

Measuring functional brain recovery in regenerating planarians by assessing the behavioral response to the cholinergic compound cytisine

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ABSTRACT Planarians are traditional model invertebrates in regeneration and developmental biology research that also display a variety of quantifiable behaviors useful to screen for pharmacologically active compounds. One such behavior is the expression of seizure-like movements (pSLMs) induced by a variety of substances. Previous work from our laboratory showed that cocaine, but not nicotine, induced pSLMs in intact but not decapitated planarians. Interestingly, as decapitated planarians regenerated their heads, they gradually recovered their sensitivity to cocaine. These results suggested a method to assess planarian brain regeneration and a possible way of identifying compounds that could enhance or hold back brain regeneration. In the present work, we demonstrate that the cholinergic agent cytisine is a suitable reference compound to apply our method. Cytisine induces pSLMs in a concentration-dependent manner in intact (but not decapitated) planarians of the species *Girardia tigrina*. Based on our data, we developed a behavioral protocol to assess planarian brain regeneration over time. We tested this method to measure the effect of ethanol on *G. tigrina*'s brain regeneration. We found that ethanol slows down the rate of planarian brain regeneration in a concentration-dependent manner, consistently with data from other research groups that tested ethanol effects on planarian brain regeneration using different behavioral protocols. Thus, here we establish a general method using cytisine-induced pSLMs as an indicator of brain regeneration in planarians, a method that shows potential for assessing the effect of pharmacologically active compounds in this process.

KEY WORDS: *brain, regeneration, pharmacology, cytisine, planaria*

Introduction

Several species of freshwater planarians are established model invertebrates in regeneration and developmental biology research (Cebrià *et al.*, 2002, 2018, 2019; Ivankovic *et al.*, 2019; Gentile *et al.*, 2011; Li *et al.*, 2015). One of the most distinct advantages of the planarian model system is that many planarians species display a remarkable ability shared by very few other organisms: the capacity for complete (and correct) regeneration of their nervous system upon injury or even full decapitation (Cebrià, 2007, 2008, 2019; Gentile *et al.*, 2011; Owlarn and Bartscherer, 2016; Ross *et al.*, 2017; Umesono *et al.*, 2011). The nervous system in these organisms is controlled by a *bona fide* brain that displays many

features in common with vertebrate brains. For example, genes related to the planarian central nervous system are highly conserved across phyla (Cebrià *et al.*, 2002a,b; Mineta, *et al.*, 2003; Umesono and Agata, 2009), and planarian neurons are similar to vertebrate nerve cells in terms of morphology and other cytological characteristics (Pagán, 2014; Sarnat and Netsky, 1985, 2002). Additionally, many flatworm species possess many well-characterized neurotransmitter systems (Buttarelli *et al.*, 2008; Nakazawa *et al.*, 2003; Ribeiro *et al.*, 2005). These facts established the usefulness of the freshwater planarian as an animal model for the study of

Abbreviations used in this paper: APW, artificial pond water; pSLM, planarian seizure-like movement.

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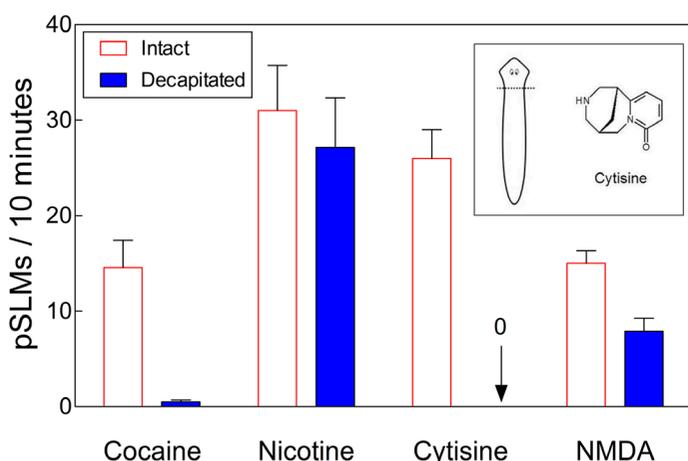


Fig. 1. Compounds capable of inducing at least 15 seizure-like movements (pSLM)/10 minutes in intact or decapitated planarians at a concentration of 1 mM, as indicated. *N* = 6-19 worms per bar (see text and Table 1). The error bars represent the standard error of the mean. Inset: Outline of a planarian showing the point of decapitation (dotted line); drawing courtesy of Mr. Alexis G. Pagán, and cytosine structure.

nervous systems in general (Pagán, 2014, 2017, 2019; Pellettieri, 2019; Roberts-Galbraith et al., 2015, 2016; Ross et al., 2017; Umesono et al., 2011).

