Planarian behavioral screening is a useful invertebrate model for evaluating seizurogenic chemicals

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Highlights

- Classic seizurogenic chemicals induce convulsions in planarians
- A 48-well exposure paradigm allows for rapid testing of seizurogenic compounds
- Automated analysis replaces manual scoring of seizure-like planarian behavior
- Acute parathion exposure causes seizure-like activity in planarians

Keywords

Dugesia japonica; *Dugesia dorotocephala*; seizures; parathion; PTZ; behavior

Abstract

Detecting adverse health effects of drugs and other chemicals early during chemical/drug development saves significant time and resources. Freshwater planarians are an emerging invertebrate model for rapid, cost-effective neurotoxicity screening. Because planarians exhibit seizure-like behavior when exposed to chemicals that cause seizures in mammals, such as N-methyl-D-aspartate (NMDA) and picrotoxin, they could be a useful first-tier model for seizure

screening and thus reduce the need for slow and expensive mammalian tests. However, planarian seizure studies to date have been low-throughput and lacking the necessary standardization and automation to make this model a viable screening solution. Here, we present results from medium-throughput behavioral testing conducted in 48-well plates using two popular models for planarian pharmacological and toxicological studies: *Dugesia japonica* and *Dugesia dorotocephala*. Planarian behavior was scored using automated image analysis, measuring both translational behavior and body shape changes. We found that known seizurogenic compounds in mammals (NMDA, nicotine, picrotoxin, pilocarpine, and pentylenetetrazole (PTZ)) induced seizure-like behavior in both planarian species within 30 min of exposure. We also tested three pesticides (parathion, carbaryl, and permethrin). Parathion and carbaryl, but not permethrin, caused planarian seizure-like activity. While the planarian species responded similarly to most compounds, some compounds showed potency differences of 10-100-fold (pilocarpine and nicotine, respectively). *D. dorotocephala* planarians were generally more sensitive, but *D. japonica* planarians displayed more reproducible behaviors. By standardizing both experimental approach and analysis methods and making them available, this work can serve as a framework for future testing of chemicals for seizurogenic potential in planarians.

1. Introduction

Drugs that target the central nervous system (CNS) or non-CNS drugs that can cross the blood brain barrier may cause adverse outcomes on brain function, such as the induction of seizures. In fact, a recent study (Behl et al., 2025) reports that failure during seizure liability testing accounts for 67% of failures of CNS-drugs during pre-clinical drug development, based on survey results by the Safety Pharmacology Society in 2015 (Authier et al., 2016). Seizures can be induced through different molecular initiation events (Behl et al., 2025; Jett, 2012; Tukker and Westerink, 2021). For example, the well-known seizurogenic chemicals pentylenetetrazole (PTZ) and picrotoxin (PTX) cause seizures by inhibiting the $GABA_A$ receptor (Squires et al., 1984) while acute toxic exposure to organophosphorus nerve gases and pesticides causes seizures due to inhibition of acetylcholinesterase (AChE) (Kozhemyakin et al., 2010). Thus, seizure liability testing is an important step during pre-clinical drug development for drugs that enter the CNS or possess structural similarity to known seizurogenic compounds (Behl et al., 2025; Easter et al., 2009; Roberts et al., 2021).

Detection of potential seizurogenic compounds has traditionally relied on mice and rat *in vivo* and *ex vivo* models in during late stage preclinical studies (Easter et al., 2009; Tukker and Westerink, 2021). However, there is increasing awareness that testing in rodents is too expensive and slow to keep up with the demands for chemical screening and may be limited in its predictive capabilities for human health (Behl et al., 2025; Smirnova et al., 2014; Van Norman, 2019). Therefore, multiple *in silico* and *in vitro* assays have been developed in recent years to fill the need to more quickly and efficiently identify seizure liability (Behl et al., 2025; Belair et al., 2025; Easter et al., 2009; Grainger et al., 2018; Tukker and Westerink, 2021). Specifically, micro-electrode arrays (MEAs), particularly those utilizing human induced pluripotent stem cell derived neuronal /astrocyte co-cultures, have emerged as a promising new approach method (Belair et al., 2025; Grainger et al., 2018; Tukker and Westerink, 2021). In these assays, whole network function can be quantified and specific changes in neuronal activity have been shown to be indicative of *in vivo* seizures (Ishii et al., 2017). However, cell systems, including 3D cultures (Pelkonen et al., 2020), lack the complexity and metabolic capacity necessary to fully evaluate the effects of chemicals on brain function in a living organism (Hogberg and Smirnova, 2022). Developing zebrafish allow for rapid and cost-effective screening in an intact organism and therefore have become a popular complementary test system, especially for rapid screening of anti-seizure compounds (McGraw and Poduri, 2025; Milder et al., 2022; Whyte-Fagundes et al., 2025).

No model can 100% predict human *in vivo* effects and therefore modern toxicology aims to leverage the evidence gained from multiple complementary models via integrated approaches to hazard and risk assessment (Hogberg et al., 2022; Jaworska et al., 2016; Rockley et al., 2023). Freshwater planarians are invertebrates that have been utilized for pharmacological studies for over a century (Buttarelli et al., 2008; Ireland and Collins, 2022; Raffa, 2008). Planarians have recently gained recognition for their amenability to rapid behavioral testing of toxicants and neuroactive drugs (Ireland et al., 2025a; Ireland and Collins, 2022, 2023). Planarians are sensitive to chemical exposure and respond with stereotypical behavioral responses that can be measured. Because planarians live in water, chemical exposure is facile and behavioral experiments can be conducted easily and inexpensively (Ireland and Collins, 2022). Planarians share key neurotransmitter systems with humans, including GABA, glutamate, serotonin, dopamine, and acetylcholine (Ross et al., 2017), thus making them a relevant neurobiology model. Different species have been used for chemical exposure experiments for different reasons, such as commercial availability, size, and more recently

genome availability and amenability to high-throughput studies in multi-well plates (Ireland and Collins, 2023; Wu and Li, 2018).

Studies focused specifically on seizure-like behavior in planarians (**Figure 1**) have primarily used the commercially available *Dugesia dorotocephala* (DD) and *Dugesia tigrina* (DT) species and found that these species are sensitive to known seizurogenic compounds such as N-methyl-D-aspartate (NMDA) (Rawls et al., 2009), nicotine (Bach et al., 2016; Pagán et al., 2015; Rawls et al., 2011), and pilocarpine (Miller et al., 2025). When exposed to these compounds, planarians display stereotypical behaviors. Because it has not yet been shown that the behavioral phenotypes induced by these compounds are due to seizures through EEG or other means to visualize neuronal activity, we will refer to them as 'planarian seizure-like activity (pSLA)', a term originally introduced by (Rawls et al., 2009) and defined as "asynchronous paroxysms (C-shape, twitching behavior)".

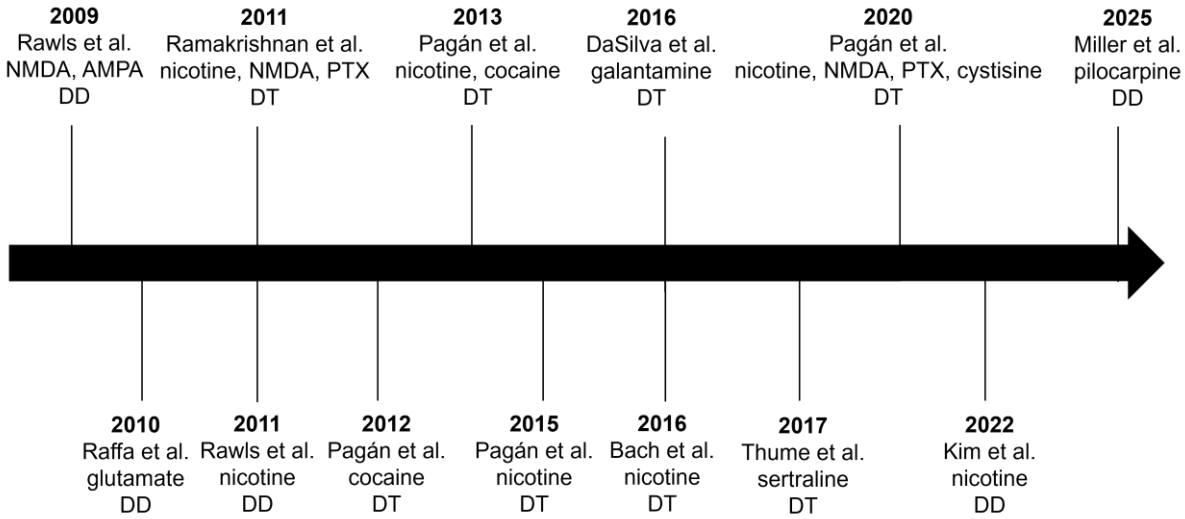


Figure 1. Overview of planarian studies on seizurogenic chemicals in the last 25 years (2000-2025). Tested seizurogenic compounds are listed. Some of the studies also tested other compounds, including therapeutics to treat seizures, which are not listed here. Abbreviations indicate the species tested: DD – *Dugesia dorotocephala*; DT – *Dugesia tigrina*.

Other researchers have referred to pSLA as planarian seizure-like motility (pSLM) (Pagán et al., 2013), defined as "vigorous writhing and bending of the body" (Bach et al., 2016). A clear definition of what these terms encapsulate does not exist but they seem to denote rapid shape changes – each lasting approximately 1 second (Rawls et al., 2009) - that include C-shape,

writhing, head flailing, dorsal oscillation, corkscrew, among others. Some of these shapes are well-defined (e.g., corkscrew), while others are ambiguous, e.g., “dorsal oscillations” (Miller et al., 2025). While object tracking has been employed to characterize the spatial behavior of planarians exposed to seizurogenic chemicals (Miller et al., 2025), pSLA thus far has been scored manually, by counting the number of abnormal behavioral changes during a 5-10 min recording period. Manual pSLA scoring relies on qualitative criteria about shape changes, which may differ between laboratories and even researchers. Moreover, behavioral data is intrinsically noisy due to the biological variability of organisms; thus, standardization of methods to minimize variability and automated pSLA analysis is indispensable to overcome these limitations and guarantee reproducibility and transferability of results.

