with the high affinity of PnTx for these two receptor subtypes  
380 (Table S2, Supplementary information), and with previous embryo labeling.

381 Furthermore, to assess the specificity of [ $^3\text{H}$ ]-PnTx-G binding to nAChR subtypes in various adult  
382 rat brain regions, *in vitro* competition experiments were performed on brain tissue sections in the  
383 absence or presence of various specific antagonists of nAChR subtypes: unlabeled PnTx-G, the  
384 selective  $\alpha 7$  nAChR antagonist MLA, and A-85380, a selective binder of the  $\alpha 4\beta 2$  receptor subtype  
385 (Sullivan et al., 1996) (Table S2, Supplementary information). Here again, it is worth mentioning that  
386 *in vitro* brain imaging reproduced what was observed after *in vivo* exposure, suggesting, along with  
387 competition experiments (see below), that the labeling is due to intact PnTx-G. Non-radioactive PnTx-  
388 G completely abrogates the labeling of the radioactive toxin, demonstrating the specificity of this  
389 interaction in the central nervous system (Fig. 6b). Using MLA, a large part of the [ $^3\text{H}$ ]-PnTx-G  
390 labeling was abolished, especially in hippocampus and hypothalamus regions, revealing a selective  
391 toxin- $\alpha 7$  receptor interaction in these regions, as well as the high content of this receptor subtype in  
392 these brain areas (Fig. 6b). In the SuG region, MLA was less efficient than A-85380 in decreasing the  
393 [ $^3\text{H}$ ]-PnTx-G-associated radioactivity, suggesting the prevalence of  $\beta 2$ -selective nAChRs in this area,  
394 and in particular the  $\alpha 4\beta 2$  subtype, as previously reported (Baddick and Marks, 2011; Marks et al.,  
395 2011). In contrast, A-85380 was inefficient in abolishing the labeling in hippocampus and  
396 hypothalamus regions (Fig. 6b). These results highlight the prevalence of [ $^3\text{H}$ ]-PnTx-G labeling for  $\alpha 7$   
397 nAChR in brain, even if other subtypes, such as the  $\alpha 4\beta 2$  subtype, also seem to be recognized, but to a  
398 lesser extent.

399

#### 400 **4. Discussion**

401 PnTx belong to the CI toxin family, an emerging class of more than 40 different marine toxins  
402 produced by dinoflagellates and distributed worldwide. Interestingly, among all the CI toxins, PnTx  
403 display severe toxic effects after oral administration to mice, associated with piloerection, prostration,  
404 decreased mobility (occasionally preceded by an initial phase of hyperactivity immediately following  
405 administration), hypothermia, hind leg paralysis, abdominal breathing and cyanosis, and finally death  
406 by respiratory arrest (Arnich et al., 2020; Delcourt et al., 2019; Munday et al., 2012; Sosa et al., 2020).  
407 These neurotoxic properties have been correlated with their high potency to block various central and  
408 peripheral nAChR subtypes (Aráoz et al., 2011; Delcourt et al., 2019; Molgó et al., 2017).  
409 Nevertheless, no human poisoning has yet been associated with their ingestion via contaminated  
410 shellfish. This may be because recent monitoring shows that reported PnTx-G levels are below the  
411 threshold value proposed by Anses (2019) of 23 µg/kg of total meat for contaminated shellfish in  
412 various European shellfish production areas (Kvrgić et al., 2021; Lamas et al., 2019; Moreiras et al.,  
413 2019; Otero et al., 2019; Otero et al., 2020; Rambla-Alegre et al., 2018; Varriale et al., 2021). On the  
414 other hand, in places where PnTx-G levels were high, as in the Mediterranean Ingril lagoon (Anses,  
415 2019; Aráoz et al., 2020; Hess et al., 2013), wild shellfish collection has been banned (since January 1,  
416 2020) by the health authorities because of the possible health risk to shellfish consumers. Interestingly,  
417 a recent publication suggests a possible link between PnTx and portimine release during a *V. rugosum*  
418 bloom and acute dermal irritation in sixty people (mostly children) who had prolonged contact with  
419 the dinoflagellate bloom in Cuba's Caribbean waters (Moreira-González et al., 2021).