The contributions of planarians to biological research are not limited to the neurosciences and to developmental/regeneration biology. Since the mid-1970s planarians are proving themselves as relevant animal models in pharmacology and toxicology, on account of the aforementioned neurochemical similarities with vertebrates (Hagstrom et al., 2015, 2016, 2019; Ireland et al., 2020; Pagán, 2014, 2017, 2019; Poirier et al., 2019; Raffa and Rawls, 2008; Wu and Li, 2018; Zhang et al., 2019). Another reason for their usefulness in this regard is that planarians display a diverse

repertoire of drug-induced quantifiable behaviors amenable to pharmacological manipulation. One of these behaviors is the planarian seizure like movement (pSLM). This behavioral response has also been referred to as a C-like hyperkinesia (Palladini et al., 1996; Pagán et al., 2008) and is expressed as a series of sudden contractions/twitches of the anterior section while the tail is usually anchored to the bottom of the container, as opposed to the passive gliding that these animals normally display (please see Supplementary Video 1). pSLMs are easily quantified visually, are concentration-dependent, and have been used to document the behavioral effects of a variety of psychoactive agents, including cocaine, nicotine, and opiates, among other compounds (Bach et al., 2016; Bezerra da Silva et al., 2016; Pagán et al., 2008, 2012, 2013, 2015; Raffa and Rawls, 2008; Ramakrishnan and Desaeer, 2011; Ramakrishnan et al., 2013; Ramoz et al., 2012, Rawls et al., 2009, 2010, 2011; Tallarida et al., 2014).

Previous work from our laboratory showed that nicotine is able to induce pSLMs in intact or decapitated planarians. In contrast, cocaine-induced pSLMs are only expressed in intact planarians; if a planarian brain is surgically disrupted in any way, the ability of cocaine to induce pSLMs is significantly reduced, or upon decapitation, completely eliminated (Pagán et al., 2013). Furthermore, we also showed that decapitated planarians gradually recovered their sensitivity to cocaine as their central nervous system regenerated (Pagán et al., 2013). This recovery of cocaine sensitivity correlated closely with anatomical observations of the timeline of planarian brain regeneration using histochemical methods (Cebrià, 2007; Fraguas et al., 2012). Our results suggested a general method of behaviorally assessing the regeneration of the planarian brain, and also suggested a way to identify substances capable of delaying or enhancing planarian brain regeneration. Cocaine, being a controlled substance, is an impractical reference compound in this regard. Therefore, we performed a limited screening of various substances capable of inducing pSLMs only in planarians with intact brains (Table 1). Out of 19 substances tested, we identified

TABLE 1

SCREEN OF COMPOUNDS SELECTED FOR THEIR ABILITY TO INDUCE PLANARIAN SEIZURE-LIKE MOVEMENTS (p-SLM) AT A CONCENTRATION OF 1 mM IN INTACT OR DECAPITATED PLANARIANS

