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Long-range Neural and Gap Junction Protein-mediated Cues Control Polarity During Planarian Regeneration

Néstor J. Oviedo[1,2], **Junji Morokuma**[1], **Peter Walentek**[1,3], **Ido P. Kema**[4], **Man Bock Gu**[5], **Joo-Myung Ahn**[5], **Jung Shan Hwang**[6], **Takashi Gojobori**[6], and **Michael Levin**[1,*]

[1]Center for Regenerative and Developmental Biology and Department of Biology, Tufts University. Suite 4600, 200 Boston Avenue, Medford. MA 02155, USA [4]Department of Pathology and Laboratory Medicine University Medical Center Groningen, University of Groningen The Netherlands [5]College of Life Science and Biotechnology, Korea University. Seoul, Republic of Korea [6]Center for Information Biology and DNA Data Bank of Japan National Institute of Genetics. Yata 1111, Mishima Shizuoka 411-8540. Japan

SUMMARY

Having the ability to coordinate the behavior of stem cells to induce regeneration of specific large-scale structures would have far reaching consequences in the treatment of degenerative diseases, acute injury, and aging. Thus, identifying and learning to manipulate the sequential steps that determine the fate of new tissue within the overall morphogenetic program of the organism is fundamental. We identified novel early signals, mediated by the central nervous system and 3 innexin proteins, which determine the fate and axial polarity of regenerated tissue in planarians. Modulation of gap junction-dependent and neural signals specifically induces ectopic anterior regeneration blastemas in posterior and lateral wounds. These ectopic anterior blastemas differentiate new brains that establish permanent primary axes re-established during subsequent rounds of unperturbed regeneration. These data reveal powerful novel controls of pattern formation and suggest a constructive model linking nervous inputs and polarity determination in early stages of regeneration.

Keywords

gap junctions; neural signals; regeneration; polarity; planaria

INTRODUCTION

Regeneration, or the ability to functionally restore structures lost to injury or disease, is widely distributed throughout metazoan phyla (Brockes and Kumar, 2008). A fundamental aspect of

*Corresponding author: michael.levin@tufts.edu Telephone number: (617) 627-6161 Fax number: (617) 627-6121.
[2]Current address: School of Natural Sciences, University of California, Merced. 5200 North Lake Road. Merced, CA 95343, USA
[3]Current address: Institute of Zoology, University of Hohenheim. Stuttgart, Germany

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regeneration is the ability to sense the loss of specific components and activate cellular mechanisms that integrate new tissues within the organism. Understanding the signals that precisely orchestrate this morphogenetic process is a key prerequisite for unlocking the full potential of regenerative medicine.

Planarians possess an accessible adult somatic stem cell population (neoblasts) that maintains differentiated tissues during physiological cell turnover. Upon amputation, neoblasts proliferate and migrate to restore missing parts by forming a regeneration blastema that differentiates into missing structures (Reddien and Sánchez Alvarado, 2004). Individual cell behavior must be continuously coordinated with the large-scale morphogenetic state of the organism. This requires the stem cells to integrate information about the location/position of the damage (short-range) and recognition of remaining pre-existing tissue within the whole fragment (long-range). This type of modulation (by signals from the microenvironment and nervous/immune system activity) occurs *in vivo* to regulate stem cell behavior in a variety of species and tissues (Jones and Wagers, 2008; Mendez-Ferrer et al., 2008). The planarian is thus a highly tractable system within which to dissect signaling processes of very broad relevance to regenerative patterning.

How does the tissue remaining after an injury know what is missing and thus decide what must be rebuilt (e.g., head or tail)? Early molecular events during planarian regeneration are mostly unknown but the initial processes involve wound closure, dorsal-ventral interactions, and neoblast proliferation preceding blastema formation (Reddien and Sánchez Alvarado, 2004). Establishment of anterior-posterior (A/P) polarity during regeneration is affected by modulation of the *Wnt* signaling pathway (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008) or treatment with gap junction inhibitors, chick embryo extracts, colchicine, chloramphenicol, or etionine (Brøndsted, 1969; Nogi and Levin, 2005; Rodriguez and Flickinger, 1971). However, no existing molecular model integrates these data as a constructive algorithm for rebuilding large-scale morphology after amputation (Meinhardt, 2009). This is of particular relevance as the influence of systemic signals (e.g.: immune, nervous, and metabolic) is now beginning to be appreciated as an important regulator of stem cell behavior (Hsu and Drummond-Barbosa, 2009; Spiegel et al., 2008). Critical fate and axial polarity decisions around the injury site take place during the first day of regeneration and involve signals from both local and very distant tissue. Thus, molecules associated with rapid, long-range transmission of information are likely to be involved.

Gap junction (GJ) proteins are plasma membrane channels involved in direct cell-cell communication via physiological signals, regulating embryonic development, neoplasm, and tissue function in diverse contexts (Levin, 2007a; Phelan, 2005). GJ protein-mediated signals are known to regulate behavior of embryonic and adult stem cells (Wong et al., 2008) probably by modulating the exchange of information between undifferentiated cells and their surrounding microenvironment (Elias and Kriegstein, 2008; Oviedo and Levin, 2007b). Such short-range stem cell regulation by GJ proteins is essential for regeneration of complex structures in both vertebrates and invertebrates (Hoptak-Solga et al., 2008; Oviedo and Levin, 2007b).