Here, we present a medium-throughput method for screening chemicals for seizurogenic potential in two planarian species that are popular models in toxicology and pharmacology research: *Dugesia japonica* (DJ) and DD planarians. Screening is performed in 48-well plates. Scoring of pSLA is obtained from automated image analysis using a Motility Index (MI) metric that uses a standard image subtraction algorithm, similar to methods routinely used to evaluate zebrafish behavior (Bruni et al., 2016), including specifically for seizures (e.g., (Afrikanova et al., 2013; Whyte-Fagundes et al., 2025)). Using this method, we quantified the behavior of DJ and DD planarians to 9 test compounds. Four of the compounds have previously been shown to induce pSLA in some planarian species (NMDA, nicotine, picrotoxin, pilocarpine; Figure 1) and were confirmed as pSLA inducers by our automated analysis. PTZ, which is used to create rodent and zebrafish seizure models (Dhir, 2012; Gawel et al., 2020; Milder et al., 2022; Shimada and Yamagata, 2018; Yang et al., 2023), also induced pSLA in both planarian species. We also evaluated allyl isothiocyanate (AITC) as a control compound that induces “scrunching”, a well-characterized planarian behavior that shows oscillatory body shape changes (Cochet-Escartin et al., 2015; Sabry et al., 2019), and show that our analysis can distinguish it from pSLA. The other test compounds were three pesticides with seizurogenic potential in mammals, namely two acetylcholinesterase inhibitors (parathion and carbaryl) and the pyrethroid permethrin (Jett, 2012). Permethrin, tested to the solubility limit, did not cause effects but parathion and carbaryl induced pSLA, thus demonstrating that pSLA can be triggered through different pathways in planarians, similar to the situation in mammals.

155 **2. Methods**

156 ***2.1. Planarian species and maintenance***

157 The freshwater planarian species DJ and DD were used in this study. DJ planarians have been
158 maintained in the laboratory for > 15 years on organic liver (beef and chicken). DD planarians
159 were obtained commercially from Carolina Biologicals (Cat #132954) and then maintained on
160 frozen bloodworms (San Francisco Bay Brand, #88055) in the laboratory. All species were
161 maintained in planarian water (0.21 g/L Instant Ocean salts [Spectrum Brands, Blacksburg, VA,
162 USA], 0.83 mM $MgSO_4$, 0.9 mM $CaCl_2$, 0.04 mM $KHCO_3$, 0.9mM $NaHCO_3$), fed 1-2x per
163 week and cleaned on the day of feeding and 2 days following feeding. Adult intact planarians
164 which had been fasted 4-9 days were selected for experiments. If amputated planarians were
165 used in an experiment alongside intact planarians, they were selected to be of similar size to
166 the matched intact ones. Amputations were performed 4 hours prior to chemical exposure with
167 an ethanol-sterilized razor or scalpel blade between the auricles and pharynx.

168

169 ***2.2. Chemicals and preparation***

170 Chemicals used in this study are listed in Table 1. Depending on water solubility, chemical
171 stock solutions were prepared either in planarian water or in 100 % dimethyl sulfoxide (DMSO)
172 in a fume hood. For all chemicals prepared in DMSO, the final DMSO concentration in the 1x
173 solution was 0.5%. Because DMSO alone can affect planarian behavior (Pagán et al., 2006),
174 we tested DMSO up to 5% for acute behavioral effects. No overt qualitative behavioral
175 phenotypes or pSLA were found for DMSO at concentrations <4% in DJ or <5% in DD
176 (Supplemental Figure S1). Chemicals prepared in planarian water were used the same day,
177 while stocks made in DMSO were aliquoted and stored at -20°C or -80°C until use. Frozen
178 stocks were thawed a maximum of 3 times. Depending on chemical solubility, chemicals were
179 added as 1x, 2x or 10x solutions to the planarians in the wells (Table 1).

180 pH measurements were taken with an Apera PH60-MS pH Tester kit (Apera Instruments,
181 Columbus, OH) after allowing the probe to stabilize >10 minutes. The pH of the highest tested
182 concentration of each chemical is listed in Supplemental Table S1. To obtain planarian water
183 at acidic pH, dilute hydrochloric acid was added until the desired pH was obtained and pH was
184 verified immediately before use.

185

186 **Table 1. Overview of chemicals and concentrations used**

Chemical	CAS #	Tested concentrations[1]	Vendor	Purity (%)

allyl isothiocyanate (AITC)[2]	57-06-7	25 μM (DD); 100 μM (DJ)	Sigma-Aldrich	95%
carbaryl[3]	63-25-2	10-75 μM	Sigma-Aldrich	≥98.0 %
dimethyl sulfoxide (DMSO)[2]	67-68-5	0.1%-5% (DD); 0.5-5% (DJ)	Sigma-Aldrich	≥99.7%
N-methyl-D-aspartate (NMDA)[4]	6384-92-5	3 mM (DD); 15 mM (DJ)	Tocris Bioscience	≥99% (HPLC)
(−)-nicotine[2]	54-11-5	10-50 μM (DD); 50-1000 μM (DJ)	Sigma-Aldrich	≥99% (GC)
parathion[3]	56-38-2	10-50 μM (DD); 10-75 μM[6] (DJ)	Sigma-Aldrich; ChemService	≥98.0% (HPLC); 98.8% (GC/FID)
pentylenetetrazole (PTZ)[5]	54-95-5	5- 20 mM	Cayman Chemical	≥98%
permethrin (mixture of cis and trans isomers) [3]	52645-53-1	100 μM; 1 mM[6]	Sigma-Aldrich	>97% (29.8% cis, 67.8% trans isomers)
picrotoxin, *Anamirta cocculin* (PTX)[4]	124-87-8	5 mM[6]	Sigma-Aldrich	≥98% (mixture of picrotoxin and picrotin, HPLC)
(+)-pilocarpine hydrochloride[5]	54-71-7	2-6 mM (DD); 6-100 mM (DJ)	Cayman Chemical	≥98%

[1]if the same concentration or concentration range was used in both species, there is no species reference; else, it is indicated in parentheses which species was tested at this concentration.
[2] indicates highest concentration stock was 10x of the nominal, prepared in planarian water.
[3] indicates highest concentration stock was 200x of the nominal, prepared in DMSO and diluted to an intermediate 2x or 10x.
[4] indicates highest concentration stock was 1x of the nominal, prepared in planarian water.
[5] indicates highest concentration stock was 2x of the nominal, prepared in planarian water.
[6] indicates solubility issues with the 1x stock in planarian water.

2.3. Plate setup and acute recording

Planarians were loaded into the wells of a tissue culture treated 48-well plate (Genesee Scientific, El Cajon, CA), one planarian per well, using a p1000 pipettor with the pipette tip slightly trimmed with a clean razor blade to avoid damage to the planarians. The planarians were loaded in planarian water and an appropriately concentrated chemical stock was added such that the total volume of 1x solution in each well equalled 200 μL. Chemical addition was performed in the fume hood. The chemical stock was added using a multichannel pipettor (CAPP Aero 12-Channel Pipette, 5-50 μL), the plate was sealed using thermal film (Excel

Scientific, Victorville, CA, USA), and behavior was recorded, with the recordings beginning 2-6 minutes after chemical addition. Four conditions (3 test conditions and 1 vehicle control) were tested per plate, with each condition comprising two columns of wells, with n=12 planarians per condition (Figure 2A). If any chemical in the plate was prepared in DMSO, the vehicle control consisted of 0.5% DMSO, else it contained untreated planarian water. As needed, independent replicates were performed to increase the sample size.

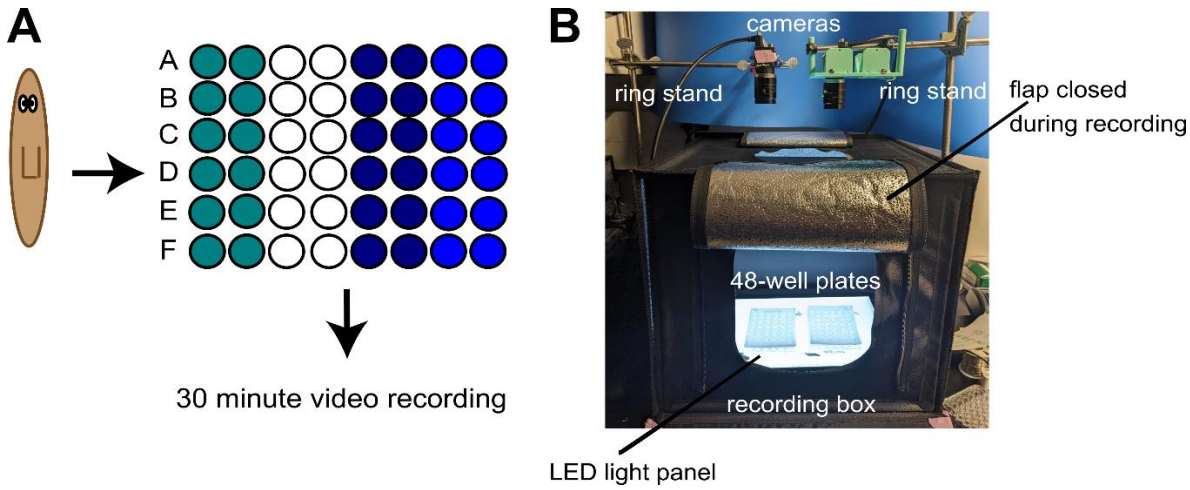


Figure 2. Schematic of 48-well plate and behavioral imaging setup. A) Testing was performed in 48-well plates with one worm per well. Each chemical condition (different colors) was tested across 2 columns of wells (n=12). Every plate contained one population of vehicle controls (white wells). B) Custom imaging setup allowing for recording of 2 plates simultaneously. Details of the components used are provided in Supplemental Table S2.