Compound	Main pharmacological activity	pSLM/10 minutes induced by 1 mM (± SEM) - Intact planarians	pSLM/10 minutes induced by 1 mM (± SEM) - Decapitated planarians	Reference
Cocaine	Dopamine transporter antagonist / Local anesthetic	14.6 ± 2.8; N = 12	0.5 ± 0.2; N = 8	This work
Nicotine	Cholinergic agonist	31.0 ± 4.7; N = 7	27.1 ± 5.2; N = 7	This work
Cytisine	Cholinergic partial agonist	26.0 ± 3.0; N = 12	0 ± 0; N = 6	This work
N-methyl-D-aspartic acid	Glutamatergic agonist	15.0 ± 1.3; N = 19	7.9 ± 1.4; N = 19	This work
Amphetamine	Dopamine transporter antagonist	1.4 ± 0.5; N = 8	-	Pagán et al., 2012
Dopamine	Dopaminergic agonist	4.0 ± 2.0; N = 13	-	This work
Lidocaine	Local anesthetic	3.9 ± 2.0; N = 5	-	Pagán et al., 2013
Procaine	Local anesthetic	8.8 ± 0.9; N = 6	-	This work
Procainamide	Antiarrhythmic agent / Local anesthetic	6.4 ± 2.1; N = 9	-	This work
Carbamylcholine	Cholinergic agonist	5.8 ± 3.3; N = 4	-	This work
Dihydro-beta-erythroidine	Cholinergic antagonist	4.8 ± 1.4; N = 4	-	This work
Cotinine	Cholinergic antagonist	0.3 ± 0.3; N = 4	-	Bach et al., 2016
d-Tubocurarine	Cholinergic antagonist	7.5 ± 1.5; N = 8	-	Pagán et al., 2015
L-glutamic acid	Glutamatergic agonist / Natural amino acid	11.8 ± 1.5; N = 10	-	Pagán et al., 2012
D-glutamic acid	Glutamatergic agonist / Natural amino acid	10.9 ± 1.9; N = 10	-	Pagán et al., 2012
Serotonin	Serotonergic agonist	1.0 ± 0.6; N = 3	-	This work
Caffeine	Adenosine receptor antagonist	0.5 ± 0.5; N = 2	-	This work
Picrotoxin	GABA antagonist	2.3 ± 2.2; N = 4	-	This work
L-alanine	Natural amino acid	3.7 ± 1.2; N = 3	-	This work

cytisine, a cholinergic compound (Fig. 1), as a concentration-dependent pSLMs inducer in intact—but not decapitated—planarians. Based on our data, we developed a behavioral method to assess planarian brain regeneration as described above. We validated this method by measuring the effect of ethanol on the planarian *Girardia tigrina*'s brain regeneration.

Results

Candidate compounds to study pSLMs in planarians

Table 1 shows a series of compounds of various pharmacological specificities, that we tested at a concentration of 1 mM for their ability to induce pSLMs, as we reasoned that this is a physiologically-relevant concentration. We had two main criteria for selecting a specific candidate compound for further study: (1) that at 1 mM concentration the candidate chemical would be capable of inducing at least 15 pSLMs in ten minutes in intact planarians, just like cocaine did (as reported in Pagán *et al.*, 2013), and (2) that said candidate compound at the same concentration (1 mM) would be unable to induce pSLMs in decapitated planarians. We found four candidate compounds that fit the first criterion: cocaine, nicotine, cytisine (a cholinergic agonist), and NMDA (N-methyl-D-aspartic acid), a glutamatergic agonist (Table 1, Fig. 1). In the cases of cocaine and nicotine, our results replicated our previous findings (Pagán *et al.*, 2013). Of the two previously untested compounds, only cytisine fulfilled the second criterion, as decapitation completely abolished its pSLM-inducing ability, while decapitation decreased the NMDA-induced pSLMs by roughly 50 % (Table 1, Fig. 1). Thus, we chose cytisine as our candidate compound to assess planarian brain regeneration. In this work, we used cytisine to measure the delay of the reacquisition of behavioral responses by ethanol in regenerating worms. Ethanol has been found to delay the recovery of locomotor behavior in decapitated planarians of the species *Schmidtea mediterranea* (Lowe *et al.*, 2015).

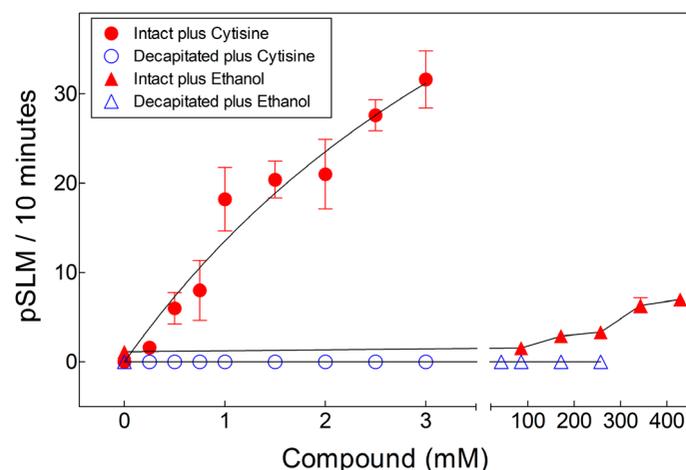


Fig. 2. Cytisine and ethanol induced seizure-like movements in intact (but not decapitated) planarians in a concentration-dependent manner, as indicated. $N = 4-8$ worms per symbol. The error bars represent the standard error of the mean. Whenever error bars are not showing, it means that they are smaller than the symbol size.