The classical literature also suggests the nervous system as an essential component of vertebrate and invertebrate regeneration (Brøndsted, 1969; Singer, 1952). Despite recent advances (Kumar et al., 2007), the molecular connection and reciprocal influence between nerves and regeneration remains poorly understood. The planarian central nervous system (CNS) consists of a brain and a pair of longitudinal ventral nerve cords (VNC) running along the A/P axis (Supplemental Figure 1A) (Agata and Umesono, 2008; Cebrià, 2008). The planarian CNS has been suggested to control morphogenesis of posterior areas (Best et al., 1969; Flickinger and Coward, 1962; Lender, 1960). However, the mechanisms of such signaling are unknown.

Understanding the flow of long-range patterning information during regenerative morphogenesis is crucial for developing rational strategies to control stem cell behavior *in vivo*. First, while it was recently suggested that gap junctional communication (GJC) may be required for A/P patterning in planaria (Nogi and Levin, 2005), it is completely unknown which specific innexins (invertebrate GJ proteins) or mechanisms are involved in this process. Moreover, it is not known what tissue or structure transmits morphogenetic information from the organism to the neoblasts during regeneration. Our data revealed the existence and properties of instructive, long-range signals from the central nervous system that control pattern formation, and molecularly identified the specific GJ proteins that mediate this process. Strikingly, large-scale alterations in the animals' body-plan, induced by short-term physiological modulation, persist across multiple cycles of regeneration, uncovering a mechanism by which physiological signals re-specify anatomy. We synthesize these data into a constructive, binary model integrating neural inputs and fate determination in early stages of regeneration.

MATERIAL AND METHODS

Planarian culture

A clonal strain of *Dugesia japonica* (GI) were kept and maintained as in (Oviedo et al., 2008a).

Antibody labeling and image collection

Planarians were processed for immunostaining as in (Oviedo et al 2008b). Primary antibody dilution: α-phosphorylated histone H3 (Upstate) 1:250, α-arrestin (gift from K. Watanabe) 1:10000, and synapsin 1:50 (Developmental Studies Hybridoma Bank). Incubation with 2^{ry} antibody was performed overnight in goat anti-mouse Alexa488, 1:400. Representative images for each condition were processed as previously described (Oviedo and Levin, 2007b).

Whole-mount in situ hybridization

Animals were processed for whole-mount *in situ* hybridization as in (Nogi and Levin, 2005).

Drug treatment and amputations

Octanol solution (8-OH) was prepared by directly diluting 10μl of 1-Octanol (Sigma-Aldrich) into 500ml of commercial natural spring water (PS, Poland Spring Water), resulting in a final concentration of 127μM. Worm fragments were transferred into drug solutions immediately after amputation in PS.

dsRNA synthesis and microinjections

dsRNA synthesis and microinjections were performed as in (Oviedo et al., 2008a). Microinjection schedules extensively optimized until reproducible results were obtained and injections were performed as in (Oviedo et al., 2008b). Triple RNAi worms were injected with a mix (1:1) of *Dj-Inx-5 + −13* dsRNA for three consecutive days followed by rest of 14 days and then two consecutive days of injections with *Dj-Inx-12* dsRNA. For *Dj-ßcatenin-B (RNAi)*, worms were injected for three consecutive days and amputations were performed one week after first injection.

Octanol incorporation in planarian tissue

Levels of octanol in planarian tissues were measured by gas chromatography mass spectrometry as previously described (Zada et al., 2002).

Mutagenesis analysis

Mutagenesis analysis was performed by luminometry as previously described (Min et al., 1999).

RESULTS

Long-range information is provided to wounded areas through the ventral nerve cord and gap junctions

Though not previously revealed by any pharmacological treatment (Brøndsted, 1969; Nogi and Levin, 2005) or genetic approaches (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008), non-local patterning cues have been long sought for building theoretical models of morphogenesis (Meinhardt, 2009) because neighboring cells adopt radically different fates (head or tail) after surgical bisection. We began by pharmacological loss-of-function studies targeting gap junctional communication as a primary candidate for long-range patterning signals during establishment of regenerative polarity.

