Ion Channel and Pump Function in Planarian Regeneration

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Abstract

Regeneration is the process in which lost tissues are recovered through differentiation of stem cells kept in storage. A pharmacological screen in the organism planaria identified several channels as requisite for normal regeneration. Inhibition of *eag* potassium channels was shown to disrupt the initiation of regeneration, whereas inhibition of the H^+/K^+-ATPase pump and the minK voltage-dependent potassium channel caused eye defects in regenerates. Together, these results suggest an important role for potassium signalling and potassium-induced electrical potentials in regeneration.

1 Introduction

Complex structures are generated from fertilized eggs during the process of animal development. Key events, such as establishment of the left-right body axis, hinge on genetic cascades which commit cells to certain fates and determine their temporal and spatial position. Regeneration is a process similar to development in which lost tissues are replaced by the differentiation of stem cells maintained in the adult. It may utilize the same molecular mechanisms involved in embryonic development. Regeneration is well-documented in flatworms, earthworms, and frogs but has also been shown to occur in humans in limited cases [2]. Research into regeneration in model organisms would provide invaluable information for stem cell therapeutics, which aims to prompt regeneration from either pluripotent adult cells or totipotent stem cells. However, much has to be learned about the events upstream of the genetic cascades.

Electrical polarity on the organismic level is known to be present during regeneration and development [2]. Cells as diverse as amphibian neural crest cells [22], fish keratocytes [23], bone cells [24], and cornea epithelial cells [21] have been shown to respond to small electrical fields of the mV/mm order. Studies as early as 1979 on frogs with inserted subdermal shunts have shown that an endogenous electric field is necessary for proper regeneration [5]. Reversal of the endogenous electrical field also causes a reversal in morphology during regeneration [6]. Reports of corneal cells cleaving perpendicular to the electric field direction are especially intriguing as they show that some developmental mechanisms are responsive to, and perhaps dependent on, electrical fields.

Such an electrical polarity arises from assymetric distributions of ions and also possesses field properties carrying positional and directional information. Ion fluxes, or electrical currents, are requisite to maintain this polarity and were first observed by Jaffe and Nuccitelli in 1977 [19]. It was hypothesized by Morgan as early as 1905 that gradients of diffusible

determinants guide proper execution of body plan by specifying spatial information [7]. However, the discovery of homeobox genes; elucidation of morphogens such as BMP; and recent reports of developmental dependency on ion pumps and gap junctions in model organisms such as *Drosophila* have increased the complexity of the basic model [2, 8, 9]. More recent reports utilizing molecular biology techniques have begun to elucidate the crucial upstream role of electrical potentials in directing proper developmental patterning by influencing the complex actions of differentiation, migration, and intercellular attaching [2, 3].

Ion gradients may induce assymetric gene expression through several possible mechanisms. First, the ion concentration or the resultant electrical field may act as the carrier of positional information. Second, ion gradients may electrostatically attract charged morphogens such as calcium or inositol triphosphate through gap junctions. Lastly, ion assymetries could activate voltage-gated gap junctions and allow intercellular communication to take place [27]. Whatever the initiating factor, the resultant morphogens then initiate a cascade that eventually results in differential gene expression. All of these steps require intensive intercellular communication and coordination. Therefore, we wish to test the hypothesis that ion pumps, ion channels, and gap junction channels play critical roles in regeneration.

Unfortunately, classical models of development such as *Drosophila* and *C. elegans* either have limited regenerative capabilities or lack them altogether [10]. A better model for extensive studies of regeneration would be planaria. Planaria, free-living flatworms, are one of the simplest animals that exhibits bilateral symmetry and cephalization and the weight ratio of brain to body is similar to that of a rat [28]. Due to these attributes, planarian research would elucidate many mechanisms that should be conserved in higher organisms. Planaria are well suited for detailed molecular studies of regeneration as they are easy to maintain and normally regenerate within one week after being cut. Although frogs are commonly used for regeneration studies, they require more than a month to regenerate, which is in stark contrast with the planaria's ability to regenerate within a week [10]. Additionally, planaria

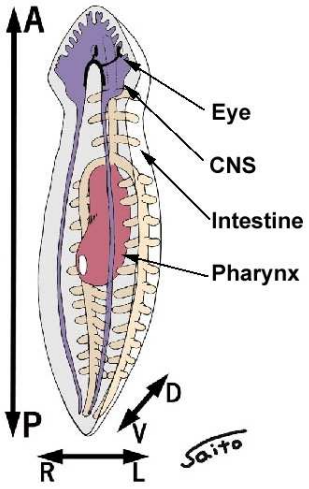


Figure 1: Planaria possess a simple central nervous system (CNS), digestive system, and cephalization. The double-sided arrows indicate the anterior-posterior, dorsal-ventral, and right-left axes. Image courtesy of Levin, M.

are amenable to many modern techniques such as RNAi, fluorescent imaging, and *in situ* hybridization. Regeneration may be artificially induced via cutting. After cutting, wound closure starts immediately and completes within hours. By the second day after cutting or fissioning, blastemas (structures where stem cells differentiate) can be seen and eyes appear on regenerating trunk segments as early as the third day.