420 In the present study, we addressed more precisely the toxicokinetic properties of PnTx-G, how  
421 PnTx interact *in vivo* with their central and peripheral receptor targets, and how easily they cross  
422 physiological barriers. First, the rapid blood clearance of [<sup>3</sup>H]-PnTx-G ( $t_{1/2} = 13$  min) was shown, as  
423 well as its distribution in several peripheral organs and in the brain. In addition, using digital  
424 autoradiographic approaches in the adult rat, we provide evidence that after oral gavage or i.v.  
425 injection, [<sup>3</sup>H]-PnTx-G crosses the intestinal and blood-brain barriers to target skeletal muscle and  
426 brain neuronal nAChR subtypes, respectively. Despite possible minor metabolization of PnTx-G in the  
427 brain, the labeling of the main brain structures by the intact toxin was supported by autoradiographic  
428 competition experiments performed on brain tissue sections. Indeed, to determine the subtypes of

429 neuronal nAChRs involved, coronal rat brain sections were incubated with [<sup>3</sup>H]-PnTx-G alone, or in  
430 the presence of selective nAChR ligands, highlighting the major target role of the  $\alpha 7$  nAChR subtype  
431 in the central nervous system. The ability of CI toxins to cross the blood-brain barrier was first  
432 suggested by transcriptional and immunohistochemical analysis showing that neuronal nAChRs were  
433 upregulated after intraperitoneal spirolide administration to rats (Gill et al., 2003). More recently, mass  
434 spectrometry analyses revealed the ability of 13-desmethyl spirolide C to cross the blood-brain barrier  
435 in a transgenic mouse model (Alonso et al., 2013), but without any precise information on the brain  
436 areas and nAChR subtypes recognized in the central nervous system. Interestingly, the role of  $\alpha 7$   
437 nAChR in receptor-mediated transcytosis across the blood-brain barrier was already suggested, and  
438 exploited for brain-targeted drug delivery (Huey et al., 2017; Wei et al., 2015). This process could  
439 explain the ability of PnTx-G to cross so efficiently the blood-brain barrier, and eventually the other  
440 physiological barriers.

441 Second, biodistribution analyses on full embryo sections after i.v. administration of [<sup>3</sup>H]-PnTx-G to  
442 pregnant rats, associated with 3D reconstruction imaging, revealed the ability of the toxin to cross the  
443 placental barrier and to distribute into various peripheral and central organs of the embryos.  
444 Intriguingly, the embryonic brain stem regions labeled by the toxin (spinal cord, medulla oblongata,  
445 pons) are not similar to those highlighted in the adult rat (hippocampus, SuG, hypothalamus). This  
446 observation seems to be in line with the timing of our observation (E19) and the timeline of brain  
447 regional neurogenesis in rat in which the spinal cord, medulla and pons mature early (from gestational  
448 days 11 to 16), while hippocampus neurogenesis starts later and continues until postnatal days 16-19  
449 (Rice and Barone, 2000). Furthermore, the embryo regions labeled by the toxin superimposed nicely  
450 with those described in the eMouse Atlas, which are known to over-express the  $\alpha 7$ - and  $\alpha 1$ -nAChRs  
451 genes in central and peripheral regions of the mouse embryo (Armit et al., 2017). In line with the  
452 ability of PnTx-G to cross the placental barrier in the rat, similar results were established using a  
453 human placental perfused cotyledon model. A facilitated transfer of the toxin was observed in this  
454 human placental model, suggesting a possible role of nAChRs in this process. This hypothesis seems  
455 to reinforce the view of the reported expression of various nAChR subtypes, including  $\alpha 7$ , in the  
456 placenta (Machaalani et al., 2018).

457 In conclusion, the collective results from this study show that when PnTx-G was ingested, the toxin  
458 crossed the intestinal and blood-brain barriers and reached specific brain regions. Our results also  
459 demonstrate the placental transport of PnTx-G, following single low-dose exposure of pregnant rats  
460 and in a human placental cotyledon model. The significance these outcomes may have for pregnant  
461 women exposed to PnTx-G by ingestion of contaminated shellfish is at present unknown. Likewise,  
462 the consequences of mammalian fetal exposure to PnTx-G remain to be determined. Nevertheless, a  
463 preliminary study tackling the potential developmental toxicity of PnTx in chick embryos has been  
464 reported (Couesnon et al., 2014). These results raise many additional questions on the mechanisms by  
465 which PnTx target the nervous and impact on the musculoskeletal systems, and on the potential risk  
466 associated with long-term consumption of contaminated shellfish on human health.

467

#### 468 **Figure Legends**

469 **Fig. 1.** Toxicokinetics, organ distribution and elimination of [<sup>3</sup>H]-PnTx-G in male rats. (a) Blood  
470 PnTx-G β-radioactivity, expressed as a % of the single i.v. dose (3 μg/kg) administered to rats (n=3),  
471 as a function of time. Each symbol in the curve represents the mean ± SEM. (b) PnTx-G β-  
472 radioactivity in organs, urine and feces, expressed as a % of the administered dose (i.v., 3 μg/kg). Data  
473 were determined 15 min (organs and urine) or 4 h (feces) after single i.v. administration to rats  
474 (n=3)(mean ± SEM).