GJ blockers have been extensively used to characterize functional roles of gap junctional signaling in the absence of general disruption of membrane integrity of cell housekeeping functions (Abdelmohsen et al., 2005; Burnside and Collas, 2002; Jin et al., 2008; Levin and Mercola, 1998; Levin and Mercola, 1999; Levin and Mercola, 2000; Schilling et al., 2008). Among n-alcohols, heptanol and octanol are best known for their potent and specific inhibition of GJC in vertebrate and invertebrate models (Adler and Woodruff, 2000; Anderson and Woodruff, 2001; Brooks and Woodruff, 2004; Chanson et al., 1989; Christ et al., 1999; Ehrlich and Diez, 2003; Garcia-Dorado et al., 1997; Mire et al., 2000; Momose-Sato et al., 2005; Waksmonski and Woodruff, 2002; Weingart and Bukauskas, 1998). In planarians, treatment with heptanol or octanol, but not hexanol (which does not block GJC), leads to a consistent alteration of A/P polarity during regeneration, suggesting that the effects of these compounds on planarian regeneration correlate with their ability to block GJs ((Nogi and Levin, 2005), Supplemental Figure 1C). By optimizing an octanol dose that induced consistent alterations of A/P polarity during regeneration but did not shut down all gap junctions simultaneously, we avoided generalized toxicity and alteration of neoblast maintenance (Oviedo and Levin, 2007b), allowing us to specifically investigate patterning phenotypes in the context of normal neoblasts (Supplemental Figure 1B). Because of the unique suitability of pharmacological compounds for testing spatio-temporal properties, we used this loss-of-function reagent for the initial characterization of GJC's role in regeneration; molecular validation using RNAi (which cannot be targeted or turned off in timing experiments) is presented further below.

A series of transverse amputations, resulting in 2 blastemas, were performed at different levels along the A/P axis followed by octanol exposure (Figure 1A and 1B). Both untreated and octanol-exposed amputated fragments developed normal anterior blastemas that differentiated into heads. However, fragments treated with octanol often formed ectopic anterior blastemas at posterior-facing wounds that developed into heads, resulting in animals with heads at both ends of the A/P axis (i.e.: bipolar head regeneration). This abnormality in polarity was never observed in untreated animals, and the fragments were much bigger than the thin slices that have been reported to sometimes exhibit A/P abnormalities. Furthermore, susceptibility to developing the bipolar-headed phenotype gradually increased as the plane of amputation (posterior wound) was moved towards the posterior end, reaching maximum effect (~100%) at the post-pharyngeal area (Figure 1A). Thus, dependence of the blastema upon GJ-mediated signals exists as a gradient along the A/P axis, being highest at the post-pharyngeal region.

The planarian brain is capable of preventing regeneration of secondary head within the same animal (Flickinger and Coward, 1962; Lender, 1960). Our data, showing that the head fragment

produced the fewest bipolar-headed animals, support this model. However, the mechanism of this inhibition is not known. We sought to determine whether pre-existing head tissues in regenerating fragments could prevent regeneration of an ectopic head in the presence of GJC inhibitor. Cuts along the A/P axis of octanol-treated animals, always including the original head region (Figure 1B), resulted in >95% single-head (normal) animals. Although posterior regenerating fragments require GJs for correct assignment of blastema identity, GJ inhibition in fragments containing brain does not result in bipolar head regeneration. Thus, we sought an additional mechanism by which the blastema can sense the presence of the head at long distances.

Neural projections of the planarian CNS reach all parts of the worm (Cebrià, 2008). To test the hypothesis that the CNS may mediate long-range signaling, we made cuts that did or did not disrupt ventral nerve cord (VNC) continuity along the A/P axis (Figure 1C, specificity of cuts confirmed in Supplemental Figure S2). All (octanol treated and untreated) animals formed blastemas, suggesting that damage recognition and basic wound healing were not altered by this procedure. Strikingly, VNC-interrupted worms treated with octanol developed multiple A/P axes (Figure 1C and 1D). In all (3/3 conditions) where tissue removal did not affect VNC contiguity, regeneration proceeded normally (classes I, II and X in Fig. 1C). However, when amputation produced disruption of VNC integrity (7/8 conditions), regeneration of ectopic heads, pharynxes, and abnormal protrusions occurred (Figure 1C and 1D), revealing a new set of 6 phenotypes including some with multiple A/P axes (Figure 1D). For a direct comparison of the effects upon regeneration pattern, please compare classes II, VI and VIII (Figure 1C). Thus, disruption of VNC continuity, together with GJ blockade, result in the loss of correct blastemal A/P identity (formation of ectopic heads) in fragments containing an intact brain.

These results show that sensing the presence of brain tissue at long distances by the blastema can occur if the VNCs is un-interrupted or if GJC is functional. Taken together, these data reveal two parallel pathways by which a blastema surveys the remaining anatomy within the pre-existing tissue for instructive cues during regenerative morphogenesis.

CNS- and GJ-mediated inputs function during very early regeneration

To characterize the temporal properties of this novel signaling system, we performed experiments combining disruptions of GJ signaling (octanol) and VNC continuity (surgical cuts) at specific time points during regeneration (Figure 2). Uncoupling GJC with octanol is known to happen very fast (< 1 min after exposure) and the effects are reversible after the reagent is removed from the media (Chanson et al., 1989). Analyses of the incidence of A/P polarity duplications were performed in two different fragments (with or without pre-existing head) (Figure 2). Regenerating tissue without head (post-pharyngeal fragment) was treated with GJ inhibitor at different time-points after amputation (Figure 2A). Starting the exposure at progressively later intervals is a technique that does not rely on drug wash-out, and revealed that the most significant effect results from starting GJ blockade within the first 3-6 hours post-amputation. The effect on A/P patterning dropped significantly (<60%) for treatments beginning >12 hours of regeneration, and reached insignificant levels thereafter. We conclude that GJC-mediated signals are fundamental for establishment of polarity within post-pharyngeal area, and that the primary role of GJ-mediated information occurs within 12 hours of amputation.