As shown in Figure 2B, recordings were taken with a custom imaging setup inside a NEEWER Photo Studio Shooting tent (Amazon.com, Seattle WA) that allows for recording in a controlled lighting environment. All components used in this set up are described in Supplemental Table S2. In brief, the plates were set on top of an LED light panel with brightness set to 1350 to 1450 lux, as measured with a lux meter. A Fresnel lens was placed on top of the plate to minimize imaging aberrations. Recordings were 30 minutes in length, except where indicated, and taken at 5 frames per second using a Point Grey Flea3 camera (FLIR, Wilsonville, OR) using Flycap software. This frame rate was chosen because we wanted to compare pSLA to other hyperactive behaviors, such as scrunching, which requires a minimum 5 fps frame rate for detection (Cochet-Escartin et al., 2015; Sabry et al., 2020), and we found that higher frame rates did not improve signal detection (Supplemental Figure S2).

231

232 ***2.4. Automated image analysis***

233 Analysis of acute behavioral recordings was performed in MATLAB (2025a, MathWorks,
234 Natick, MA) using custom scripts. Any worm that crawled out of the water during recording
235 was excluded from analysis. To create a standardized image of the background to be using for
236 masking the individual wells and removing background noise, the average projection of the
237 first 2 min of recording was used. For determination of overall activity and changes in body
238 shape, we calculated the absolute value of the difference of consecutive frames divided by the
239 absolute value of the sum of the two images. The difference image was then binarized using a
240 noise threshold determined from recordings of plates containing water only under the same
241 imaging conditions. The motility index (MI) was then calculated for each well and frame as
242 the sum of pixels that changed between the two images above the noise threshold. The area of
243 each worm was calculated via thresholding of the image following subtraction by the average
244 projection background image. As optical aberrations during imaging create some "blind spots"
245 in which the planarian can become lost, any frames where no worm was found were excluded
246 in the MI calculations. As individual bouts of pSLA have been previously described as lasting
247 approximately 1 second (Rawls et al., 2009), the median MI score for 1 sec (5 frame) bins was
248 calculated. These scores were then normalized by the median worm area in the first 2 min of
249 recording, where the worms are most likely to be moving the most and thus have elongated
250 shapes.

251 To correlate normalized MI scores with the presence of pSLA, we manually evaluated the
252 recording of 10-12 planarians exposed to NMDA (3 mM in DD, 15 mM in DJ) across 3
253 independent plates to identify pSLA events. For each analyzed worm, 1 minute of recording
254 (300 1 sec bins) was chosen, which exhibited high pSLA activity qualitatively by eye and 1 sec
255 bins containing pSLA were marked. For each species, we then evaluated the distribution of
256 normalized MI scores across all identified pSLA events (Supplemental Figure S3). The 10th
257 percentile of each species' pSLA distributions (DD: 0.11; DJ: 0.21) was then used as a
258 threshold, where normalized MI scores above this threshold were counted as pSLA events.

259

260 MI provides information about the general activity of a planarian but gives no context to the
261 spatial displacement of the worm. Thus, we also quantified the translational activity of each
262 planarian over 20 sec (100 frame) bins. This bin size was chosen because a normal gliding
263 planarian can circle around the outside of a well of a 48-well plate in about this time. For each
264 bin, the minimum intensity projection was determined. The average projection of the first 2

min of recording was subtracted from the minimum intensity projection to remove the background. The resulting image was binarized using a pre-determined threshold. The translational activity of each worm was calculated as the sum of pixels in this filtered image (thus representing the motion of the worm) divided by the median area of the worm during the first 2 min of recording.

2.5. Lethality analysis

To determine if the presence of acute pSLA is correlated with subsequent lethality, some plates were removed after the initial acute recording and stored in a dark 20 °C incubator until the following day. Plates were recorded for 5 min at 21-27 hours after plate setup. From the recordings, each planarian was manually scored as alive, dead, "crawl-out" when the worm crawls out of the solution in the well, or "sick" when the worm was alive/moving but displayed abnormal morphology, indicated by head regression, pharynx extrusion, lesions/disintegration or C-shape body postures.

2.6. Statistical analysis

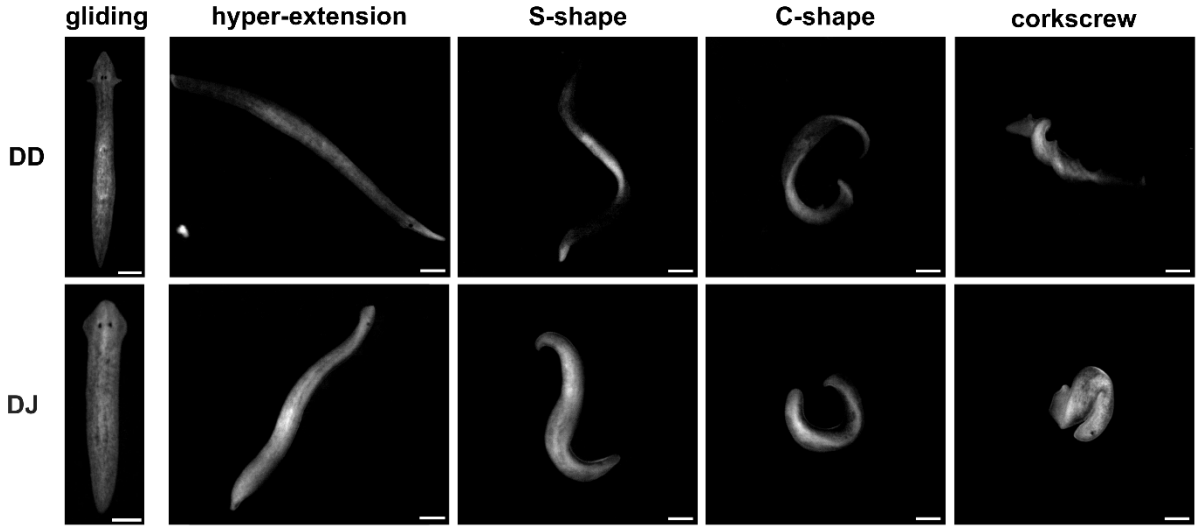
The number of pSLA events, defined by each species threshold (see section 2.4), over the entire 30 min recording were counted and are provided in Supplemental File S1. Statistical analysis was performed in R (4.5.0, (R Core Team, 2016)). For each compound, data were compiled across all independent replicates and compared with the compiled in plate vehicle controls (planarian water or 0.5% DMSO) across the same replicates. A negative binomial generalized linear model was performed using the MASS package (Venables and Ripley, 2002) to determine an effect of condition on the number of pSLA events. Post hoc pairwise comparisons of the estimated marginal means of the model was performed using the contrast function of emmeans ((Lenth, 2023) using the Benjamini & Hochberg (BH) p-value adjustment method for multiple comparisons.

3. Results

3.1. Defining pSLA events using prototypical concentrations of NMDA

The term pSLA was first introduced in 2009 by Rawls and co-authors to describe planarian behavior that looked convulsive and indicative of seizures in response to NMDA treatment (Rawls et al., 2009). However, pSLA identification was qualitative and the manual counting of pSLA is extremely time consuming and susceptible to bias. To overcome these limitations and

298 thus make rapid screening of potential seizurogenic compounds in freshwater planarians
299 possible, we developed a pipeline to robustly and quantitatively identify pSLA.
300
301 Because we needed a prototypical example of pSLA to build our analytical pipeline, we first
302 identified a NMDA concentration that qualitatively induced robust pSLA in both planarian
303 species within 30 minutes of exposure. Using the published data as a guide (Rawls et al., 2009),
304 we tested 3 mM NMDA and found that this concentration immediately induced convulsive
305 behavior in DD. However, robust convulsions were not observed in DJ until 15 mM NMDA
306 was used. At these selected concentrations, NMDA-induced various paroxysms, including head
307 flailing, turning, rapid twitching and convulsions, over-extension and C-shapes, corkscrews
308 and spiral curling in both species (Supplemental Videos S1 and S2), as previously described in
309 the literature. Figure 3 shows example high magnification images of the different shapes and
310 behaviors that we observed in both species.