475 **Fig. 2** Biodistribution of [<sup>3</sup>H]-PnTx-G either after oral gavage (100 μg/kg) (a) or i.v. injection (3  
476 μg/kg) (b) of the toxin to rats. Rats were euthanized 6 h (oral gavage, n = 3) or 15 min (iv, n = 3) after  
477 exposure to the radiolabeled toxin, and perfused with a solution of NaCl to remove the radioactivity  
478 from blood, preceding the radioactivity count of individual organ tissues. The images represent middle  
479 sections of peripheral organs and are located at Bregma -5.52 mm in the brain. The intensity scale  
480 (blue to red) is proportional to the increase in radioactivity levels (in counts/pixel). The time for image  
481 acquisition on the β-Imager was 1 h for peripheral organs and 24 h for the brain. Images are  
482 representative of at least 24 sections from 3 rats.

483

484 **Fig. 3** (a) Radio-HPLC analyses of [<sup>3</sup>H]-PnTx-G (15 nCi control) and soluble extracts from spleen,  
485 brain and liver of rats injected with the toxin. (b) Radio-HPLC analyses of [<sup>3</sup>H]-PnTx-G (control) and  
486 soluble extracts from rat embryo extracts. Soluble extracts of spleen, liver, brain and whole embryos  
487 were obtained from rats euthanized 30 min post-i.v. injection, and were prepared using a homogenizer  
488 and centrifugation. Supernatants were collected, analyzed with a radio-HPLC column (Kromasyl C18  
489 4.6 x 150 mm), and compared to control analysis of [<sup>3</sup>H]-PnTx-G.

490 **Fig. 4** Biodistribution of [<sup>3</sup>H]-PnTx-G in rat embryos, 30 min after i.v. injection of toxin in pregnant  
491 rats. (a) Optical (left) and autoradiographic images (right) of rat embryo sagittal sections. Two slices  
492 (spaced by 80 μm) representative of the 66 sections from each of the three different embryos were  
493 analyzed by digital autoradiography. The intensity scale (blue to red) is proportional to the  
494 radioactivity levels (in count/pixel). (b) 3D reconstruction images of [<sup>3</sup>H]-PnTx-G labeling from 66  
495 embryo sections (see protocols in Methods section) highlighting the regions with the higher or lower  
496 radioactivity levels detected using respectively high and low thresholds defined by experts  
497 (corresponding respectively to 89% and 83% of the maximal radioactivity value). In blue and green,  
498 respective [<sup>3</sup>H]-PnTx-G labeled regions with α7- and α1-nAChR enriched gene expression (Armit et  
499 al., 2017). Tongue in cyan expressed both receptor subtypes and liver and other brain areas are colored  
500 in gray.

501

502 **Fig. 5** Maternal-fetal transfer of PnTx-G in a human perfused *ex vivo* cotyledon model. (a) Mass  
503 spectrometry analysis of PnTx-G standard. (b) Calibration curve of PnTx-G that was spiked at  
504 different known concentrations in placental perfusates, and submitted to an extraction protocol before  
505 analysis by MALDI-TOF spectrometry (mean ± SD, n = 4-6). (c) Measurement in maternal (Perfusate  
506 Placenta Maternal (PPM) with maternal recirculation) and fetal (Perfusate Placenta Fetal (PPF)  
507 without fetal recirculation) compartments of PnTx-G concentration, from time 0 (T0) to time 90 (T90),  
508 in min (mean ± SD, n = 3). (d) Schematic view of the human perfused cotyledon model, and (e) fetal

509 transfer rate of antipyrine (ATP) and kinetics of clearance index (PnTx-G transfer/antipyrine transfer  
510 ratio) of PnTx-G (mean  $\pm$  SD, n = 3).