We next investigated the temporal properties of GJ/VNC-mediated signals during regeneration of fragments with a pre-existing head (Figure 2B). Planarians were treated with octanol and amputated at post-pharyngeal level (time 0), followed by VNC disruption at different time points. These experiments revealed a dramatic decrease (4-fold) in the incidence of A/P defects if the VNC was disrupted after the first 3 hours post-amputation. These data suggest that information provided to the blastema via the CNS (existence of the head at long range,

indicating a tail fate for new tissue) is largely transferred very early after injury (~6 hours) and is consistent with timeframe of CNS mediated inputs in regenerating post-pharyngeal fragments shown in Figure 2A.

Innexin genes (*Dj-Inx-5, −12 and −13*) mediate information crucial for morphogenesis and polarity determination during regeneration and tissue maintenance

Although previous work demonstrated planarian innexins to be important for stem cell function and blastema formation (Oviedo and Levin, 2007b), it is not known which (or how many) of the dozen innexins actually mediate instructive A/P patterning information during regeneration. Compensation among GJ protein family members has made it difficult to identify phenotypes associated with individual connexins or innexins in mammalian models (Elias and Kriegstein, 2008; Levin, 2007a) and few studies have targeted more than one GJ gene at the same time. We used gene-specific knockdown to abolish the expression of multiple innexin genes simultaneously. Our microinjection strategy involved more than 600 animals and targeted, via RNA interference (RNAi), up to four innexins simultaneously (Supplemental Table S1). No phenotype was found with single RNAi of CNS-associated innexins. Permutations with simultaneous down-regulation by RNAi of two innexins revealed that 4 of 12 (4/12) combinations led to abnormal behavior characterized by abnormal response to light, locomotion problems, and ruffled edges and regeneration-associated phenotypes. All experiments (n=5) in which three innexins were down-regulated simultaneously by RNAi consistently led to such phenotypes in intact and regenerating animals (Supplemental Table S1). We therefore focused on characterization of simultaneous RNAi of three innexins, as macroscopic phenotypic abnormalities were consistent with those induced by octanol treatment.

Triple RNAi of *Dj-Inx-5*, *−13*, and *−12* led to the most consistent and strong phenotypes (Supplemental Table S1). Endogenous expression of these 3 transcripts is mostly found overlapping within the CNS as well as in sub-epithelial cell populations throughout the animal, and is up-regulated within regeneration blastemas ((Nogi and Levin 2005), Supplemental Figure S3). Sequential down-regulation of these genes was obtained from an optimized double-stranded (dsRNA) microinjection schedule (five dsRNA microinjections over two weeks, Figure 3A). dsRNA microinjections of *DJ-Inx-5 and 13* were administered together for 3 consecutive days followed by a resting period of two weeks and two more microinjections of dsRNA of *DJ-Inx-12* [i.e.: *Dj-Inx-5+13, −12(RNAi)]*. About 30 days after the first dsRNA microinjection, intact planarians showed behavioral changes (see Supplemental Movie 1) characterized by slow movements and random contractions (~95%, n=137) similar to those observed in intact animals after octanol treatment.

Molecular analyses with markers of CNS, neoblasts, and intestine clearly demonstrated the presence of ectopic brain tissue, multiple pharynxes, and altered distribution of neoblasts and digestive system (Figure 3B). Ectopic brain and photoreceptors generally developed in the post-pharyngeal area. Remarkably, in all cases (n=14), two new pharynxes appeared, and were aligned correctly with respect to the new brain. One of the two ectopic pharynxes showed a characteristic inversion of A/P polarity with respect to the original pre-existing axis, indicating that not only ectopic pharynxes, but also changes in polarity, were induced. All of these morphogenetic changes occurred in intact animals (i.e., no amputation-induced regeneration was involved), suggesting that this signaling system is required for complex tissue maintenance. We conclude that the down-regulation of 3 specific innexins leads to the development of ectopic primary axes and organs with consistent internal A/P polarity but exhibiting a failure to conform to the normal (1-headed) pattern of the organism. This is consistent with the phenotype observed in regenerating fragments after pharmacological

blockade of GJ, confirming the importance of GJs in the establishment of large-scale A/P patterning and identifying a specific subset of the innexin family involved in this process.

In control animals, proliferative neoblasts are scattered throughout the body but absent in tissue in front of the photoreceptors and around the pharynx (Newmark and Sanchez Alvarado, 2000). We found that this arrangement was consistent in *Dj-Inx-5+13,+12(RNAi)* worms; however, the presence of two ectopic brains and pharynxes revealed additional areas without mitotic activity surrounding them (Figure 3B). The normal pattern of the digestive system was also dramatically altered, exhibiting fusion of posterior branches (post-pharyngeal area) followed by bifurcation around the new pharynxes, suggesting that modifications in this tissue also resulted from signals from the new morphogenetic arrangement during tissue maintenance. Further, amputation of *Dj-Inx-5+13,+12(RNAi)* animals (Figure 3C,D) led to worms with inverted A/P axis and bipolar head after regeneration from pharyngeal (20%, n=21) and post-pharyngeal (80%, n=18) fragments, which is anatomically consistent with pharmacological inhibition of GJ or RNAi of these innexins in intact animals. These results demonstrate that the GJ-dependent signaling system is required for proper A/P establishment during regeneration and firmly implicate three specific CNS-associated GJ genes (*DJ-Inx-5, −12* and *−13*) as mediating important morphogenetic signals and polarity determination during tissue maintenance and regeneration (Figure 3E).