311
312 **Figure 3. High magnification examples of abnormal body shapes and behaviors observed**
313 **during NMDA-induced pSLA.** Normal gliding behavior (left) as observed in planarian water
314 exhibits relatively fixed body lengths and straight motion. During NMDA exposure (3 mM in
315 DD and 15 mM in DJ), planarians change rapidly between different abnormal body shapes.
316 Scale bars: 0.5 mm.
317
318 Next, to allow for rapid behavioral screening of multiple conditions simultaneously, we
319 developed a medium-throughput screening set-up using 48-well plates as explained in Methods
320 Section 2.3 and shown in Figure 2. Each well contained one planarian, with 2 columns per
321 treatment condition, for a total of 12 planarians per condition per plate, allowing for

322 simultaneous testing of 4 conditions in the same plate (Figure 2A). The use of commercial
323 components (provided in Supplemental Table S2) for the behavioral imaging box (Figure 2B)
324 allows for replication of this method in other laboratories. Using this set-up, chemically treated
325 planarians were imaged for 30 minutes immediately (within 2-6 min) following exposure. To
326 quantitatively identify pSLA, we measured the motility index (MI, see Methods Section 2.4),
327 normalized by worm area, for each planarian over the 30 min recording period. As individual
328 bouts of pSLA have been previously described as lasting approximately 1 sec (Rawls et al.,
329 2009), we calculated the median MI per 1 sec (5 frames) bins for each worm. Species-specific
330 pSLA thresholds were then determined based on the distribution of normalized MI scores for
331 a subset of representative NMDA-exposed planarians (see Methods Section 2.4, Supplemental
332 Figure S3), where normalized MI scores per 1 sec bin above this threshold were identified as
333 potential pSLA events. We then applied this analysis to all NMDA data.
334
335 We found that NMDA showed significantly more pSLA events than the respective controls in
336 each species (Figure 4A-B). Because these NMDA concentrations had low pH (~3,
337 Supplemental Table S1), we also tested the effect of pH alone using planarian water at
338 approximately the same pH as NMDA. Acidic planarian water induced significantly more
339 pSLA events than the respective non-adjusted controls, though not to the same extent as with
340 NMDA. However, in DJ, the number of pSLA events in the acidic pH sample was not
341 significantly different from NMDA. We conclude that pH is a contributing factor to NMDA-
342 induced pSLA in both species.
343
344 While the total number of pSLA events over the entire recording time allows classification of
345 a chemical as seizurogenic or not, there is additional information about the chemical's action
346 in the dynamics of the response, which can be evaluated from the normalized MI scores as a
347 function of time (Figure 4C-D). In both species, NMDA induced high activity within 0-2 min
348 of recording time, which dissipated within ~5 (DD) or ~10 (DJ) min. After this time, NMDA-
349 exposed planarians became contracted and immobile and/or crawled out of the NMDA
350 solution. As 0 or nearly 0 MI scores are seen with both normal gliding behavior and immobility,
351 we also quantified the translational activity of the planarians (see Methods Section 2.4). These
352 translational activity plots show that the decrease in MI scores in NMDA-exposed planarians
353 is concomitant with decreased motion around the well (Figure 4E-F). This decreased motility
354 may indicate the toxic nature of NMDA as almost all worms in both species were dead or had
355 crawled out of their wells by ~24 h (Supplemental Figure S4).

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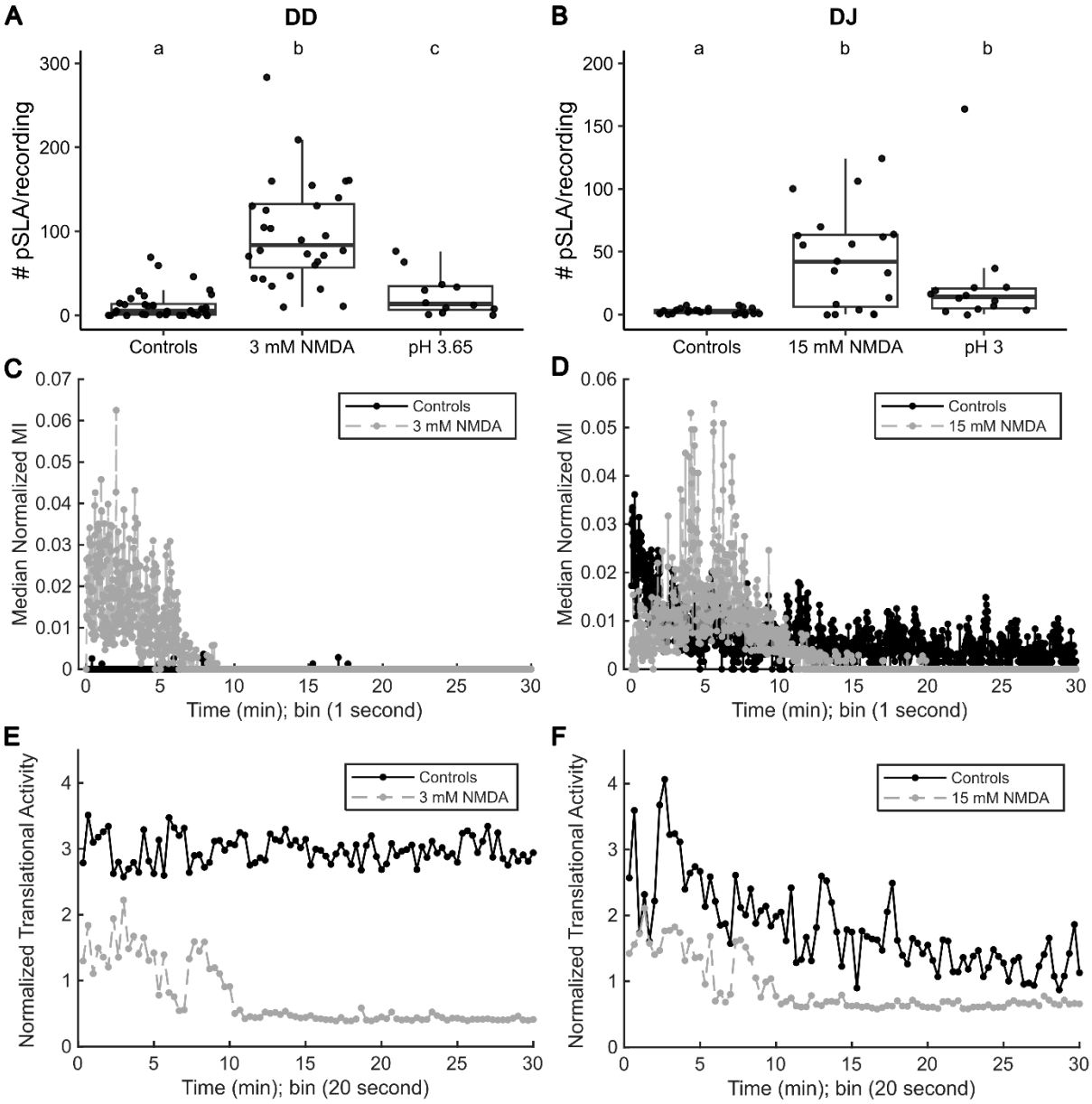

358

359 **Figure 4. Validation of quantitative analysis of pSLA activity using NMDA.** (A-B)
360 Boxplots of the number of pSLA events per 30 min recording in A) DD and B) DJ exposed to
361 NMDA and planarian water at the same pH as the respective NMDA concentration. Dots
362 represent individual planarians. Statistical significance was determined using pairwise
363 contrasts (with a Benjamini & Hochberg p-value correction) of the estimated marginal means
364 of a negative binomial generalized linear model of condition versus number of pSLA events.
365 Different lower-case letters indicate the groups are statistically significantly different ($p<0.05$).
366 (C-D) Median normalized MI for all C) DD or D) DJ planarians exposed to their prototypical
367 NMDA concentration and their respective in plate vehicle controls as a function of time. (E-F)

368 Median normalized translational activity of all E) DD or F) DJ planarians exposed to their
369 prototypical NMDA concentration and their respective in plate vehicle controls as a function
370 of time. Sample sizes are listed in Supplemental Table S3.
371
372 ***3.2. MI analysis distinguishes pSLA from other hyperactive behaviors***
373 To verify that our analysis method is specific to pSLA, we exposed DD and DJ planarians to
374 AITC, which has been shown to activate the planarian Transient Receptor Potential Ankyrin
375 channel (*Dj*TRPAa) and induce forward scrunching, a periodic muscle-based form of
376 locomotion (Sabry et al., 2019). AITC concentrations that induced scrunching within a few
377 minutes of exposure (25 μM for DD and 100 μM for DJ) had increased normalized MI scores
378 (Figure 5A-B) and showed differences in translational activity compared to controls (Figure
379 5C-D). However, the MI scores were not high enough to be detected as significant pSLA
380 activity (Figure 5E-F). AITC behavior was only analyzed for 10 minutes following exposure
381 as scrunching did not persist past this time.