511

512 **Fig. 6** Digital autoradiography of sagittal sections of rat embryos (a) and adult rat coronal brain slices  
513 (b) incubated with [<sup>3</sup>H]-PnTx-G alone or in the presence of selective nAChR ligands. (a) Sagittal  
514 sections of rat embryos were prepared and incubated for 90 min with [<sup>3</sup>H]-PnTx-G (0.3 nM) with or  
515 without the different competitors (unlabeled 0.3  $\mu$ M PnTx-G, 0.25  $\mu$ M  $\alpha$ C-conotoxin PrXA, 100 nM  
516 MLA). Each image is representative of one of the 16 slices of each embryo, (n = 3). (b) *In vitro*  
517 competition experiments on adult rat coronal brain sections incubated for 90 min with [<sup>3</sup>H-PnTx-G]  
518 and various nAChR antagonist ligand competitors (cold 0.3  $\mu$ M PnTx-G, 100 nM MLA, 5 nM A-  
519 85380). Each image is representative of one of the 42 sections of each brain (bregma B:-6.12) (n = 3).  
520 All the sections were analyzed by digital autoradiography after 24 h (embryos sections) or 64 h (brain  
521 sections) of acquisition on the  $\beta$ -Imager. The intensity scale (blue to red) is proportional to the  
522 increase in radioactivity level (in counts/pixel).

523

#### 524 **CRedit authorship contribution statement**

525

526 **Denis Servent:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing -  
527 original draft. **Carole Malgorn:** Investigation. **Mylène Bernes:** Investigation. **Sophie Gil:**  
528 Investigation, Formal analysis, Writing – review & editing. **Christelle Simasotchi:** Investigation.  
529 **Anne-Sophie Hérard:** Investigation, Formal analysis, Writing – review & editing. **Thierry**  
530 **Delzescaux:** Investigation, Formal analysis, Writing – review & editing. **Robert Thai:** Investigation.  
531 **Peggy Barbe:** Investigation. **Mathilde Keck:** Investigation, Formal analysis. **Fabrice Beau:**  
532 Investigation. **Armen Zakarian:** Investigation, Funding acquisition, Writing – review & editing.  
533 **Vincent Dive:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing –  
534 review & editing. **Jordi Molgó:** Conceptualization, Investigation, Formal analysis, Supervision,  
535 Writing – review & editing.

536

537 **Declaration of competing interests**

538 The authors declare that they have no known competing financial interests or personal relationships  
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546