Previously, a pharmacologic screen successfully identified H^+/K^+-ATPase as being critical for asymmetric electric potentials upstream of left-right patterning in *Xenopus* embryos [3]. Using this technique, we wish to determine which pumps are crucial to regeneration and also to validate the necessity of gap junction channels in regeneration. The pharmacologic screen enabled the exposure of large numbers of regenerating planaria to a wide variety of specific pharmacologic agents, and the observation of results within two weeks or less. Additionally, the planarian regeneration process is very robust, and thus is suitable for exposure to drugs that are oftentimes toxic at high concentrations. These combined factors enable planarian pharmalogical screens to achieve medium throughput at a relatively low

cost. Each of the chosen drugs acted specifically on one of the following: V-ATPase pumps, KvLQT channels, Kv channels, *eag* channels, K_{ATP} channels, H^+-ATPase pumps, I_{Ks} channels, H^+/K^+-ATPase pumps, ductin subunits of H^+-ATPase, or gap junction channels.

Target	Drug
V-ATPase	Skelid
	NeM
	SB-242784
KvLQT	HMR-1556
K_v	THB
eag	DMT
K_{ATP}	PHM
K_{ATP} opener	Diazoxide
	Pinacidil
I_{Ks} activator	DIDS
H^+/K^+-ATPase	PG
Ductin	DCCD
Gap Junctions	Glyc. Acid
	Lindane
	Arach. Acid

Figure 2: A brief list of the drugs used and their targets, for a more detailed list including the full names of the drugs and concentrations used see Appendix A.

2 Methodology

2.1 Planarian Care

Isogenic *Dugesia japonica* planaria derived from a single worm were used for this screen. The planaria were kept in 20cm×12cm×6cm plastic containers at 22°C in spring water (Poland Spring) and fed organic chicken liver twice a week. The planaria were allowed to feed for three hours, after which excess liver was removed and the water then exchanged for fresh spring water.

Figure 3: Planaria were cut into head (left), trunk (middle), and tail (right) segments to initiate regeneration.

2.2 Drug Treatment and Scoring

A broad range of drugs was used in this screen to determine which ion channels and pumps were necessary for regeneration. Based on an earlier, preliminary screen, pharmacological agents were chosen to target H^+ and K^+ pumps and channels. Many of the drugs used in this screening were previously developed for and successfully characterized in neuroscience studies. The drugs were initially tried at concentrations known to not be toxic to *Xenopus* embryos and/or at the maximum non-toxic concentration of DMSO (dimethylsulfoxide; used for vehicle to dissolve certain water-insoluble drugs). Concentrations were then lowered if toxic, and raised if not toxic or if no phenotype was observed (See Appendix A for a comprehensive list of the different concentrations) [3]. Drugs giving rise to aberrant regeneration in the preliminary screen were then given to a larger sample of worms for more detailed analysis.

Mature (non-regenerating) planaria were starved for at least four days, cut with a sterile razor blade into head, pharynx, and tail pieces on ice (which initiated regeneration) and then placed in spring water or 0.125% DMSO to serve as controls. Batches of 15 or more worms were similarly prepared and placed in 40 mL petri dishes containing drugs dissolved in spring water. In all cases where DMSO was necessary for dissolving water insoluble drugs, the DMSO level never went above 0.125%, a concentration known to allow normal regeneration [T. Nogi, personal communication]. Drugs were diluted from stock solution and then vortexed. The planaria were starved and incubated at 22°C in drug solution for at least three days, after which the drug solution was changed for spring water if dirty. Planaria were

visually inspected for signs of abnormal regeneraiton under a dissecting microscope daily. Planaria were placed on ice and imaged with a SMZ1500 microscope (Nikon) with coolsnap cf digital camera (Photometrics) and captured with OpenLab 3.1.2 software (Improvision). Images were then processed using The GIMP (GNU Image Manipulation Program) when necessary to enhance contrast and detail.

3 Results

3.1 DMSO controls

DMSO was shown to produce a small but significant (χ^2 analysis, $p<0.05$) level (4%, n=25) of eye abnormalities in regenerating planaria. However, DMSO was ruled out as a confounding factor for the results of DMT, PG, and HMR-1556 treated planaria. In the case of DMT, the scored phenotype, inhibition of regeneration, did not involve aberrant eye regeneration. PG did not require DMSO as a vehicle and therefore the eye defects seen in PG-treated planaria could be attributed to the effects of PG alone. Although eye defects were scored for HMR-1556, planaria were exposed to a level of vehicle an order of magnitude lower than that required to produce eye defects, therefore involvement of DMSO in abnormal regeneration could be ignored.