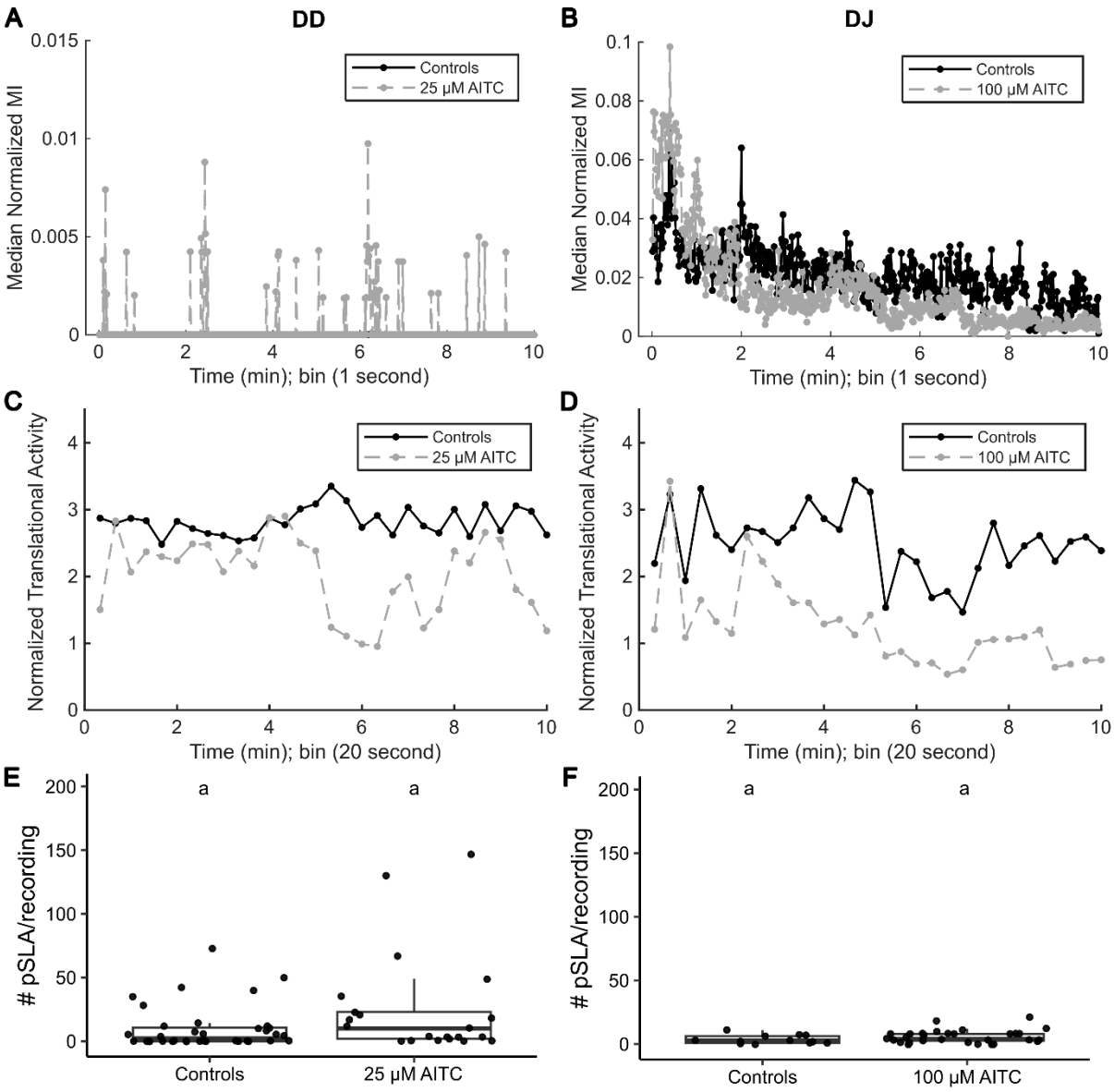

Figure 5. Quantitative MI analysis can distinguish pSLA from scrunching behavior induced by AITC. (A-B) Median normalized MI for all A) DD or B) DJ planarians exposed to AITC and their respective in plate vehicle controls as a function of time. Note that in A the control curve is not visible as all scores were 0. (C-D) Median normalized translational activity of all C) DD or D) DJ planarians exposed to AITC and their respective in plate vehicle controls as a function of time. (E-F) Boxplots of the number of pSLA events per 10 min recording in E) DD and F) DJ exposed to AITC and the respective in plate vehicle control. Dots represent individual planarians. No significant differences were found between controls and AITC in each using pairwise contrasts (with a Benjamini & Hochberg p-value correction) of the estimated marginal means of a negative binomial generalized linear model of condition versus

393 number of pSLA events. Note the y-axes are scaled differently in the various plots to best reflect
394 each data set. Sample sizes are listed in Supplemental Table S3.
395
396
397 ***3.3. Compounds known to induce pSLA in planarians are detected via MI analysis***
398 Next, we tested other compounds that have previously been reported to induce pSLA in
399 planarians (Table 2).
400
401 **Table 2. Chemicals and concentrations reported to induce pSLA in planarians.**

Chemical	Species	Concentration range	References
Nicotine	DT; DD	10-2000 µM (DT) 1-5 mM (DD)	(Bach et al., 2016; Pagán et al., 2015; Ramakrishnan and DeSaer, 2011; Rawls et al., 2011)
Pilocarpine	DD	1,2,3,4,6 mM	(Miller et al., 2025)
PTX	DT	0.01-5 mM	(Ramakrishnan and DeSaer, 2011)

402
403 *3.3.1 Nicotine*
404 Studies by different research groups have reported hyperactivity and pSLA in DD and DT
405 planarians upon acute nicotine exposure in the µM to mM range (Figure 1 and Table 2).
406 Nicotine binds to and activates nicotinic acetylcholine receptors. While there is supporting
407 pharmacological evidence for nicotinic acetylcholine receptors in DD planarians (Rawls et al.,
408 2011), the receptors have not yet been characterized. No significantly increased pSLA events
409 were observed in DD planarians exposed to 10 – 50 µM nicotine (Figure 6A) although we
410 qualitatively observed hyperactivity and pSLA-like events in DD planarians exposed to 10 µM
411 nicotine immediately following exposure. This hyperactivity was slower and less intense than
412 pSLA seen with 3 mM NMDA, as supported by lower amplitudes in the MI versus time plot
413 for nicotine (Supplemental Figure S5) compared to NMDA (Figure 4C). In contrast to 3 mM
414 NMDA, no lethality was observed at ~24 h with 10 µM nicotine (Supplemental Figure S4).

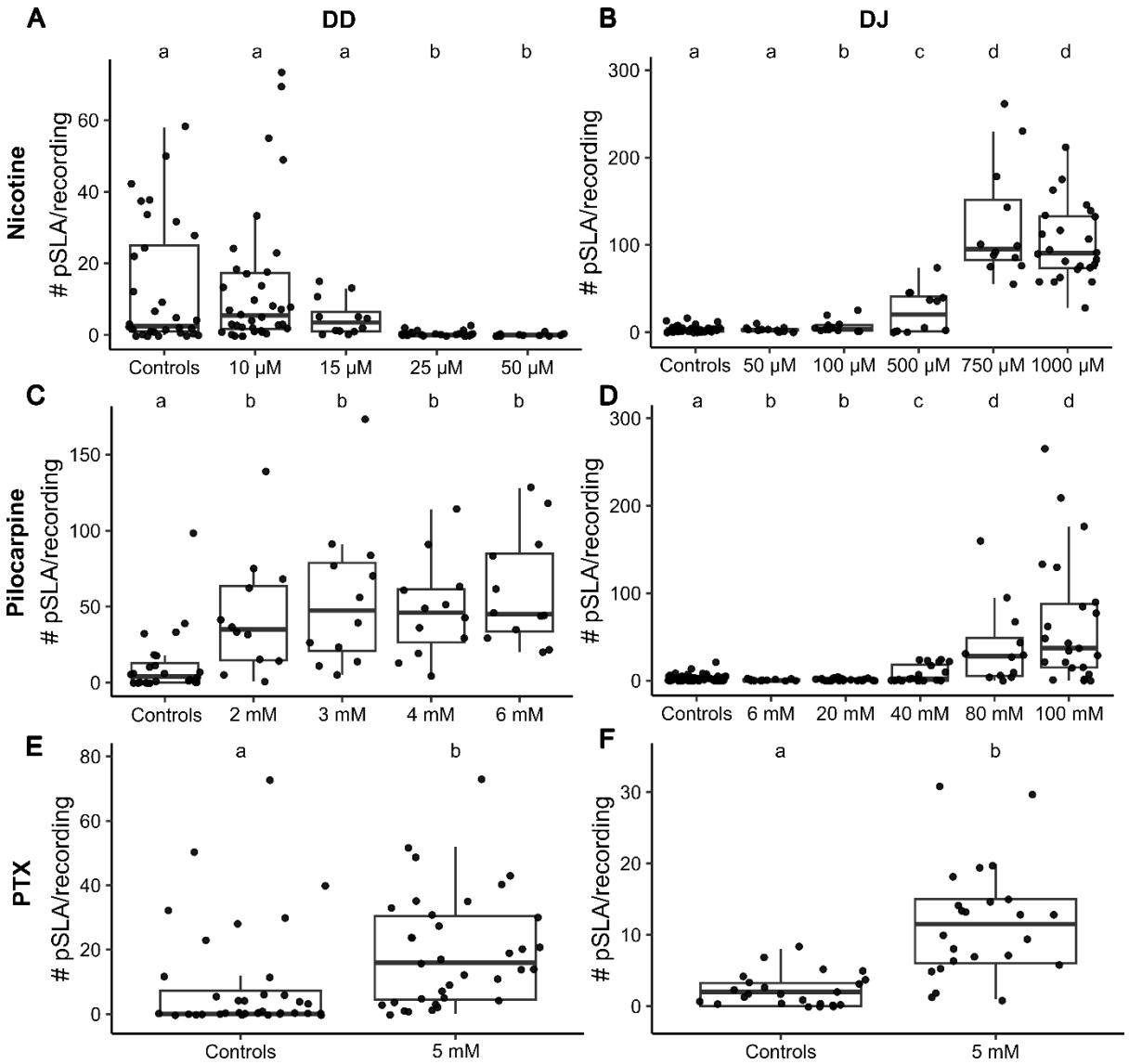

Figure 6. MI analysis of known seizurogenic compounds in planarians. Boxplots of the number of pSLA events per 30 min recording in (A-B) Nicotine, (C-D) Pilocarpine, and E-F) PTX in DD (left column, A, C, E) and DJ (right column, B, D, F). Dots represent individual planarians. Sample sizes are listed in Supplemental Table S3. Statistical significance was determined using pairwise contrasts (with a Benjamini & Hochberg p-value correction) of the estimated marginal means of a negative binomial generalized linear model of condition versus number of pSLA events. Different lower-case letters indicate the groups are statistically significantly different ($p<0.05$). Note the y-axes are scaled differently in the various plots to best reflect each data set.