547 **Supplementary Material**

548 Additional supporting information to this article may be found online at:

549

550 **References**

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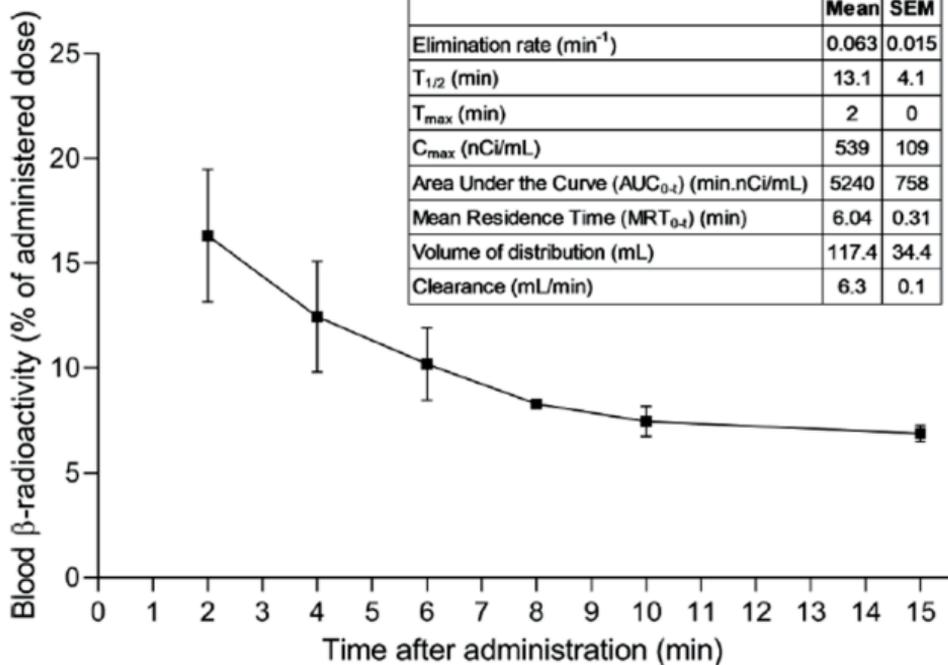
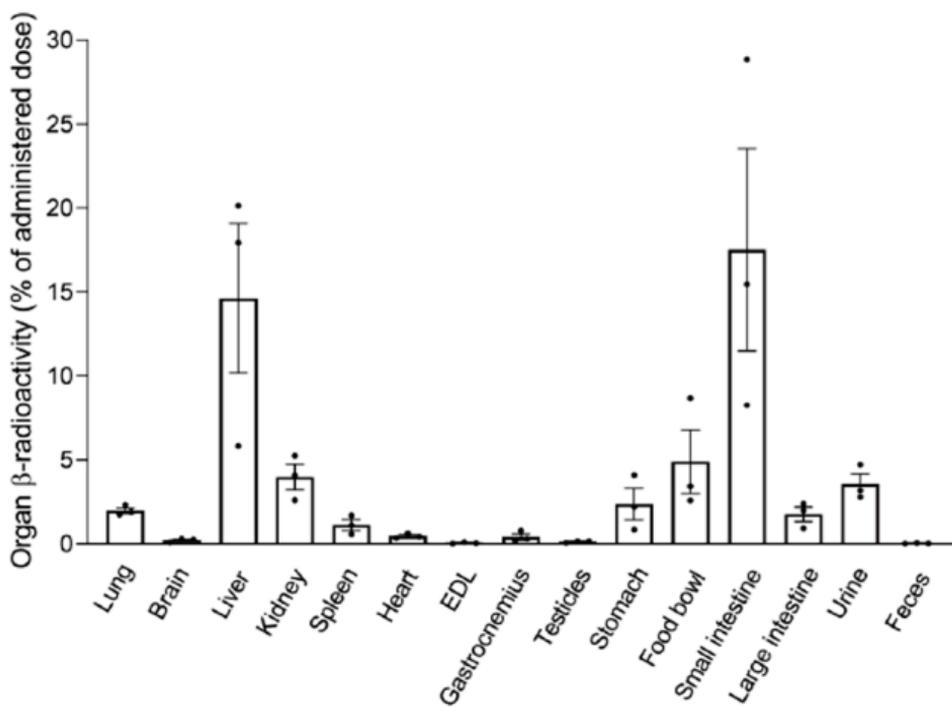
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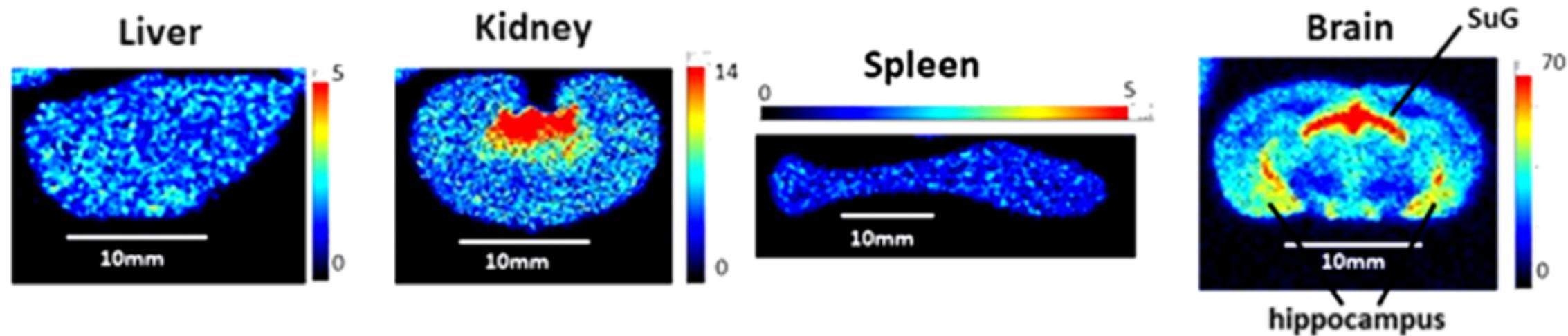
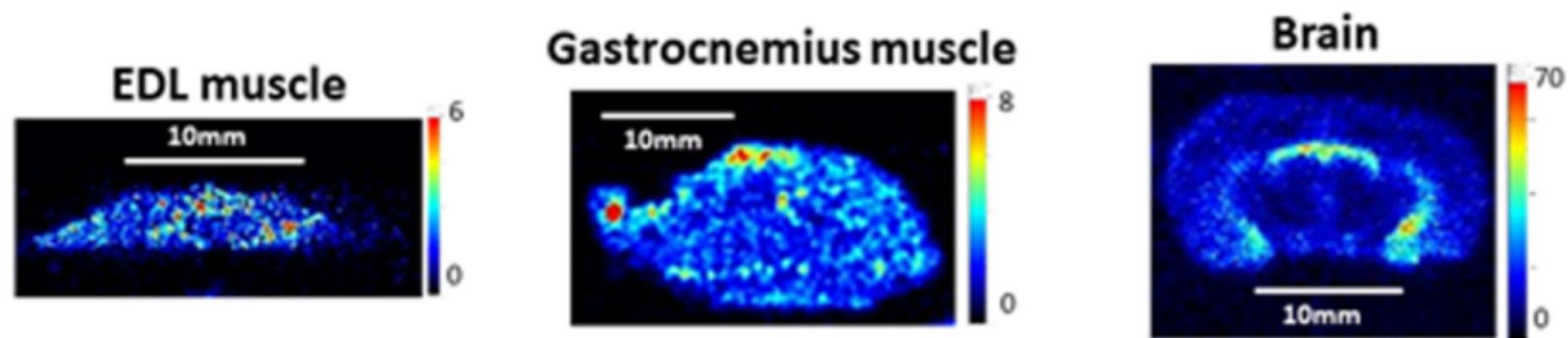