Polarity in regenerating lateral wounds is also controlled by CNS/GJ signaling

We asked whether information provided by the CNS was also used by posterior-facing wounds. Post-pharyngeal fragments were treated with octanol and single or multiple cuts were produced laterally so as to disrupt VNC integrity or leave it intact (Supplemental Figure S4A). Each amputation resulted in blastema formation; unexpectedly, all of these organisms (n>100) exhibited behavioral changes (i.e.: multiple sides pulling in different directions) and the appearance of photoreceptor pigmentation in two, three, or four different blastemas within the same fragment, suggesting that the alterations of normal behavior result from ectopic anteriorization of multiple areas. Indeed, these animals ultimately formed triple or quadruple heads (Figure 4A). These striking morphogenetic changes were more frequent in those fragments with simultaneous lateral cuts (Figure 4A). Although the majority of untreated post-pharyngeal regenerating fragments formed single headed worms (77-94%), in some cases (6-24%) duplication of head structures (side head) were observed (Figure 4A). Interestingly, none of these resulting animals displayed reversal of polarity (see side head in Figure 1D). These results in both treated and untreated post-pharyngeal fragments are consistent with our observations of two different mechanisms involving GJ proteins and the ventral cord respectively.

Analysis with molecular markers revealed differentiation of ectopic brains and pharynxes, and changes in neoblast distribution and digestive system morphology (Figure 4B and Supplemental Figure S4B, C). The number of ectopic organs and their orientation was always consistent with the number of heads that establishes new A/P axes, suggesting that similar key morphogenetic regulation observed during regeneration of single animals occurred when multiple axes formed in the same body. Taken together, these results indicate that CNS/GJ-mediated signaling supplies crucial cues to both posterior and lateral wounds, informing blastemas about the presence of anterior structures (brain) within the pre-existing tissue and during early stages of regeneration.

Morphogenetic changes persist for several rounds of regeneration after GJ inhibition

Animals with bipolar, triple, and quadruple heads are viable, providing a unique opportunity to ask whether it is possible to permanently re-set the target morphology (i.e., the complex 3-dimensional pattern that is recreated after injury and determines the termination point of

regenerative processes). While it has been suggested that induction of the head could serve as a permanent organizing center, no systematic analysis of this type of signaling has been published. We performed 2 types of amputations on multi-headed animals without further octanol treatment: (i) removing one, two, three, or four heads simultaneously (regeneration of multiple anterior wounds), and (ii) longitudinal cut along the A/P axis providing a long regenerative surface (Figure 5 and Supplemental Figure S5). In all cases (>200 amputations) involving anterior and longitudinal wounds, the new (ectopic head) state of the worm was regenerated.

The use of a pharmacological GJ blocker enabled time-limited exposure experiments, which are not feasible with RNAi. Two more rounds of re-amputation of octanol-induced multiple headed animals were performed in plain water (no further octanol exposure). We used gas chromatography mass spectrometry (GC-MS) to directly assay the presence of octanol in worm tissues after the initial exposure (Supplemental Figure S6). Octanol levels in worm tissues decreased rapidly, and reached undetectable levels (orders of magnitude below those needed to induce the phenotypes), within mere hours after removing the animal from octanol-containing media. Strikingly, these multiple-headed planarians re-created the altered axial configuration of the animal (Figure S5A) at each round of amputation, consistent with permanent establishment of new axes by a one-time inhibition of GJ-dependent signals (Figure 5). The amputations were carried out 3-6 weeks after octanol removal, demonstrating that altered A/P organization was re-created by the blastemas at a time long after the last detectable traces of blocker compound left the animal. Thus, once altered by an early, temporary blockade of GJ-dependent signals, the new large-scale axial morphology persists through further rounds of regeneration.

We next asked whether this permanent change of large-scale morphology in the worms was likely due to alteration of DNA sequence by octanol. A standard mutagenicity assay (Min et al., 1999) indicated that the octanol concentration used in all experiments was not mutagenic (Supplemental Figure S7), suggesting strongly that the observed long-term effects could not have been due to changes in DNA. The above data reveal a remarkable phenomenon: a brief inhibition of a signaling system (GJC) results in the alteration of A/P polarity that is re-asserted upon subsequent episodes of regeneration weeks after the GJ inhibitor is removed. These data identify a novel physiological system that permanently alters the target morphology that guides (must be restored by) regeneration.