Nicotine-induced pSLA quickly dissipated within the first 2 minutes, followed by a return to gliding behavior (Supplemental Figure S5 and S6). For these reasons, and because of the noise in the controls, no statistical significance was found when considering the number of pSLA

429 events over the entire 30 min recording. When we focused on only the first 5 min of recording,
430 we found significant pSLA for 10 μM nicotine (Supplemental Figure S7). Thus, our analysis
431 shows that nicotine induces pSLA, in agreement with published data, which only scored 5 min
432 of exposure (Ramakrishnan and DeSaer, 2011; Rawls et al., 2011).
433
434 Interestingly, pSLA was not observed in higher concentrations (15 and 25 μM) which largely
435 displayed normal gliding. Exposure to 50 μM nicotine resulted in contraction and paralysis
436 over the course of the 30 min recording (Supplemental Figure S8A). In contrast, in DJ
437 planarians, significantly increased pSLA was observed starting at 100 μM nicotine, with the
438 most pSLA events observed in 750 μM, which was highly hyperactive and demonstrated
439 NMDA-like pSLA consisting of head flailing, convulsions and dynamic changes between S-
440 and C-shapes (Figure 6B). In 750 μM nicotine, pSLA activity dissipated/slowed after 5 min of
441 recording and was replaced by contraction with some sporadic, slow convulsive events
442 (Supplemental Figure S9) and very little translational motion (Supplemental Figure S10).
443 Contraction and further morphological abnormalities such as C-shapes and lesions were
444 observed at ~24 h of exposure to 750 μM nicotine, although little lethality was observed
445 (Supplemental Figure S4).
446
447 *3.3.2 Pilocarpine*
448 It has recently been shown that a muscarinic receptor agonist, pilocarpine, can also trigger
449 pSLA in DD. Using particle tracking to quantify the time spent in the center and the periphery
450 of a well of a 12-well plate and manual scoring of different behaviors such as "dorsal
451 oscillations", "head flick", "C-shape", and others, concentrations of 4-6 mM pilocarpine were
452 found to be significantly different from controls (Miller et al., 2025). Thus, we first tested 2-6
453 mM in DD planarians. Using MI analysis, we found that all tested concentrations of pilocarpine
454 induced statistically significant pSLA in DD (Figure 6C), in agreement with the study by Miller
455 et al. (Miller et al., 2025). No lethality was observed at 24 h (Supplemental Figure S4).
456 However, DJ planarians only displayed significantly increased pSLA at ≥ 40 mM pilocarpine,
457 with the most pSLA events observed in 100 mM pilocarpine (Figure 6D), which also caused
458 100% lethality at 24 h (Supplemental Figure S4). Thus, the two species show clear differences
459 in sensitivity to pilocarpine, which may be due to differences in uptake, molecular targets, or
460 metabolism.
461
462

463 *3.3.3 PTX*

464 The $GABA_A$ receptor antagonist PTX has been shown to induce pSLA in DT planarians
465 (Ramakrishnan and DeSaer, 2011) but no studies have yet been conducted in DD or DJ
466 planarians. Because of limited water solubility, the highest concentration of PTX that could be
467 tested was 5 mM. We found that 5 mM PTX induced hyperactivity and pSLA within 30 min of
468 recording in both species (Figure 6E-F). However, in contrast to the other chemicals tested,
469 which induced pSLA within the first few minutes, 5 mM PTX induced pSLA was only observed
470 after 20-25 min of recording, following initial gliding behavior and sporadic bouts of head
471 twitching and dorsal oscillations (Supplemental Figures S4 and S8), suggesting that this
472 concentration of PTX was at the potency threshold. PTX did not induce lethality in either
473 species at ~24 hours (Supplemental Figure S4), but the worms were elongated and were still
474 exhibiting hyperactive behaviors.

475

476 ***3.4. PTZ can induce pSLA***

477 Similar to PTX, PTZ also antagonizes the $GABA_A$ receptor (albeit through a different
478 mechanism) and is frequently used to generate animal seizure models (Afrikanova et al., 2013;
479 Chitolina et al., 2023; Dhir, 2012; Milder et al., 2022; Shimada and Yamagata, 2018; Yang et
480 al., 2023). We therefore tested varying concentrations of PTZ for its ability to induce pSLA in
481 both DD and DJ planarians. We found that concentrations exceeding 10 mM (DD) or 5 mM
482 (DJ) PTZ significantly induced pSLA (Figure 7A-B). PTZ-induced pSLA was characterized by
483 an initial phase of head wiggling and turning, followed by random twitching and convulsions
484 that included longitudinal over-extension and C-shapes, corkscrews and spiral curling. In DD,
485 these hyperactive behaviors slowed down over the 30 min of recording and some of the
486 planarians moved into a contracted state, indicating muscle paralysis (Supplemental Figures
487 S5-6). In contrast, in DJ planarians, hyperactive behavior persisted throughout the entire 30
488 min recording (Supplemental Figure S9). Both species were impacted at 24 hours with high
489 numbers of death, sickness and crawl-out behavior, though the exact mix of these three
490 abnormal conditions differed across the species (Supplemental Figure S4)

491

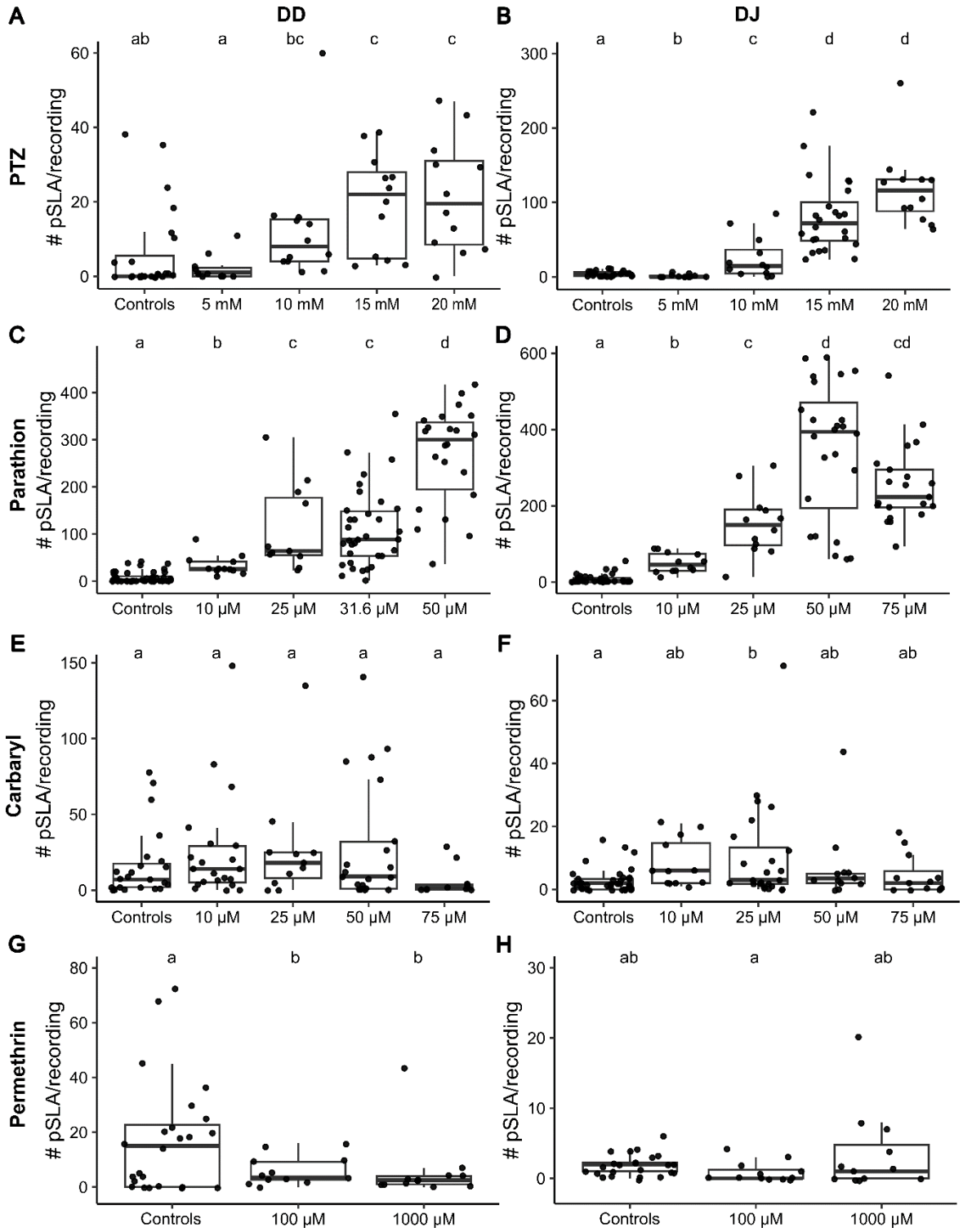

Figure 7. PTZ and parathion induce pSLA in DD and DJ planarians. Boxplots of the number of pSLA events per 30 min recording in (A-B) PTZ, (C-D) parathion, (E-F) carbaryl, and (G-H) permethrin in DD (left column) and DJ (right column). Dots represent individual planarians. Sample sizes are listed in Supplemental Table S3. Statistical significance was determined using pairwise contrasts (with a Benjamini & Hochberg p-value correction) of the estimated marginal means of a negative binomial generalized linear model of condition versus