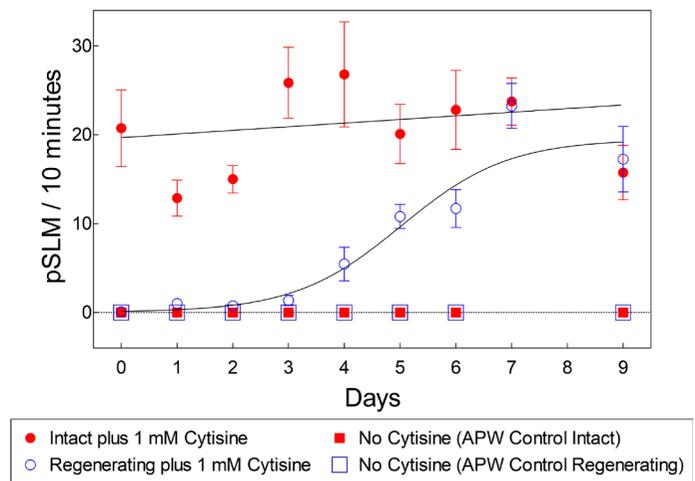


Fig. 3. Cytisine (1 mM)-induced seizure-like movements in intact and regenerating planarians over a period of nine days. $N = 4-11$ worms per symbol for the cytisine-exposed planarians, $N = 4$ for controls (APW-exposed planarians). Intact planarians exposed to cytisine displayed a constant response independent of the day of the experiment (Slope not different from 0; $p = 0.499$). The cytisine-exposed intact and regenerating planarian groups were significantly different from each other ($p < 0.001$ by 2-way ANOVA). Planarians gradually recovered their ability to react to cytisine as their heads regenerated. The error bars represent the standard error of the mean. Whenever error bars are not showing it means that they are smaller than the symbol size.

Cytisine, ethanol and pSLMs

Fig. 2 shows that both cytisine and ethanol induce seizure-like movements in intact but not decapitated planarians in a concentration-dependent manner. However, ethanol displayed significant lesser potency to induce pSLMs, since even at concentrations between 300-400 mM ethanol only induced about 6 ± 2 pSLMs over a period of ten minutes, in contrast to the 18 ± 8 pSLMs/10 minutes induced by our reference cytisine concentration (1 mM; $p = 0.002$, unpaired t-test). These 1 mM cytisine-induced pSLMs are not significantly different from the value for 1 mM cytisine shown in Fig. 1 (26.0 ± 3.0 pSLMs/10 minutes; $p = 0.160$; unpaired t-test). Based on this information, we chose a standard concentration of 1 mM cytisine to conduct the rest of our experiments.

Seizure-like movements in intact and regenerating planarians induced by 1 mM cytisine

Fig. 3 illustrates that intact planarians react to 1 mM cytisine in a constant manner over a period of nine days, as indicated by the slope of the line of pSLMs/10 minutes vs days, which was not different from 0 ($p = 0.499$, F-test); the average 1 mM cytisine-induced pSLMs per ten minutes from days 0 to 9 was 20.4 ± 1.7 , consistent with the values observed for 1 mM cytisine under the same conditions in Figs. 1 and 2. In contrast, regenerating planarians began displaying cytisine-induced pSLMs by day 4, which correlated closely with morphological observations of planarian brain regeneration obtained by a research group that worked on the planarian *Schmidtea mediterranea* (Fraguas *et al.*, 2012). In our study, regenerating planarians recovered their full sensitivity to cytisine treatment by day 7 (Fig. 3). Intact or decapitated worms exposed to artificial pond water (APW) alone did not display SLMs (Fig. 3, as indicated).