DISCUSSION

In order to re-establish pre-existing morphology, regenerating fragments require that the wound site have access to 2 pieces of information: what shape is the animal supposed to be “target morphology” (the morphology that must be achieved before regeneration can terminate), and what shape it is now (what has been removed by injury and what’s left). Our data address both components, revealing novel molecular pathways of remote sensing of the presence of head tissues, and illustrating how target morphology can be re-set by transient modulation of physiological signals.

Central nervous system and gap junctions direct the establishment of anterior-posterior polarity

Correct morphogenesis (avoiding ectopic head formation) requires either the presence of an un-interrupted ventral nerve cord or normal gap-junctional communication. Only when both are inhibited do ectopic heads appear (despite the presence of brain tissue within the regenerating fragment), suggesting that blastemas receive information via at least two complementary pathways. The presence of overlapping, complementary pathways are consistent with the remarkably robust regenerative abilities in this model.

What instruction is being passed to morphogenetic organizers such as the blastema during regeneration? The data suggest that these long-range pathways are signaling the presence or nascent formation (as a very early event during regeneration) of head elsewhere in the animal (Lender, 1960). Both posterior and side wounds make use of this information very early during the regenerative process; this is reasonable as decisions of fundamental identity needs to be made in the blastema before significant morphogenesis occurs. Previous work in planarians (Cebria et al., 2002) suggested that FGF signaling may participate in a brain-inducing circuit. However, this phenotype has not led to insight into several of the issues addressed here: polarity establishment, time-course of brain signaling to adjacent tissues, and long-range signaling between brain and other regions. While FGF-related signals seem to restrict brain to anterior areas, they do not affect polarity of any organ as does GJ blockade.

Our data also illustrate a link between position along the A/P axis and polarity (direction). Posterior areas have higher tendency to develop A/P polarity problems when GJ-dependent signals are blocked, revealing that the degree of dependence of a blastema's head/tail identity upon GJ signals is a function of position along the A/P axis. A gradient has not yet been observed in either pharmacological treatments or genetic manipulations. This provides a tractable entrypoint into the mechanisms that link position to direction along a major body axis – a key puzzle in morphogenesis of many structures (Aw and Levin, 2009; French et al., 1976).

Our results suggest a model (Fig. 6) for determination of blastema identity. The default identity for wounded surfaces is "head formation". In the absence of specific signals, informing instructing the neoblasts that a head already exists elsewhere, information reaches the blastema via 2 routes: through the VNC, and innexin proteins. The latter is most likely a long-range gap-junctional transfer of small molecule signals as has been shown in other patterning systems (Levin, 2007a;Levin and Mercola, 1999).

In addition to molecular-biological induction/repression pathways, which themselves do not determine a specific morphology (could be applied to many different arrangements of cells and tissues), it is important to develop algorithmic models that illustrate how complex morphogenesis occurs. While we are still far from a detailed picture of how all types of fragments recreate an entire worm, we propose an algorithmic model that shows what decisions are made by the various blastemas in determining the identity to adopt (Supplemental Figure. S8).

Novel pathways for long-range signaling during regenerative morphogenesis

There have been no molecular data associating specific organs or tissues with polarity or fate determination in planarians. Likewise, the alteration in axial patterning resulting from grafting pieces of head tissue into the tail of a host is not understood at the molecular level (Kobayashi et al., 1999). These data extend the understanding of regenerative morphogenesis in several ways. For the first time, we demonstrate novel roles in patterning for a distinct anatomical structure (VNC) and 3 specific innexin proteins. Moreover, the data reveal that long-range signaling from differentiated tissues instructs the blastema through multiple overlapping pathways.

The GJ-dependent signals are mediated by *Dj-Inx-5, −13*, and *−12*, as their RNAi knockdown phenocopied the effects of pharmacological inhibition by octanol. Combinatorial analysis of individual innexin genes by RNAi revealed a number of phenotypes in intact and regenerating animals. The expression of most of these innexins was associated with CNS in intact animals and during regeneration (Nogi and Levin, 2005), and indeed most of the RNAi phenotypes included behavioral abnormalities. A/P patterning problems resulted only by targeting *Dj-Inx-5, −13*, and *−12* simultaneously, and did not result from targeting single innexins or from

other combinations of up to four innexins. Thus, it suggests that this phenotype is specifically related to *Dj-Inx-5, −13,* and *−12* function and that these GJ protein family members normally compensate for one another.

When do these signaling pathways operate? Nine hours post-amputation, up-regulation of *noggin-like* gene expression near the wound site is observed (Ogawa et al., 2002). Gurley et al. concluded that important fate decisions take place as early as 12 hours post amputation (Gurley et al., 2008). However, the nature of signals instructing such early gene expression was largely unknown. Time-course inhibition of GJC and disruption of VNC contiguity revealed the window (<3-6 hours post amputation) of critical fate decisions during blastema formation. These effects cannot be explained by differential permeability of the regenerating fragments to octanol because similarly-sized anterior and posterior regenerating fragments exhibit different phenotypes upon exposure to GJ inhibitors. Blocking CNS-mediated early inputs did not affect wound closure, neoblast proliferation, or blastema formation; uniquely, isolating the blastema from long-range CNS inputs altered the identity and polarity of the new tissue, removing the normal morphogenetic coordination with pre-existing structures.