499 number of pSLA events. Different lower-case letters indicate the groups are statistically
500 significantly different ($p<0.05$). Note the y-axes are scaled differently in the various plots to
501 best reflect each data set.
502
503 ***3.5. Some pesticides induce pSLA***
504 Pesticides with different molecular targets have been found to be seizurogenic in mammalian
505 models (Jett, 2012). Because both nicotine and pilocarpine, two cholinergic receptor agonists,
506 induced pSLA, we hypothesized that organophosphorus pesticides and carbamates that inhibit
507 acetylcholinesterase (AChE) in planarians as in humans (Hagstrom et al., 2017) and can cause
508 seizures in mammals at high concentrations (Jett, 2012), could trigger pSLA upon acute high
509 concentration exposure. To test this hypothesis, we tested one chemical from each class,
510 parathion and carbaryl, respectively. Concentrations of 10 μM parathion and above caused
511 significant pSLA in both planarian species (Figure 7C-D). While 50 μM caused the most pSLA
512 events in both species, 31.6 μM parathion was a better concentration to induce pSLA in DD
513 due to the high number of crawl-out events seen in this species at 50 μM. Following ~24 hour
514 exposure to these concentrations of parathion, both species showed sickness and lethality
515 (Supplemental Figure S4). Carbaryl induced much weaker pSLA events in both species, which
516 were characterized by small contractions and head twitches. While 25 μM showed significantly
517 increased pSLA in DJ planarians compared to vehicle controls, 10-75 μM carbaryl showed no
518 significant effect on the number of pSLA in DD planarians (Figure 7 E-F), despite 25 μM
519 showing increased median MI scores per bin (Supplemental Figure S5). At 75 μM, some
520 scrunching and head twitching was observed within the first 5 minutes of recording, following
521 by decreased movement and contraction (Supplemental Figure S8B), suggesting that this
522 concentration exceeded the pSLA-inducing range of concentrations. No significant lethality
523 was observed in either species exposed to 25 μM carbaryl at ~24 h (Supplemental Figure S4).
524
525 Some pyrethroid insecticides, such as permethrin, have been shown to cause seizures in rodents
526 at lethal concentrations (Staatz and Hosko, 1985) or in environmentally relevant concentrations
527 in cats (Dymond and Swift, 2008). Pyrethoids inhibit voltage-gated sodium channels, leading
528 to neuronal hyper-excitability and at high doses can inhibit GABA signalling (Jett, 2012). We
529 tested permethrin up to its solubility limit (1 mM) and did not observe significant pSLA in
530 either planarian species (Figure 7G-H). Additionally, no obvious differences in behavior could
531 be seen in the MI or translational activity plots (Supplemental Figures S5, S6, S9, S10).
532

533

534 ***3.6. The planarian brain is not required to induce pSLA in DJ planarians***

535 It had previously been shown that pSLA in headless DT planarians was similar to that in intact
536 planarians when exposed to nicotine but not to cocaine, another pSLA inducer (Pagán et al.,
537 2012). Cocaine-induced pSLA was nearly completely lost in regenerating tails and was
538 gradually restored during regeneration (Pagán et al., 2013). These results suggest that cocaine
539 sensitive receptors are primarily found in the brain, consistent with dopamine receptors in
540 vertebrates. In contrast, nicotinic acetylcholine receptors must be distributed in both the head
541 and the body because the response in headless planarians was similar to that of intact
542 planarians. Therefore, comparing responses of intact and headless planarians to seizurogenic
543 chemicals may provide insights into where the molecular targets of these chemicals are
544 expressed in planarians. Thus, we tested whether the presence of the brain was required for
545 pSLA by exposing regenerating DJ tails to each seizurogenic compound at a concentration
546 shown to significantly induce pSLA in adult/intact DJs. Exposure began 4 h after amputation
547 to allow for wound closure and to mitigate the increased mucus production following
548 amputation (Peiris et al., 2014) that may hinder chemical uptake. All chemicals except for
549 carbaryl induced pSLA in regenerating DJ tails (Table 3).

550

551 **Table 3**. **Median number of pSLA in adult or regenerating tail DJ planarians exposed to**
552 **representative concentrations of seizurogenic compounds.** Sample sizes are listed in
553 Supplemental Table S3.

Condition	Adult Median # pSLA (25th, 75th percentile)	Regenerating Median # pSLA (25th, 75th percentile)
15 mM NMDA	42 (6, 63.5)	45 (33, 68)
pH 3	14 (5, 21)	69 (24, 132)*
750 µM nicotine	96 (83, 152)	29 (11, 55)*
100 mM pilocarpine	37 (15,88)	69 (43,201)
5 mM PTX	12 (6, 15)	15 (10, 21)
15 mM PTZ	75 (49 , 100)	150 (93, 197)
50 µM parathion	394 (194,470)	59 (37, 136)*
25 µM carbaryl	3 (2, 13)	0 (0,1)

554 * significantly different from controls ($p<0.05$) using pairwise contrasts (with a Benjamini & Hochberg p-value
555 correction) of the estimated marginal means of a negative binomial generalized linear model of condition versus
556 number of pSLA events
557

558 Some compounds, including NMDA, pilocarpine, PTX, and PTZ induced similar amounts of
559 pSLA in the two developmental stages with overlapping interquartile ranges. Nicotine and
560 parathion showed significantly more active behaviors - evidenced by higher numbers of pSLA
561 - in adult DJ planarians than in tails, suggesting that the presence of the brain plays a role for

pSLA induction in DJ planarians. Interestingly, pH 3 showed significantly greater activity in the regenerating tails than in adults. Thus, in DJ planarians, while the seizurogenic compounds do not strictly require the planarian brain to induce pSLA, presence of the brain can affect pSLA dynamics depending on the compound. Carbaryl was at the detection threshold and thus a comparison may not be meaningful.

4. Discussion

4.1. MI analysis can classify seizurogenic compounds and distinguish pSLA from other behaviors

To overcome the limitations associated with manual scoring of pSLA, namely scoring bias and time effort, we created an automated MI analysis method that uses image subtraction to detect changes in body shape. Using frame subtraction to quantify activity is a commonly used method to quantify behaviors including seizures in other model systems such as zebrafish (Afrikanova et al., 2013; Bruni et al., 2016; Whyte-Fagundes et al., 2025). By using qualitative scoring of NMDA-induced pSLA as a framework, we derived a quantitative MI threshold for pSLA in DD and DJ planarians. This analysis method was able to correctly identify known seizurogenic chemicals in planarians (NMDA, nicotine, PTX, pilocarpine) and distinguish pSLA from other abnormal behaviors, such as forward scrunching, which – in contrast to pSLA – exhibits periodic changes in body shape (Cochet-Escartin et al., 2015). Complementary analysis of the translational worm activity as a function of time helped us to distinguish normal behavior from contraction and paralysis, which both show low MI scores.

With the aim of providing a simple metric for easy identification of potential seizurogenic compounds, we quantified the number of pSLA events over the 30 minutes of recording. This recording length is longer than many previous planarian seizure studies, which primarily evaluated the first 5-10 minutes after chemical exposure. We increased this testing window to provide a better opportunity to identify seizurogenic compounds without the need for precise concentration tuning, while still being short enough to allow for medium-throughput screening. However, this longer recording time also potentially decreased our detection power for transient, short-lived behaviors that did not have time to accumulate many pSLA events, as was the case for DD planarians exposed to nicotine. Thus, in some cases, looking at the number of pSLA as a function of time may be necessary to identify weaker effects.

One limitation of MI analysis is that it has difficulty to detect pSLA when the convulsive/twitching behavior is small and/or primarily in the third dimension (e.g., in the case of carbaryl), which is not well captured in the simple 2D imaging setup we used. While these behaviors could still be detected as pSLA in DJ planarians, they were not found to be significant for DD planarians because DD planarians showed more variable control behavior, including swimming, scrunching, and turning over. This variability in DD behavior is likely due to them having been obtained commercially and not having been raised for generations under controlled laboratory conditions.

4.2. MI analysis confirms that known seizurogenic chemicals identified by qualitative studies induce pSLA in planarians

We confirmed previous studies by other research groups that NMDA (Rawls et al., 2009), nicotine (Rawls et al., 2011), and pilocarpine (Miller et al., 2025) induce pSLA in DD planarians and showed that they also induce pSLA in DJ planarians. PTX had previously only been studied in DT planarians (Ramakrishnan and DeSaer, 2011); here, we show that it also induces pSLA in DD and DJ planarians. The concentrations of NMDA, pilocarpine, and PTX that induced pSLA in DD planarians here were comparable to previous studies using DD or DT planarians.

However, we found that 3 mM NMDA lowered pH to a range where pH alone caused pSLA while previous work found that 3 mM NMDA did not lower the pH as much (pH 6.6), and thus pH alone did not induce pSLA (Rawls et al., 2009). The discrepancy in these data may be due to differences in the exact water composition as the previous study used Artificial Pond Water (APW; 6 mM NaCl; 0.1 mM $NaHCO_3$; 0.6 mM $CaCl_2$; pH 7.3) (Rawls et al., 2009) which has slightly different salt composition compared to our planarian water (see Methods Section 2.1) and thus may have different buffering capacity.

For nicotine, we found that 50 µM caused paralysis in DD planarians, comparable to concentrations used in DT planarians (Ramakrishnan and DeSaer, 2011). In contrast, other studies in DD planarians have used nicotine in the mM range (Bach et al., 2016; Pagán et al., 2015, 2013; Rawls et al., 2011). The difference is likely be due to the product used. While we and (Ramakrishnan and DeSaer, 2011) used pure (-) nicotine solution, the other studies (Bach et al., 2016; Pagán et al., 2015, 2013; Rawls et al., 2011) used nicotine hydrogen tartrate salt.