3.2 DMT inhibits regeneration

Dimethadione (DMT) is a selective blocker of the voltage-gated hERG potassium channel. At 0.125%, DMT selectively caused the death of tail and head fragments but not trunk fragments, and also inhibited formation of head and tail blastemas in the surviving trunk sections. DMT is extraordinarily specific in its actions; the three-day survival rate of head and tail segments was 0% (n=40) whereas the three-day survival rate of trunk segments

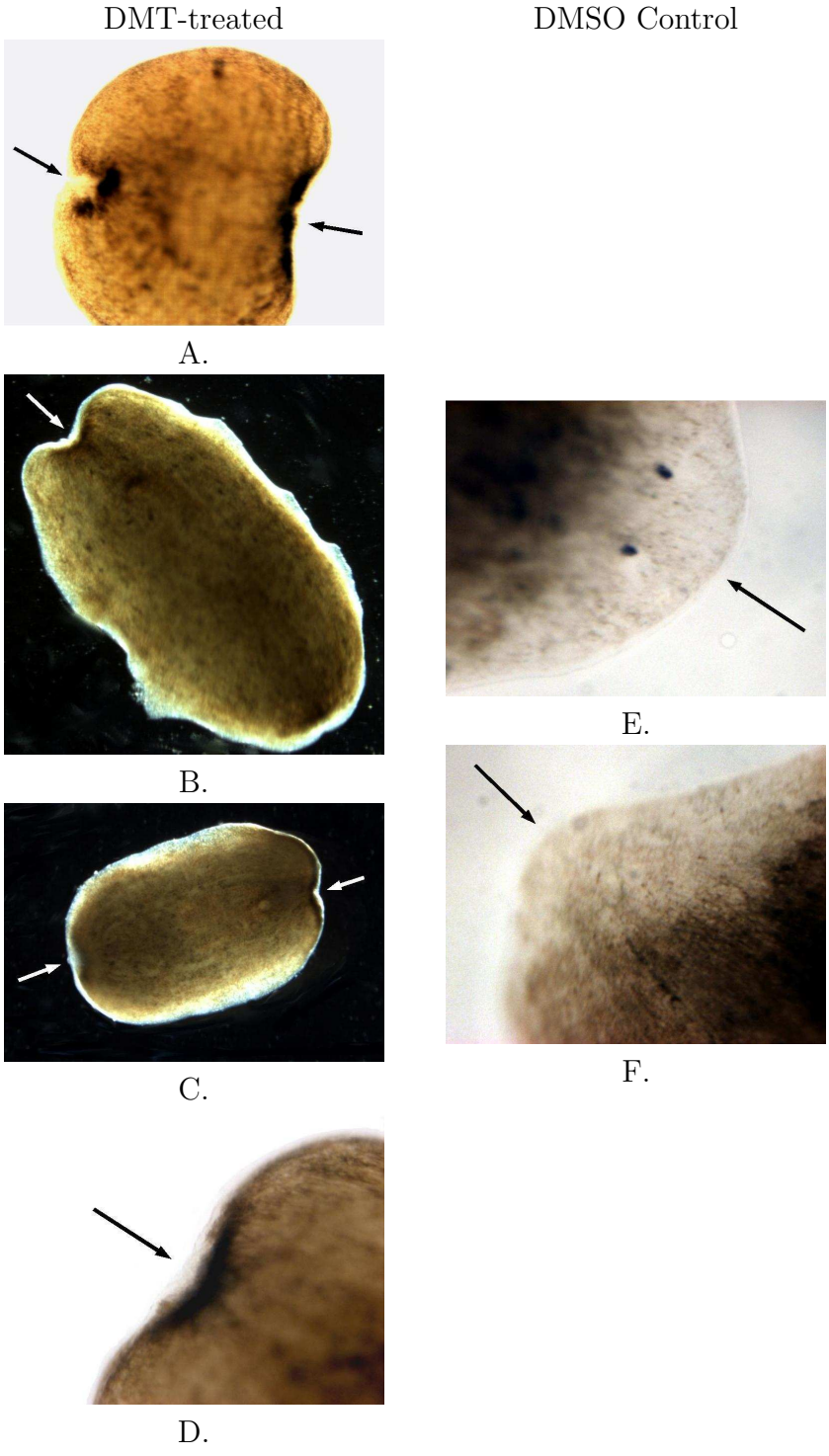


Figure 4: Dimethadione inhibits regeneration. After five days of treatment, no blastemas were observed in DMT-treated trunks (A-D), here compared with matched control head (B) and tail (C) which do possess blastema (lighter pigmented areas).