Performing *Dj-Inx-5,−13,−12(RNAi)* on intact animals led to striking morphogenetic changes characterized by the presence of additional sets of brains and pharynxes along with a new distribution of neoblasts and digestive system which are similar to changes induced with pharmacological inhibition of GJ proteins. Both RNAi and pharmacological inhibition of GJ genes indeed led to alterations in normal pattern morphology. However, the phenotypes are not identical, due to the fact that heptanol targets GJ post-translationally, in a reversible way that rapidly saturates, while RNAi results in a more permanent RNA-based down regulation of their function lasting for more than a month. Although further analysis is required to characterize the relevant GJ-permeable signaling molecules, we propose a scenario in which information mediated by this set of GJ proteins functions during tissue maintenance: a continuous “cellular inventory” is performed, allowing the animal to sense and integrate different anatomical components during morphostasis and remodeling. Therefore, it is possible that in the wounded area, absence of GJ-mediated signals associated with “presence of head” would trigger differentiation of a new brain and anterior identity. While the presence of ectopic brains is associated with the region where worms predominantly fission during asexual reproduction (post-pharyngeal area), it is unlikely that morphogenetic changes observed in this phenotype were associated with fission. During fissioning, no reversal of polarity is observed and only one brain is regenerated per animal; in contrast, in this phenotype we observed three brains within an intact animal. It is not clear what factors determine the polarity of the new brain, but it is evident that it can be established in an orientation independent of that of the host.

Recently, components of the *Wnt* signaling pathway were associated with determination of A/P polarity during regeneration and morphostasis – the first molecular information linking polarity and regeneration in planarians (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). Nevertheless, how A/P polarity is determined and maintained in adult tissues is poorly understood (Meinhardt, 2009). To determine the relationship between the GJ/VNC system and the *Wnt*-dependent signals, we identified and characterized a novel clone (*Dj-βCatenin-B*) of the Wnt signaling pathway in *D. japonica*. Its RNAi-mediated knockdown revealed the same phenotypes previously reported for its homolog in *S. mediterranea*. Importantly, these phenotypes differed significantly from those we report here for inhibition of GJC or VNC connectivity (Supplemental Note 1 and Figure 9). While both the GJ/VNC and *Wnt* pathways are required for A/P polarity, down-regulation of the physiological pathways revealed several important differences from the *Wnt* pathway phenotypes, suggesting that these are distinct regulatory mechanisms. First, the defects arising from the innexin phenotype reveal a gradient of sensitivity (maximal at post-pharyngeal areas).

In *Wnt* signaling loss-of-function, defects were observed mostly uniform along the A/P axis. Second, the post-pharyngeal area is the most susceptible to A/P mispatterning after 3 innexins are downregulated, while in *Wnt* signaling disruption, ectopic anteriorization is commonly observed at the tip of the tail and randomly in lateral edges leading to more extreme cases of radial-like hypercephalized animals that were not observed in any innexin-RNAi phenotype. Third, RNAi targeting innexins led to a general correspondence between the number of ectopic brains and pharynxes that were always aligned with each other and with similar polarity. However, in RNAi of different components of *Wnt* signaling there is no correspondence between the number of ectopic heads and pharynxes suggesting that GJ-related signals activate large-scale patterning modules (e.g., organizers) leading to well-synchronized and orchestrated downstream morphogenetic events. The ability to induce morphogenetic changes that are coherent, without having to micromanage the patterning of substructures, is a very desirable property for the purposes of regenerative intervention.

Long-term alteration of target morphology by transient, physiological modulation

Once disrupted by a transient suppression of these physiological signals, the multi-headed phenotype persists across many weeks and additional rounds of regeneration occurring in the absence of any additional reagents. Direct measurements reveal that the GJ blocker is gone from tissues within days. The striking failure of subsequent regeneration to correct the altered pattern weeks after the blockade of GJC reveals that the GJ-dependent signals have reset the "target morphology" of the animal (the pattern that serves as a comparison template for making decisions about when regeneration is to be initiated and terminated). These results cannot easily be explained by a simple induction of a head with organizer properties (Broun and Bode, 2002) because it occurs in tissue distal to the ectopic head, even after the head is removed. How biological structures encode the morphology to be recreated through regeneration is a fascinating question. These data provide the first molecular entrypoint into the physiological pathway that allows the long-term (or permanent) morphology of a complex adult bodyplan to be experimentally re-specified.

While it is unlikely that random mutagenic effects could consistently cause a formation of a perfect head, we show through a standard mutagenicity assay that the GJ blocker does not change DNA sequence. Normal GJC is known to be restored very quickly after removal of blockers (Chanson et al., 1989), and the half-life of mature gap junctions have been estimated to be less than five hours (Laird, 1996) making it extremely unlikely that any effect on GJ is present in the cuts taking place more than a month after the initial 3-day exposure to octanol. It is not yet known whether the effect is truly permanent or simply long-lived, nor how well multi-headed animals would fare in the wild. However, it should be noted that this physiological modulation results in a drastic alteration of the normal planarian body-plan and behavior that persists across the dominant reproductive mode of this animal (bisection and regeneration). These data may have significant implications for the role of epigenetic change in evolution.