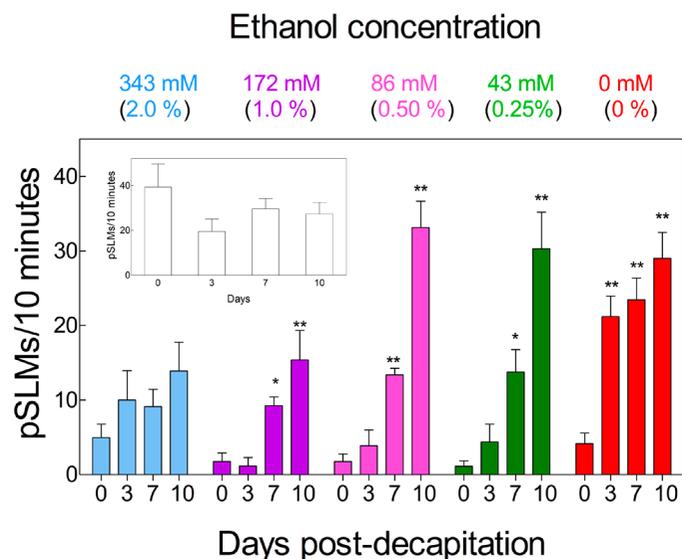


Fig. 4. Ethanol delays the onset of planarian brain regeneration in a concentration-dependent manner. $N = 7-13$ worms per bar. The error bars represent the standard error of the mean. Any symbols above the bars represent statistically significant p -values obtained through unpaired t -tests against day 0 within its own group (* $p < 0.01$; ** $p < 0.001$). Ethanol treatment significantly differs between groups and from the control group (0 mM ethanol; by Two way ANOVA, see Table 2). (Inset) Following an identical protocol as in the main figure, intact planarians exposed to 1 mM cytosine displayed a constant response independent of the day of the experiment. The average pSLM numbers for days 3, 7, and 10 were not significantly different from the pSLM values at day 0 by unpaired t -tests (p -values = 0.14, 0.51, and 0.53 respectively). $N = 9-13$ worms per bar. The error bars represent the standard error of the mean.

Ethanol delays the onset of regeneration in regenerating planarians in a concentration-dependent manner

As in Fig. 3, the inset in Fig. 4 shows that there is no difference in the response of intact planarians to 1 mM cytosine exposure as a function of days; the average 1 mM cytosine-induced pSLMs per ten minutes was 28.9 ± 4.1 , again, consistent with the values shown in Figs. 1, 2, and 3. In contrast, Fig. 4 shows that regenerating planarians exposed to various concentrations of ethanol displayed slower regeneration rates. Their regeneration rate was delayed proportionally to the ethanol concentration (two-way ANOVA, Table 2). In fact, the worms exposed to the two highest ethanol concentrations (343 and 172 mM; 2 and 1 % respectively) never recovered their full sensitivity to the exposure of 1 mM cytosine to control levels up to day 10 post-decapitation, while worms exposed to either 86 or 43 mM (0.5 and 0.25 % respectively) did recover their full sensitivity to cytosine exposure, even though their recovery was delayed compared to control worms (Fig. 4).

Discussion

Ethanol effects on planarian behavior

Ethanol has been reported to induce a variety of behavioral effects on several planarian species. One of the earliest published examples of these effects is immobilization, studied in the planarian *Schmidtea mediterranea*. In this planarian species, exposure to 3 % ethanol (approximately 500 mM) for 1 hour, immobilized planarians

for the purpose of performing live imaging studies (Stevenson and Beane, 2010). At this concentration and exposure time, ethanol did not show any effect on regeneration in *S. mediterranea* (Stevenson and Beane, 2010). Other reported effects of ethanol (at concentrations lower than 500 mM) on planarian behavior include decreased motility, as well as an increase in negative phototaxis behavior (Byrne, 2018; Ireland et al., 2020). In agreement with the aforementioned works, data from our laboratory indicate that ethanol slows down and eventually immobilizes the planarian *G. tigrina* in a concentration dependent manner (DeMichele, 2018).

Interestingly, in *S. mediterranea*, ethanol exposure induces the expression of a “drunken” phenotype, characterized by atypical gliding and “scrunching”; this latter behavior was found to be modulated by a specific potassium channel (Cochet-Escartin et al., 2016). In the planarian *Girardia dorocephala*, ethanol displayed environmental place conditioning, pSLMs, and pharmacological synergism with cocaine, but not with nicotine (Tallarida et al., 2014). In *G. dorocephala*, ethanol also seemed to induce anxiolytic-like effects (Zewde et al., 2018) and abstinence-induced withdrawal (Nayak et al., 2016).