629 ***4.3 PTZ and cholinesterase inhibitors as pSLA inducers***

630 We showed that PTZ, which is used to generate mice (Dhir, 2012; Shimada and Yamagata,
631 2018) and zebrafish (Afrikanova et al., 2013; Baraban et al., 2005; Chitolina et al., 2023; Milder
632 et al., 2022) seizure models, robustly induces pSLA in both planarian species. Thus, PTZ could
633 be used to generate a planarian seizure-like model to test the efficacy of possible anti-seizure
634 treatments. In fact, existing studies that used other compounds than PTZ suggest that pSLA in
635 planarians can be modulated by exposure to known anti-convulsant therapeutics (e.g., (Pagán
636 et al., 2015; Ramakrishnan and DeSaer, 2011; Rawls et al., 2009)). Now that we have
637 developed this automated platform, it can be used to validate existing manual studies on
638 therapeutic effectiveness. In addition, RNA interference (RNAi) could be used to verify that
639 these known seizurogenic chemicals induce pSLA through the same pathways as in mammals.
640 For example, PTX and PTZ both target the $GABA_A$ receptor, thus making it a good starting
641 point for mechanistic studies. Parathion, an organophosphorus pesticide, induced pSLA in both
642 planarian species, and carbaryl, a carbamate, caused pSLA in DJ planarians. Both compounds
643 inhibit planarian cholinesterases (Hagstrom et al., 2017; Ireland et al., 2022b); thus, RNAi
644 knockdown of the planarian cholinesterases could be used to verify that the molecular initiation
645 event of pSLA in planarians is cholinesterase inhibition, as in humans (Jett, 2012).
646
647 Interestingly, parathion and nicotine both showed significantly more pSLA events in adult
648 (intact) DJ planarians than in decapitated ones. Thus, the brain is not required for pSLA induced
649 by these compounds, as expected because cholinesterases (Hagstrom et al., 2018) and nicotinic
650 receptors (Pagán et al., 2013) are found throughout the planarian body, but is important for
651 inducing and potentially modulating pSLA. It was previously shown in DT planarians that
652 decapitation decreased the number of pSLA events in nicotine, though the effect was not
653 significant (Pagán et al., 2013). The difference between this and our study may be due to species
654 differences, different nicotine formulations as mentioned above, or analysis methods.
655
656 Finally, it is also still necessary to confirm that pSLA is indicative of seizures using measures
657 of neuronal activity. A recent study has shown that using surface wire electrodes one can
658 measure EEG activity non-invasively in planarians (Freiberg et al., 2023). Alternatively,
659 microelectrode arrays have been successfully used to measure neuronal activity *in vivo* in
660 zebrafish larvae (Tomasello and Sive, 2020) and could be adapted for planarians. These
661 verification studies will be critical for adoption of the planarian model for screening

662 seizurogenic chemicals as they would show that results found in planarians are relevant and
663 translatable to mammals and humans.
664
665 ***4.4 DD and DJ planarians respond similarly to chemicals but with different sensitivities***
666 DD and DJ planarians displayed very different sensitivities to NMDA, nicotine, and
667 pilocarpine. DD planarians were more sensitive, with the most extreme difference in sensitivity
668 of nearly 100-fold for nicotine. While NMDA and pilocarpine induced similar behavioral
669 phenotypes in the two species, there were also qualitative differences in the behaviors displayed
670 by the two species in response to nicotine as DJ planarians exhibited extensive pSLA events
671 that were never observed in DD planarians. Other studies have reported similar behavioral and
672 sensitivity differences between planarian species in response to certain chemicals (e.g., (Ireland
673 et al., 2020, p. 20; Sabry et al., 2019)). Together, these data emphasize that extrapolation
674 between planarian species is not recommended due to possible differences in chemical uptake,
675 metabolism and/or presence of targets. We also showed that DJ planarians are more
676 behaviorally homogeneous than DD planarians, which may be due to having been bred under
677 controlled laboratory conditions with standardized food for years whereas DD planarians had
678 only been maintained under these conditions for a few weeks in our study. However, the
679 increased sensitivity of DD planarians to some of the compounds tested may make them a
680 better model for screening seizurogenic chemicals. More compounds will need to be screened
681 in both species to evaluate their strengths and weaknesses for this application. A good starting
682 point would be to test chemicals that have been proposed to induce pSLA in planarians in recent
683 manual studies, such as sertraline (Thumé and Frizzo, 2017) and various cholinesterase
684 inhibitors (Bezerra Da Silva et al., 2016), which were not included here.
685
686 ***4.5 Use of planarians for screening seizurogenic chemicals and therapeutics***
687 Our results demonstrate that these two planarian species are promising models for rapid
688 screening of chemicals for seizurogenic potential. While planarians are simpler than zebrafish
689 and lack a circulatory system and blood-brain barrier (Rawls et al., 2010), they have multiple
690 advantages for early screening. They are less expensive than zebrafish, which as vertebrates
691 require animal welfare protocols for husbandry that are not necessary for invertebrates such as
692 planarians (Collins et al., 2024). While zebrafish larvae at 5 days post fertilization (dpf) are
693 primarily used for screening because at this stage experiments are not considered animal testing
694 (Strähle et al., 2012), their nervous system and metabolic machinery are still maturing at this
695 time (Menezes et al., 2014; Saad et al., 2017; Tiedeken and Ramsdell, 2010). Thus, by

696 screening compounds in adult planarians with metabolic capacity (Ireland et al., 2022a) and
697 mature neuronal function first, in conjunction with *in silico* chemical modelling and/or human
698 *in vitro* MEA assays, we can reduce cost and vertebrate use in early seizure liability testing..
699 We have previously shown that organismal planarian and zebrafish behavioral screening and
700 MEA assays were the most informative to identify developmental neurotoxicants (Ireland et
701 al., 2025b) and thus may prove to be a promising combination for identify seizurogenic
702 chemicals and anti-seizure therapeutics.
703
704 As discussed in the Introduction, all models have limitations, some of which are unique and
705 some which are shared across models. The strengths and limitations of planarians and
706 developing zebrafish (<5 dpf) for chemical screening have recently been reviewed (Collins et
707 al., 2024). One limitation shared between these organismal models and cell culture models is
708 the requirement for aqueous chemical exposure. Thus, it is challenging to test poorly water
709 soluble or volatile compounds in these systems (Collins et al., 2024) and sufficiently high
710 concentrations to induce pSLA may not be possible, as evidenced by our ability to only test
711 PTX at its seizure induction threshold. *In silico* modelling can help overcome some
712 experimental limitations, as has been shown recently for screening anti-seizure drugs in
713 zebrafish larvae (Belair et al., 2025; Cassar et al., 2017). By performing comparative studies
714 of compounds known to be seizurogenic in humans, we can determine the complementarity of
715 these rapid screening seizure models for a potential seizure liability test battery and identify
716 current data gaps and limitations, similar to current efforts in developmental neurotoxicity
717 testing (Ireland et al., 2025b; Masjosthusmann et al., 2020).
718
719 The work here can act as a starting point for future screens of seizurogenic chemicals and anti-
720 convulsants in planarians but further optimization and system validation are required to decide
721 whether planarian screening could be a valuable part of an early stage seizure liability testing
722 battery. In addition to the mechanistic studies mentioned earlier that are needed to confirm
723 molecular targets and distinguish seizures from convulsions, more compounds that have been
724 studied in cell culture and zebrafish need to be evaluated in planarians to compare system
725 sensitivity and specificity (Belair et al., 2025). To accelerate system evaluation and have
726 multiple research groups participate in characterizing the value of planarian behavioral
727 screening, the recording and analysis methods developed here are made available to other
728 laboratories. This will ensure standardized conditions that allow cross-laboratory data
729 comparisons and integration.

While the column setup that we used here was helpful for testing a few selected compounds, for new screens, we recommend a row setup, similar to what is used for rapid behavioral screening in other studies (Ireland et al., 2025a; Zhang et al., 2019), because it allows for the simultaneous testing of 5 concentrations/conditions plus a vehicle control. By using 2 plates with alternating row setup, one can control for potential edge effects (Zhang et al., 2019) and achieve n=16 per condition. There is a trade-off between screening throughput and reliability of hit calls: fewer wells per condition increase screening speed while more wells increase screen robustness and statistical power. In zebrafish, n=6 wells/condition have been used to screen chemicals for potential anti-seizure drugs (Dinday and Baraban, 2015). Here, we used a minimum of n=12 specimen per condition because of the observed variability in DD planarians, which requires larger sample sizes to have more statistical power. In terms of timing, we used 30 min to allow for throughput while simultaneously being able to detect late-onset pSLA (as in PTX). The recording period could be shortened to 5-10 min to achieve higher throughput as most chemicals induced pSLA within the first 5 min. It's also possible that effects may wear off, as for example in the case of nicotine for DD planarians, and thus shorter incubation would be helpful here. But we also showed that additional analysis incorporating the temporal dynamics can reveal such short-term effects. Our recordings began 2-6 min following chemical exposure, which was sufficient for the compounds we studied (Supplemental Figure S11), but this window could be decreased to increase the possibility of catching very short-lasting pSLA events. In addition to acute behavioral recordings, we also evaluated lethality one day after chemical exposure to determine if lethality was correlated with the presence of significant pSLA. This was not the case. Thus, lethality likely results from other toxic effects besides pSLA and thus is not an informative endpoint for this type of study.

Future work could also incorporate more sophisticated image analysis methods, incorporating machine learning, to identify specific body shape changes and use them to characterize pSLA, as has recently been done in zebrafish (Whyte-Fagundes et al., 2025). Such a tailored approach would likely be more robust than MI analysis and may reveal subtypes of pSLA, which could provide additional insight into the underlying biological mechanisms. For example, the initial behavioral effects of parathion are thought to be due to acetylcholinesterase inhibition while later effects are from secondary neurotransmitter systems such as glutamate (Jett, 2012; Kozhemyakin et al., 2010); thus, the type of convulsions may differ over time.

764 In summary, this work demonstrates that pSLA can be triggered through various pathways in
765 DD and DJ planarians, similar to in mammals. By standardizing both experimental approach
766 and analysis methods and making the tools available to the research community, this work can
767 serve as a framework for future studies of chemicals for their seizurogenic potential in
768 planarians.
769
770
771
772 **CRediT authorship contribution statement**
773 **Danielle Ireland:** Conceptualization; Software; Visualization; Validation; Formal analysis;
774 Data curation; Writing – original draft; Writing - review and editing. **Evangeline Coffinas:**
775 Investigation; Writing – review and editing. **Christina Rabeler:** Investigation; Writing –
776 review and editing. **Eva-Maria S. Collins:** Conceptualization; Software; Methodology;
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790 **Conflict of interest statement**
791 EMSC is the founder of Inveritek, LLC, which offers planarian HTS commercially. EMSC and
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795
796 **Appendix A. Supporting information**
797 Supplementary data associated with this article can be found in the online version at XXX.
798

Data availability

The datasets supporting the conclusions of this article are included within the article and available from the corresponding author upon reasonable request.

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