was 97% (n=40). Comparison with control data indicated a significant effect (χ^2 analysis, p<0.005). This phenomenon was also observed at the 0.11%, 0.1%, and 0.083% DMT levels but tapered off at 0.0556% and was not observed at the 0.042% level or below. Although trunk segments are usually larger in volume than their head and tail counterparts, trials were run with planaria of varying sizes, suggesting that the size of the head and tail segments was not a confounding factor. These results indicate that the planarian homolog of the *eag* potassium channel possibly is necessary for housekeeping functions in the tail and head, but not in the trunk. Whole planaria (n=15) exposed to DMT all died, perhaps due to apoptotic signals transmitted to the trunk by the head and trunk. Another possibility is that the regenerative process confers a protective property on the trunk segment.

The effects of DMT on regeneration seem to be reversible. In all of the trunk segments, head and tail blastemas were not produced and regeneration was inhibited. As long as the segments were maintained in DMT, regeneration did not occur. However, when placed back into spring water, regeneration occurred (Fig. 5). This may be due to the upregulation of *eag* as the cells recycle the blocked channels.

3.3 Disruption of eye formation by Prodigiosin and HMR-1556

Tail fragments subjected to prodigiosin (PG) at 0.125% regenerated with eye defects (35%, n=40), died (55%), or regenerated normally (10%), whereas head and trunk segments regenerated normally. χ^2 analysis revealed a significant effect (p< 0.005). Most of the regenerates exhibited a cyclops (a single eye situated on the anterior-posterior axis) phenotype, although other aberrant eye regenerates were also observed (Fig 8). Prodigiosin at higher levels (0.25%) was lethal, whereas lower levels (7.5×10^{-2}% and 2.5×10^{-2}%) did not produce any abnormal eye regenerates.

HMR-1556 a I_{Ks} blocker [13], showed low levels (14%, n=35) of randomized eye defects at 1.25×10^{-2}%. χ^2 analysis showed this level to be significant (p<0.05, matched with 1.25×10^{-2}