Our work explored the effect of various ethanol concentrations using the recovery of sensitivity to cytosine exposure observed as the expression of pSLMs. We interpreted this recovery as an indicator of brain regeneration. Our results are consistent with results from another laboratory (Lowe et al., 2015) where 1 % ethanol delayed the reacquisition of negative phototropic behavior of decapitated *S. mediterranea*. In full agreement with our experiments, Lowe et al., 2015 reported that decapitated planarians not exposed to ethanol began to recover their negative phototropic behavior at day 4 post-decapitation while 1 % ethanol delayed the onset of the recovery of this behavior, indicating brain regeneration. In these experiments, ethanol-exposed planarians began to recover their negative phototropic behavior at day 6 on average.

Cytisine

Based on previous work from our laboratory (Pagán et al., 2013) we knew that nicotine was able to induce SLMs in decapitated planarians, while cocaine did not. We replicated these results in this work (please see Fig. 1). These results implied that the molecular targets for nicotine were distributed throughout the planarian body while the molecular targets of cocaine are specifically localized in the planarian head, likely at the level of the brain. Furthermore, previous results from our group (Pagán et al., 2013) showed that regenerating planarians gradually displayed cocaine-induced SLMs presumably as their brains were being reconstituted, suggesting a method to assess the planarian regenerative process by observing the recovery of SLMs behavior upon brain regeneration. We found

TABLE 2

P-VALUES (TWO-WAY ANOVA) COMPARISON BETWEEN ETHANOL TREATMENTS (FROM Fig. 4)

Comparison between groups					
Ethanol (mM)	0	343	172	86	43
0	-	0.013	0.011	0.0007	0.015
343	-	-	-	-	-
172	-	0.284	-	-	-
86	-	0.0001	0.0007	-	-
43	-	0.003	0.035	0.914	-

that the cholinergic compound cytisine, a neurotoxin isolated from the *Laburnum* plant, induced a robust pSLM response in intact planarians while showing no such effect in decapitated planarians (Fig. 1), suggesting that cytisine's receptors sites in planarians are located in the head region, most likely in the brain.

Interestingly, our data also suggests that there are at least two populations of NMDA receptor targets in *G. tigrina*; based on the approximately 50 % reduction of pSLMs in decapitated worms. Our hypotheses about the possible localization of the binding sites for cocaine, nicotine, cytisine, and NMDA in the planarian nervous system are consistent with an *operational receptor model*, which preliminarily assumes the existence of specific targets for a drug based solely on any observed physiological/behavioral effects in the absence of molecular data (Black and Leff, 1983, 1985, 2010; Leff *et al.*, 1985, 1990).

Cytisine has been used with a moderate level of success as a tobacco cessation therapy; its best described effect is as a partial agonist in neuronal nicotinic cholinergic receptors in the vertebrate brains (Beard *et al.*, 2016; Etter, 2008; Gomez-Coronado *et al.*, 2018; Walker *et al.*, 2016). In vertebrates, the location of neuronal cholinergic receptors is mostly limited to the central nervous system (Bertrand and Terry, 2018; Zoli *et al.*, 2015, 2018). This fact, combined with our results, is consistent with the idea that the planarian nervous system parallels vertebrate nervous systems (Pagán *et al.*, 2013; Pagán, 2014, 2017, 2019; Sarnat and Netsky 1985, 2002).

Fig. 2 shows that the induction of pSLMs by cytisine is concentration-dependent, further reinforcing the notion of a specific receptor site in intact planarians. Cytisine was unable to induce pSLMs in decapitated planarians up to a concentration of 3 mM (Fig. 2). In contrast, ethanol was able to induce pSLMs at much higher concentrations than cytisine. For example, ethanol induced about 6 pSLMs in ten minutes at concentrations between 300 and 400 mM in intact planarians; and like cytisine, no seizure-like movements were observed in decapitated worms (Fig. 2). It is unlikely that ethanol contributed to any observed pSLMs in our experiments, since all the worms used in our experiments were washed in 1 mL APW (see methods); this wash was discarded prior to exposing the worms with APW containing 1 mM cytisine.