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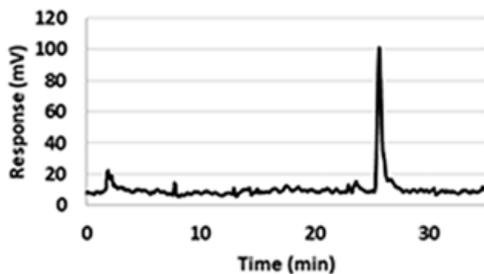
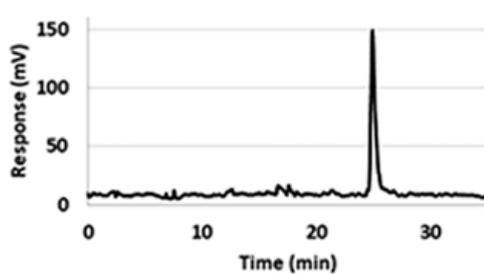
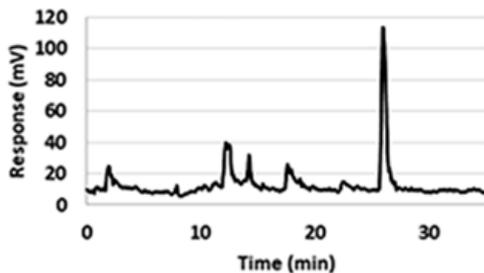
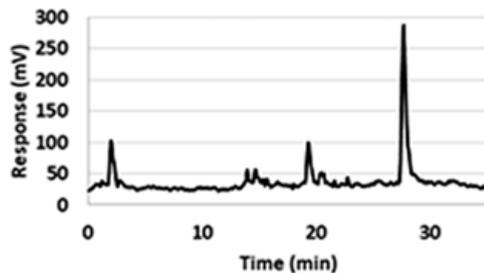
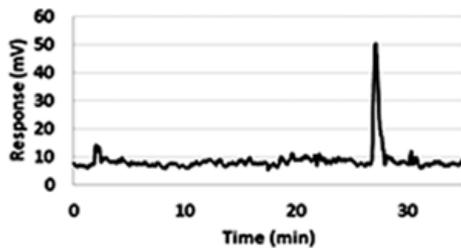
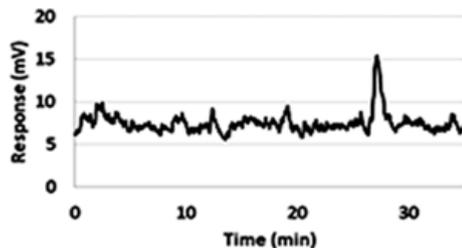
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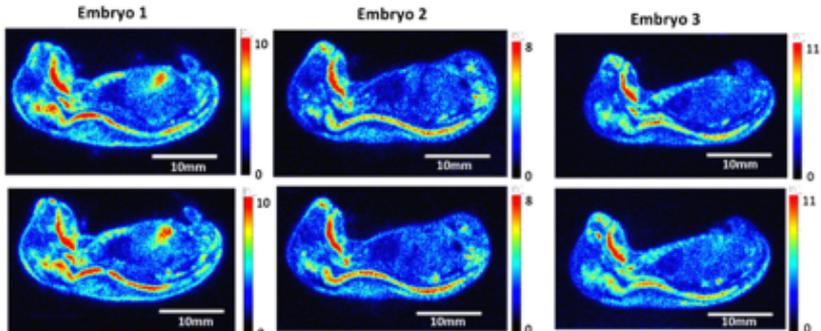
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727