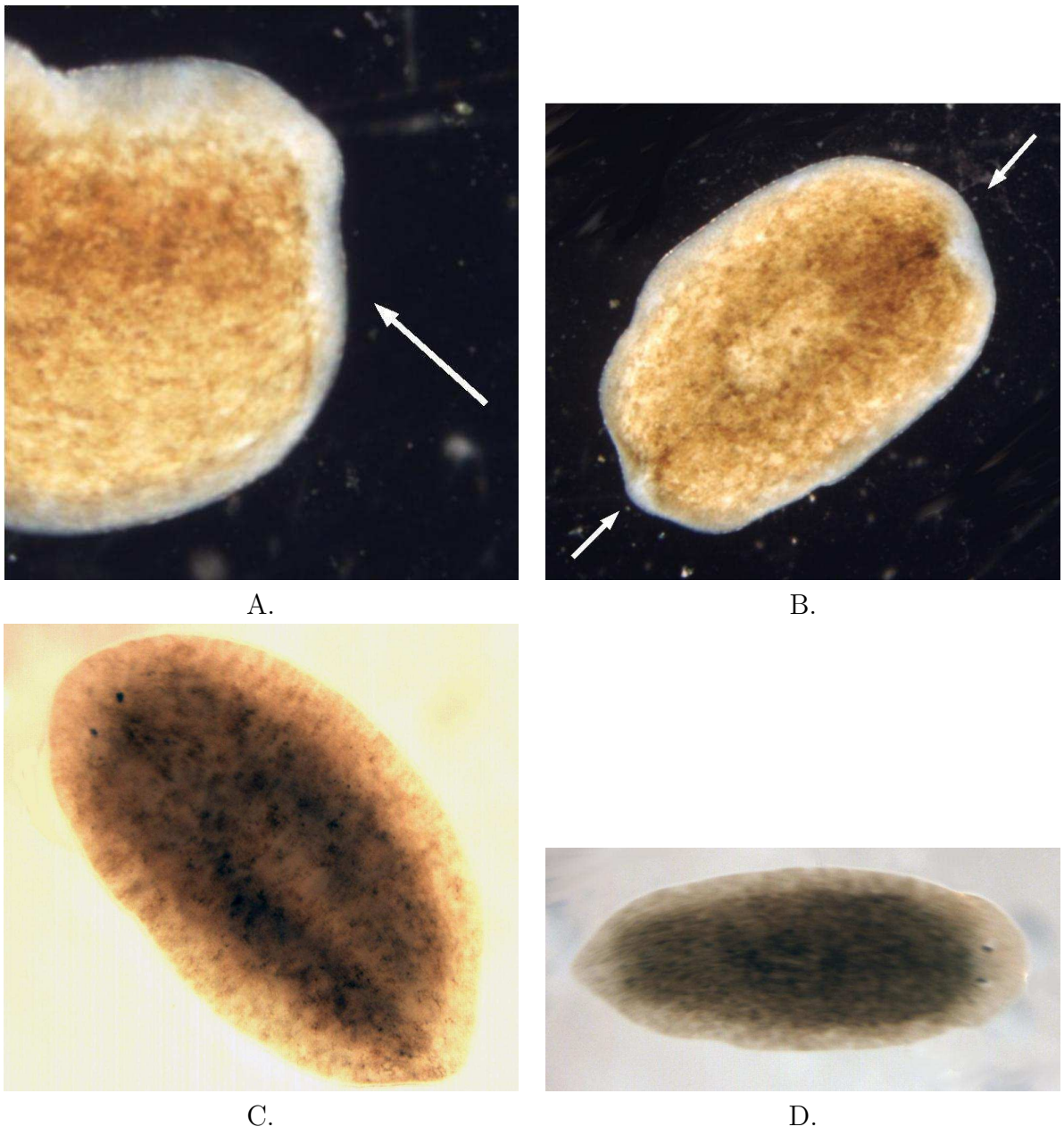


Figure 5: DMT inhibition is reversible. Three days after removal from DMT 0.125% solution, planaria developed blastema (A, B). Six days after removal, normal regeneration was observed (C, D).