The rate of brain regeneration that we observed was consistent with results from other research groups in various planarians species (Reviewed in Birkholz *et al.*, 2019; Brown and Pearson, 2017; Cebrià 2007; Cebrià *et al.*, 2018; Fraguas *et al.*, 2012; Inoue *et al.*, 2004; Ivankovic *et al.*, 2019; Ross *et al.*, 2017). Our experiments show that intact planarians, as expected, displayed a constant response to 1 mM cytisine exposure over a period of nine days, in contrast to decapitated planarians, which did not significantly respond to this cytisine treatment until day four, when they began to display pSLMs, indicating a gradual recovery of function, which was complete by day 9 (Fig. 3). The observed recovery in our experiments is consistent with morphological observations of the brain of different planarian species by other groups showing that the brain hemispheres began to recover their connectivity by approximately day four post-decapitation (Cebrià, 2007; Fraguas *et al.*, 2012).

In Fig. 4 we show that decapitated planarians exposed to ethanol display a delay in their recovery of cytisine sensitivity, which can be interpreted as a delay in the process of brain regeneration. Planarians exposed to 0.25 and 0.50 % ethanol recovered their

full brain functionality (as interpreted from the observed recovery of full sensitivity to 1 mM cytisine treatment), while planarians exposed to 1 or 2 % ethanol did not. Overall, these data suggests that our method has the potential to help identify substances influencing planarian brain regeneration based on the recovery of cytisine-induced pSLMs. Moreover, our method has the advantage that it can be easily modified to adapt it for various experimental needs. For example, instead of following regeneration over a ten day period, a specific day post-decapitation (say, 5 days) could be chosen as a reference endpoint to observe whether a specific substance delays or enhances regeneration by quantifying any pSLMs induced by 1 mM cytisine. This is only one of the possible modifications of our method that can be implemented.

Challenges and future work

Despite the potential usefulness of the cytisine method, there are certain disadvantages that must be addressed. For example, there is a distinct possibility that there are substances influencing planarian brain regeneration that also induce pSLMs by themselves, possibly confounding the interpretation of results or that antagonize cytisine's ability to induce pSLMs. The obvious compounds in this regard are other cholinergic agents, a possibility that we intend to explore through a systematic screening with cholinergic agents with different specificities, particularly cholinergic antagonists. In essence, we intend to perform a pharmacological dissection to look for cytisine antagonists, which may not be limited to cholinergic agents. For example, in vertebrate organisms the cholinergic and dopaminergic neurotransmitter systems are closely related. This relationship is being explored in the context of Parkinson's Disease (Rizzi and Tan, 2017). Interestingly, the study of acetylcholine/dopamine interaction has also been proposed in planarians (Buttarelli *et al.*, 2000; Carolei *et al.*, 1975). This possibility of cross-interaction of compounds that might confound any cytisine results can be easily prevented by changing the experimental design. For example, by exposing decapitated planarians to a candidate substance for say, three days, then removing the tested substance, allowing the planarians to regenerate normally for two days, and then test for cytisine-induced pSLMs. We are designing alternative protocols in our laboratory as suitable modifications of this method. Another possible limitation of our method is whether cytisine affects planarian regeneration by itself. We are also planning experiments to address this possibility. An exciting possible set of experiments that we intend to pursue is to explore any cytisine effects on planarian heads as opposed to their decapitated bodies; amputated planarian heads display significant motility that can be quantified (Please see Supplementary video 2); we can also follow their body regeneration in light of their sensitivity to cytisine and perform pharmacological dissection studies as outlined above. Additional future work in our laboratory will include immunohistochemical studies to assess and compare the extent of planarian brain reconstitution with the recovery of sensitivity to cytisine. Another interesting aspect of our results that can be explored is the nature of the putative distinct populations of NMDA receptors that we seem to have identified in *G. tigrina*.

The impressive regenerative abilities of planarians offer unique insights into the regain of the neuronal structural reorganization that is necessary to recover the expression of normal behavior after injury. Our results indicate that the recovery of cytisine sensitivity in regenerating planarians is an innovative, inexpensive, and use-

General Planarian Stock



Set aside 48 worms and decapitate them (Group 1)

Day 0: Record pSLMs induced by either APW (4 worms) or 1 mM cytosine (4 worms).

Set aside 48 worms and leave them intact (Group 2)

Day 0: Record pSLMs induced by either APW (4 worms) or 1 mM cytosine (4 worms).