**a****b**

**a****b**

**a****PnTx-G Ctrl****Spleen extract****Liver extract****Brain extract****b****PnTx-G Ctrl****Embryo extract**

**a**

Medulla oblongata

Trigeminal V nerve

Pons

**High Threshold**

Tongue

Liver

Cervical ganglion

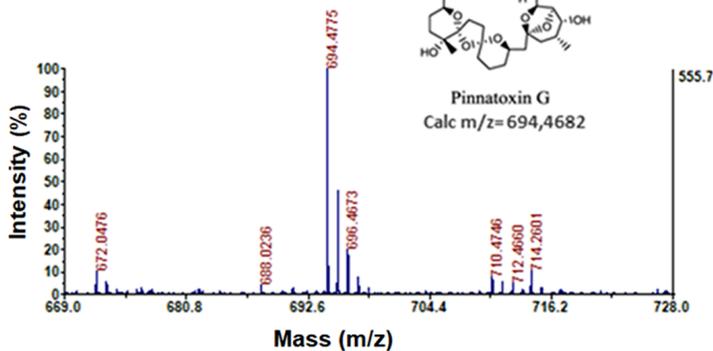
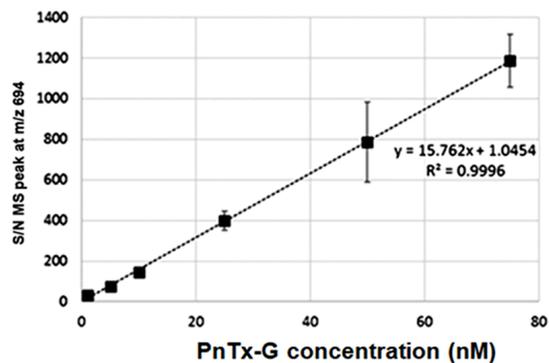
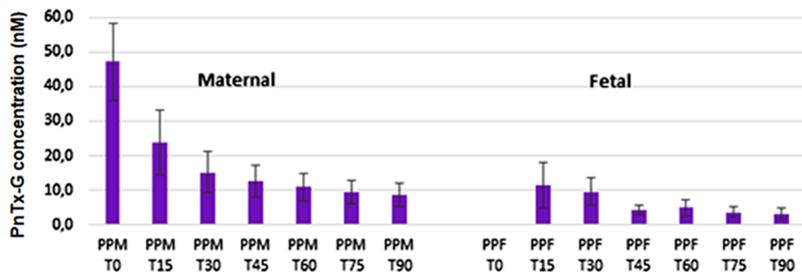
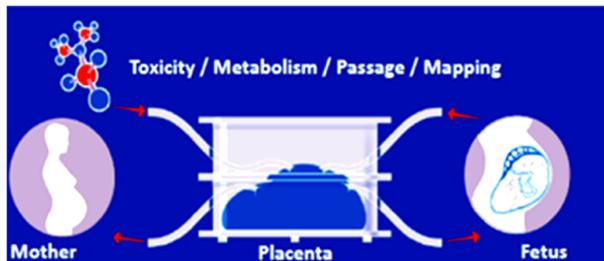
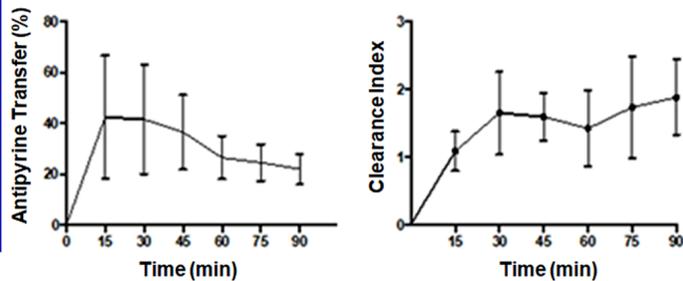
Thoracic ganglion