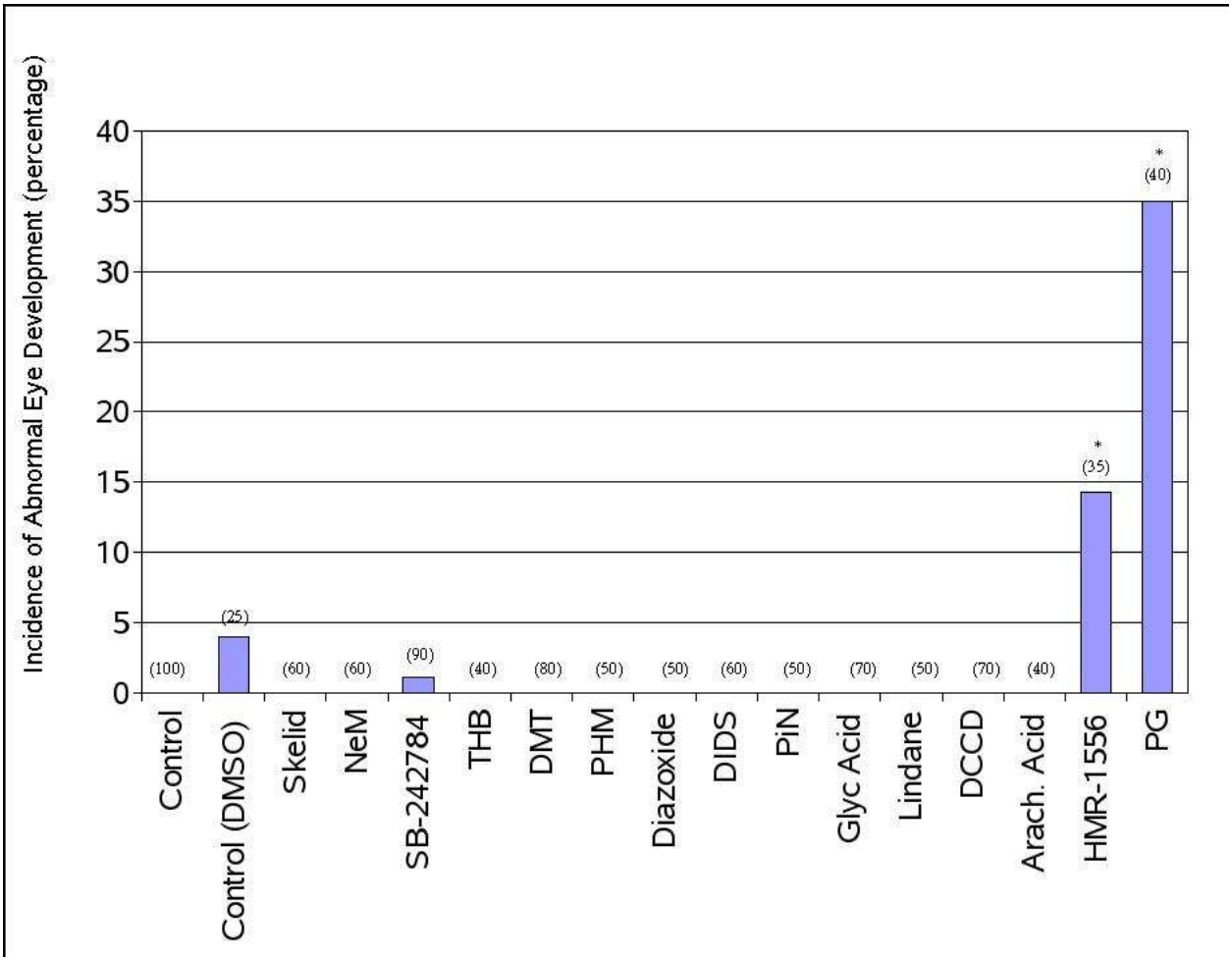


Figure 6: PG and HMR-1556 produce high incidences of abnormal eye regenerates.

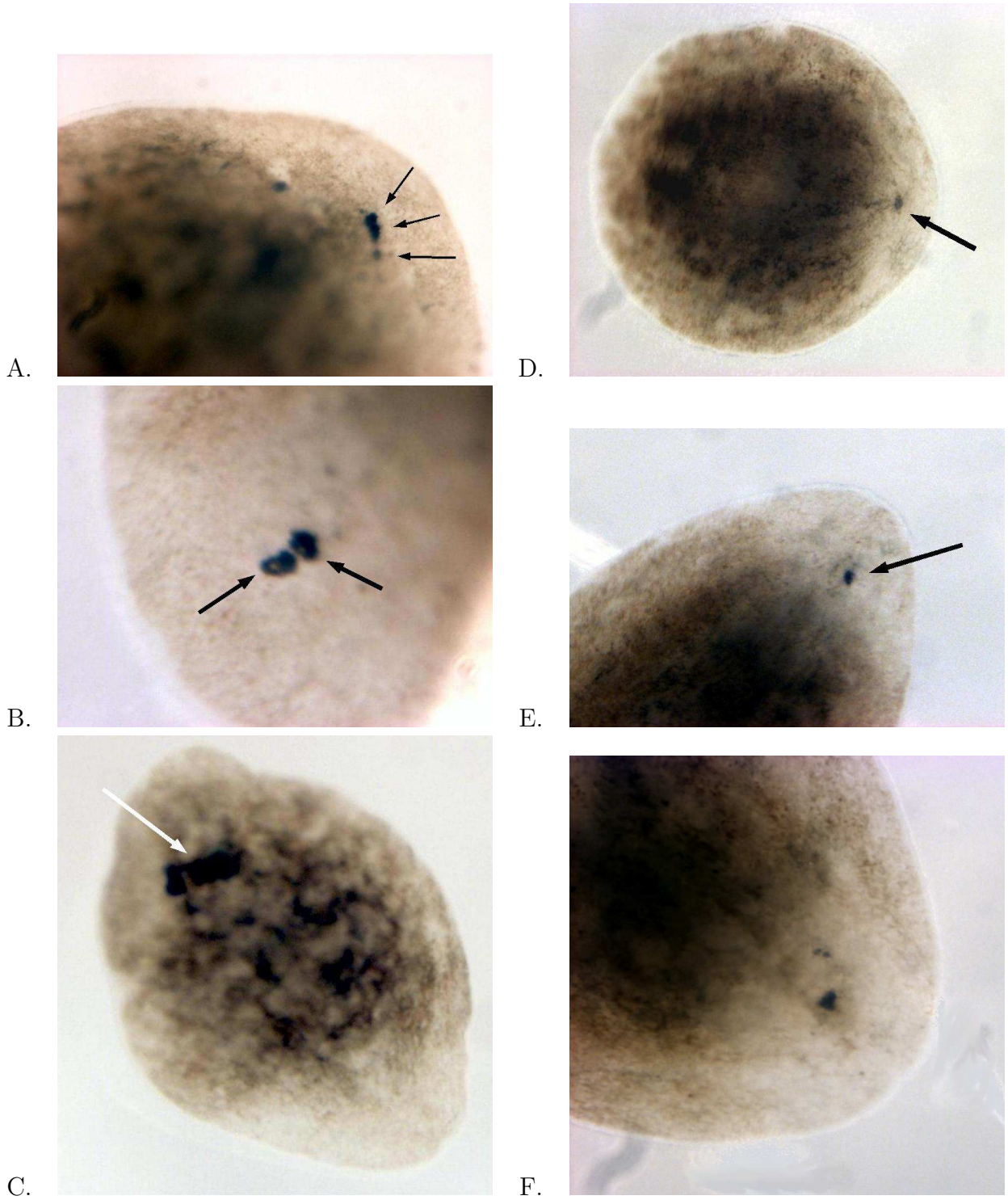


Figure 7: Prodigiosin-treated tail segments regenerated with one eye. Here A, B, and C have completed regeneration and are seen at 11 days. Regenerating tail segments mostly exhibited a cyclops phenotype seen in D, E, F.