Discard worms.
Go back to Groups 1 and 2.
Repeat for days 1, 2, 3, etc.

Fig. 5. Experimental setup used to assess the recovery of cytosine sensitivity upon head regeneration in decapitated worms (see text).

ful method to assess the effect of exogenous substances on the observed brain regeneration in this experimental organism. As a validation of our method, our results indicate that ethanol slows down the rate of planarian brain regeneration in a concentration-dependent manner, in full agreement with data from other research groups using alternate behavioral methods in a different planarian species (Lowe *et al.*, 2015). Therefore, here we establish cytosine-induced pSLMs as an indicator of brain regeneration in our experimental organism, providing a general method to assess the effect of exogenous compounds on this process. This method will likely contribute to the identification of substances capable of delaying, and perhaps even enhancing, planarian brain regeneration. Our work represents an additional tool that will likely prove useful on the integration of the fields of pharmacology, neuroscience, and regeneration/developmental biology, with planarians as a leading model organism.

Materials and Methods

Data analysis

All graphs and statistical procedures were done using the Prism software package (GraphPad Inc., La Jolla, CA, USA). Planarians (*Girardia tigrina*) were obtained from Ward's (Rochester, NY). The water in which the worms were shipped was replaced with artificial pond water (APW: NaCl, 6 mM; NaHCO₃, 0.1 mM; CaCl₂, 0.6 mM) immediately upon arrival, and the animals were allowed to acclimate to the laboratory conditions for about 24 hours before being used. All experiments were performed in APW at room temperature. Planarians between 1–1.5 cm were selected for the experiments. Cytosine was purchased from Tocris (Ellisville, MO). General laboratory materials and chemicals were purchased from Fisher Scientific (Suwanee, GA).

Induction of pSLMs

In all tests, the induction of pSLMs by cytosine was measured by exposing a worm to approximately 1 mL of APW, followed by the removal of

the APW (wash) and placing the worm inside the well of a ceramic plate (Supplementary Video 1). Then the relevant cytosine concentration was added to the well and any pSLMs were visually counted for a period of ten minutes. For an illustration of what pSLMs look like, please refer to Supplementary Video 1. All experiments were performed by at least two observers, and after counting, the worms were humanely euthanized and discarded by adding 2% hydrochloric acid to the experimental solution.

Candidate compounds to study pSLMs in planarians

We chose substances that at a concentration of 1 mM induced at least 15 pSLMs in intact planarians over a ten-minute period, following our previous results using cocaine as a reference compound (Pagán *et al.*, 2013). Upon finding such a compound, we exposed decapitated planarians to 1 mM of the candidate compound. If the planarian body did not react to this exposure, we chose the compound for further study.

Recovery of behavioral function upon brain regeneration

This procedure was first described in Pagán *et al.*, (2013), and summarized in Fig. 5. Briefly, two sets of planarians, 48 worms each, were placed in separate containers in APW. One of the groups was designated “Planarian Stock 1” (PS1), and each planarian was decapitated as shown in Fig. 2. The second group, “Planarian Stock 2” (PS2) was left intact. The APW was changed every day. Starting at the decapitation date (Day 0) eight planarians of each set were exposed to either APW or 1 mM cytosine and counted as described above. All experiments were done at room temperature and the worms were not fed at any point during the experiments. The procedure was repeated in subsequent days by going back to the PS1 and PS2 as described in Fig. 5 as well as in the “Results and Discussion” section. In this way, the gradual recovery of the pSLM response was observed as a function of days after decapitation; this is the method by which we obtained the data to construct Fig. 3. After counting, the worms were euthanized and discarded as described above.

Effect of ethanol on the recovery of cytosine sensitivity of regenerating planarians

Five sets of four 12-well polypropylene plates were washed with APW twice and the wash discarded. Each set was assigned to contain control worms (exposed to APW) or experimental worms (exposed to 0.25, 0.50, 1.0, or 2.0 % ethanol in APW). Each well contained only one decapitated planarian. Each of the four plates represented the days of exposure (0, 3, 7, or 10 days). In each of the designated days, the appropriate plate was set aside, and each individual worm was tested for the expression of pSLMs induced by 1 mM cytosine as described in the “Induction of pSLMs” section above. The data was processed and graphed as described (Fig. 4).

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