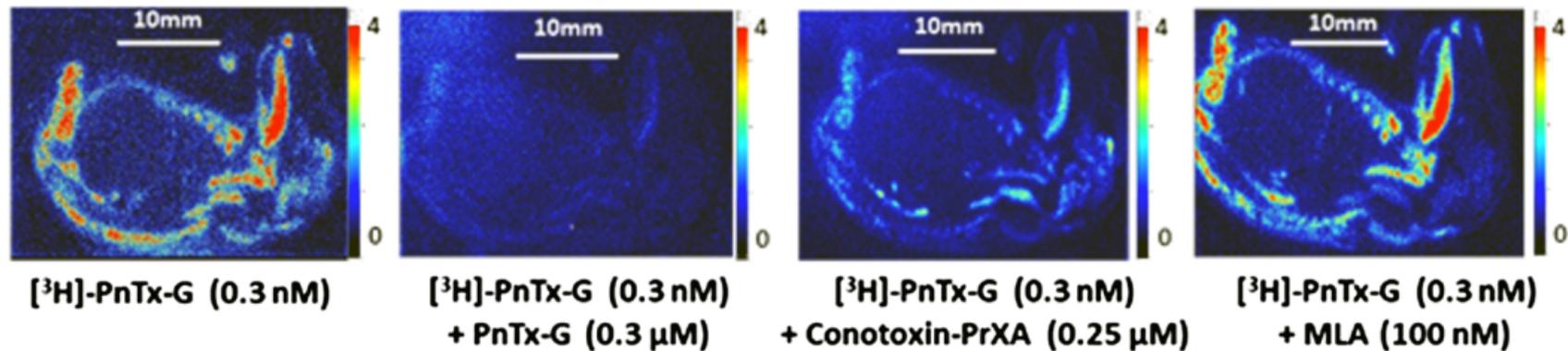
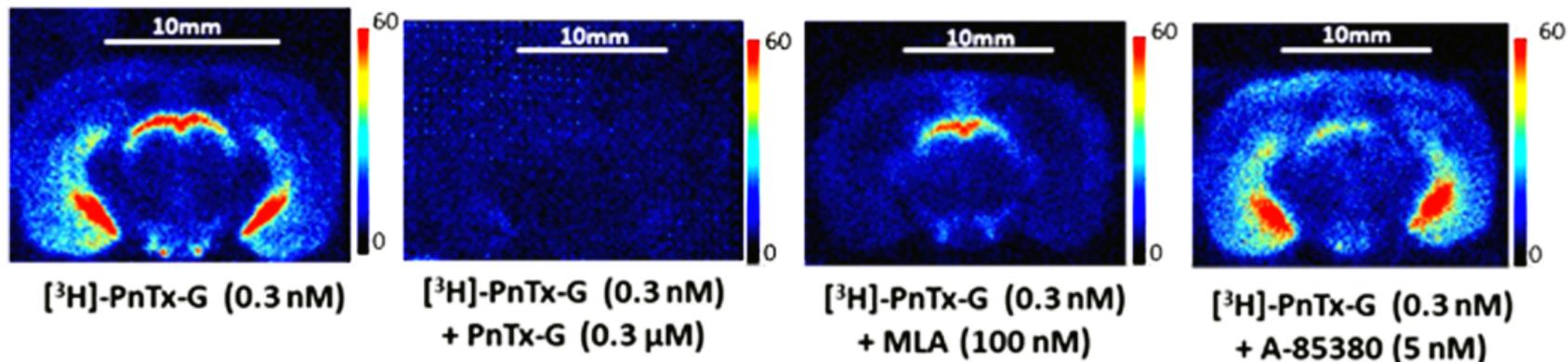
**Low threshold**

Forelimb musculature

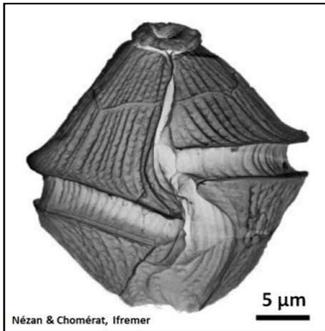
Hindlimb musculature

Spinal cord

**a****b****c****d****e**

**a****b**

*Vulcanodinium rugosum*

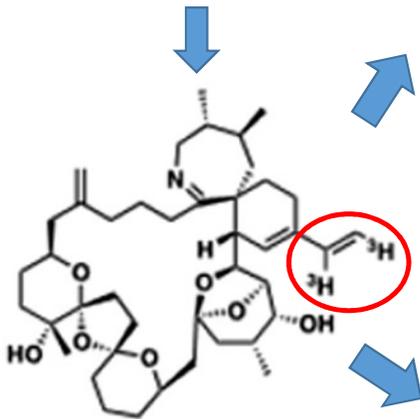
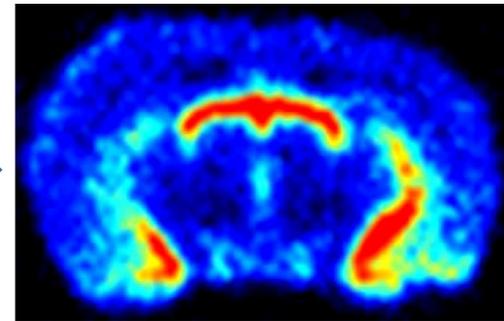


Oral gavage



Cross blood-brain barrier

Rat brain

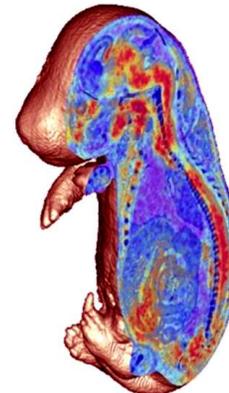


Synthesis, radiolabeling  
 $^3\text{H}$ -Pinnatoxin-G

Intravenous administration



Cross materno-fetal barrier



Rat fetus



Human cotyledon