DMSO control). Approximately equal numbers of cyclops and three-eyed planaria were Higher levels (1.56×10^{-2}% to 0.125%) showed normal regeneration, indicating that there exists an effective window of the drug outside of which no phenotype is seen.

3.4 Drugs which do not disrupt regeneration

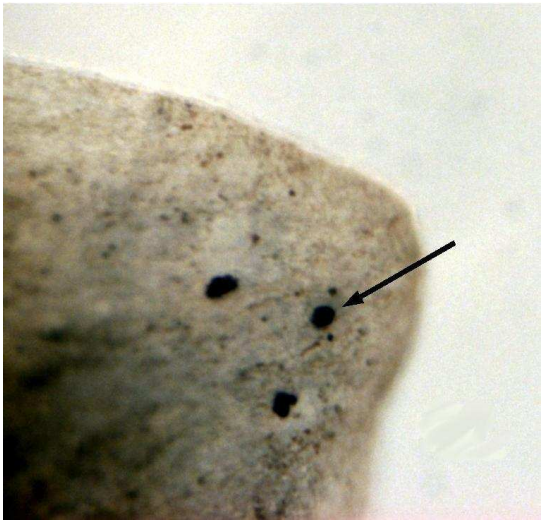
The drugs skelid, NeM, SB-242784, THB, PHM, diazoxide, pinacidil, DIDS, DCCD, glycyrrhetinic acid, lindane, and arachidonic acid did not produce aberrant regenerates. Planaria exposed to differing concentrations of the drugs either died or regenerated normally. One SB-242784-treated planaria did exhibit abnormal eye regeneration, but χ^2 analysis showed this to be insignificant ($p>0.2$).

4 Discussion

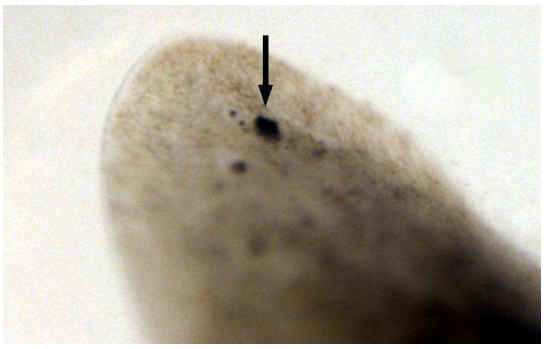
The planarian pharmacological screen is an effective, fast, and relatively inexpensive method of searching for ion channels and pumps crucial to regeneration. However, there exist several inherent disadvantages of the pharmacological screen. The action of drugs designed for vertebrate channels and pumps on invertebrate homologs has not been fully characterized, and some drugs may lose their potency when used in invertebrates. Addtionally, the pharmacological screen is a positive only test, since it relies on a premise that there is a concentration of the drug that will not be toxic, but will still perturb normal regeneration. Therefore, negative results returned by drugs such as PHM do not rule out the involvement of the associated ion channels and pumps with regeneration.

There are *Drosophila* (*eag*) and *C. elegans* (*egl-2*) homologs of hERG, and a BLAST search on the Sánchez planarian EST database (`http://planaria.neuro.utah.edu/`) with the *egl-2* cDNA sequence revealed a sequence AY068378 [25]. However, the effects of DMT, designed to block hERG, on invertebrate homologs has not been fully characterized. *eag* is an

A.



B.



C.

Figure 8: HMR-1556 treated worms

outwardly-rectifying, voltage-dependent channel. That is, it only allows outwards potassium flow and is activated only when a voltage is present.

Potassium channels other than *eag* may be involved in directing regeneration. Perhaps a potassium flux generated by *eag* channels is crucial for the global voltage gradient seen immediately after injury. This voltage gradient would then direct intercellular communication through gap junction channels and specify proper anterior-posterior regeneration. Detailed analysis of *eag* channel function with other pharmacologic agents and RNAi is necessary

PG inhibits H^+/K^+-ATPase, a pump already shown to be involved in the proper development of left-right asymmetry [3]. Here, we see that it is necessary for definition of the left-right axis in the head. H^+/K^+-ATPase may play a role in creating a gradient that is later used to properly situate eyes. Voltage imaging with DiBAC dye should be conducted to further test this hypothesis.

HMR-1556 targets the potassium channel KvLQT and caused 14% (n=35) of eye defects in tail regenerates, with the majority being the regeneration of a three-eye or cyclops phenotype. Combined with eye defects resulting from PG, it is clear that potassium flux plays an important role in direction of eye development.

Research into electrical direction of patterning may shed light on important health issues such as cancer and magnetic fields. As cancer is also a disease of morphology, investigation into the controls of cell proliferation and differentiation will provide new insight as to what goes awry in cancer. It is long known that tumor cells may be distinguished from normal cells based on their ion content [30]. Cancerous cells also differ from normal cells in the types of ion channels and pumps that they express and it was found that these membrane proteins also control cell proliferation rate [31, 32]. *eag* may be such a channel, as it has been reported that hERG potassium channels are more frequently expressed in cancerous tissue [12]. Combined with our results, it is clear that the role of normal *eag* function in controlling the pace of cellular growth and the development of correct morphology should

be investigated further.

Due to the Lorentz Force Law (in its simplest form, $\vec{F}=q\vec{v}\times\vec{B}$) magnetic fields effect a force on moving charges and may affect normal. With the advent of modern technology, magnetic fields have become pervasive in the human environment. As it is clear that ion fluxes are involved in directing growth, further research should be conducted to probe the possible health issues of magnetic fields.

5 Conclusion

This study successfully applied a pharmacological screen using 15 drugs on over 1000 planaria to study the regenerative importance of 10 pumps and channels. Several of the targeted pumps and channels were indicated to be crucial to proper regeneration, especially of the head. Three drugs indicated by the screen to have disrupted normal regeneration were dimethadione, prodigiosin, and HMR-1556. As all three inhibit a K^+ pump or channel, these results indicate an imporant role for potassium flux in the process of regeneration.

6 Acknowledgments

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A List of Drug Concentrations

Table 1: Drugs and Concentrations

Target	Drug	Concentrations
V-ATPase	Skelid (tiludronate)	0.5%
		0.35%
		0.25%
		0.05%
		5×10^{-3}%
V-ATPase	NeM	1.25×10^{-2}%
	(n-ethylmaleimide)	1.25×10^{-3}%
		2.5×10^{-4}%
		1.67×10^{-4}%
		1.25×10^{-4}%
		2.5×10^{-5}%
V-ATPase	SB-242784	1.25×10^{-3}%
		1.25×10^{-2}%
		3.125×10^{-3}%
		1.56×10^{-3}%
		1.25×10^{-3}%
		1.25×10^{-4}%
		1.25×10^{-5}%
KvLQT	HMR-1556	0.125%
		3.125×10^{-2}%
		1.56×10^{-2}%
		1.25×10^{-2}%
		1.25×10^{-3}%
		1.25×10^{-4}%
K_v	THB	1%
	(triethylhexylammonium bromide)	0.5%
		5×10^{-2}%
		5×10^{-3}%
eag	DMT	0.125%
	(dimethadione)	0.111 %
		0.1 %
		3.12×10^{-2}%
		8.33×10^{-2}%
		1.25×10^{-2}%

Table 1: (continued on next page)

Target	Drug	Concentrations
		1.25×10^{-3}%
		1.25×10^{-4}%
K_{ATP}	PHM	0.75%
	(Phentolamine mesylate)	0.5%
		0.25%
		2.5×10^{-2}%
		2.5×10^{-3}%
K_{ATP} opener	Diazoxide	0.125%
		3.125×10^{-2}%
		1.56×10^{-2}%
		1.25×10^{-2}%
		1.25×10^{-3}%
K_{ATP} opener	Pinacidil	0.125%
		0.1%
		1.56×10^{-2}%
		1.25×10^{-2}%
		1.25×10^{-3}%
I_{Ks} activator	DIDS	0.125%
	(4,4'-diisothiocyanostilbene-2,2'-disulfonic acid)	0.1%
		6.25×10^{-2}%
		3.125×10^{-2}%
		1.56×10^{-2}%
		1.25×10^{-2}%
H^+/K^+-ATPase	PG	0.25 %
	(Prodigiosin)	0.125%
		7.5×10^{-2}%
		2.5×10^{-2}%
Ductin (a subunit of H^+-ATPase)	DCCD	1%
		0.5%
		5×10^{-2}%
		5×10^{-3}%
		5×10^{-4}%
		5×10^{-5}%
		5×10^{-6}%
Gap Junction Channels	Glycyrrhetinic Acid	1.5%
		0.75%
		0.15%
		5×10^{-2}%
		5×10^{-3}%
		5×10^{-4}%
		5×10^{-5}%

Table 1: (continued on next page)

Target	Drug	Concentrations
Gap Junctioon Channels	Lindane	0.5%
		0.35%
		0.25%
		0.125%
		5×10^{-2}%
Gap Junction Channels	Arachidonic Acid	0.1 %
		1×10^{-2}%
		2×10^{-3}%
		1×10^{-3}%
DMSO control	DMSO	0.125%
		1.25×10^{-2}
Control	Spring water only	