The results highlight the importance of systemic signals for the regulation of stem cell behavior in adult tissues. Moreover, our findings also indicate that GJ proteins regulate stem cell behavior not only through short-range signaling (Oviedo and Levin, 2007a), but also through long-range signals involving neural tissue, allowing the dissection of the long-sought neural regulation of regeneration. Because the complexity of tissue regeneration cannot be fully understood from the perspective of transcriptional networks alone, the novel physiological modulation we reveal here opens novel avenues in which to explore these signals in the complexity of the whole animal.

Our findings provide mechanistic insights into the initial process of regeneration. The data reveal long-range communication between stem cells (neoblasts) and differentiated tissues

mediated by the central nervous system and gap junction coupled cells throughout the worm. Similar long-range regulatory mechanisms have been recently demonstrated to actively modulate the natural microenvironment of bone marrow stem cells (Mendez-Ferrer et al., 2008; Spiegel et al., 2008). The characterization of physiological signals, and their influence upon regenerative morphogenesis, offers unprecedented opportunities for rational modulation of somatic and stem cell behavior in guiding pattern formation in biomedical settings (Levin, 2007b; Sundelacruz et al., 2008; Zhao et al., 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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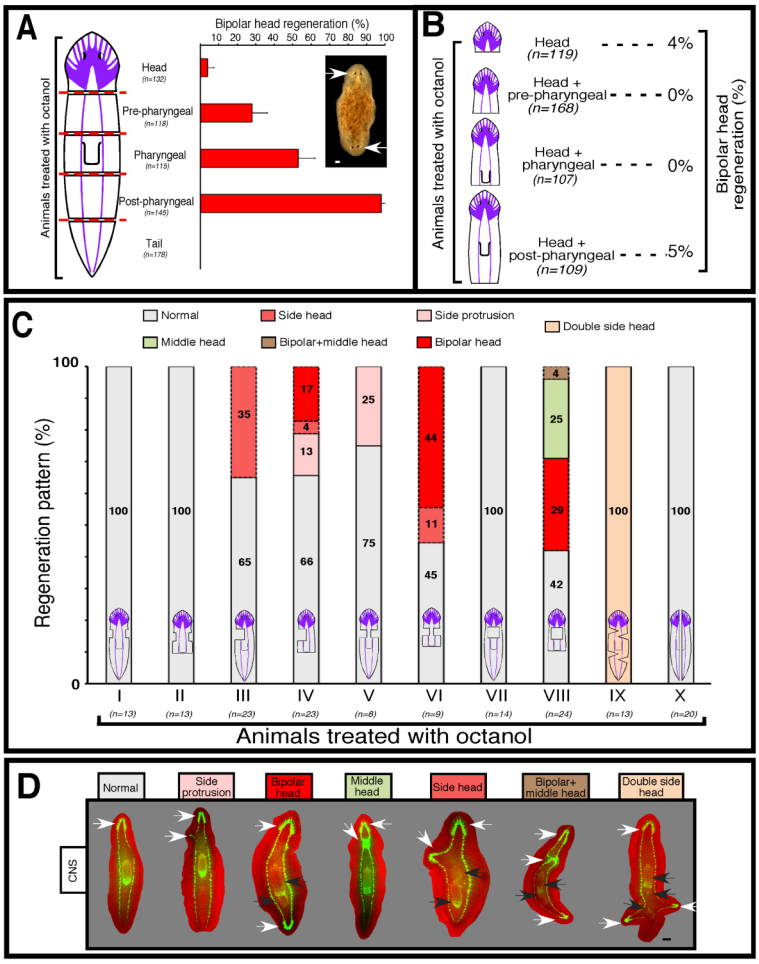

FIGURE 1. Neural cues mediate polarity during regeneration

(A) Schematic of transverse amputations in the presence of octanol. The incidence of bipolar head phenotype gradually increases toward posterior areas. Inset is representative of bipolar-head regenerate (white arrows indicate heads). Red dotted lines indicate level of amputation. Data are presented as mean +/− confidence intervals (95%). **(B)** Schematic representation of transverse amputations along the A/P axis to obtain fragments with a pre-existing head (left). Fragments generated after amputations exhibited very few bipolar-head regenerate. **(C)** Planarians were dissected in different ways (illustrative representation is shown for each case within columns) and the regeneration pattern was recorded after 2 weeks of regeneration. Animals reestablishing the original pattern were considered normal, while worms developing abnormalities were classified into seven categories by macroscopic observations, A/P polarity, and CNS morphological abnormalities. Seven abnormalities were: middle head; side head; bipolar + middle head; side protrusion; bipolar head and double side head (color-coded at the top of the graph). Percentage of animals regenerating each pattern is displayed along with the number of animals (within parentheses) assayed in each condition (Roman numbers). Untreated animals always regenerated normally (not shown for simplicity). Most cases (3/4) where dissections did not disrupt VNC integrity at pre-pharyngeal level regenerated 100% normal animals (conditions I, II, VII and X). Conversely, almost all cases (7/8) where VNC integrity was disrupted in pre or post-pharyngeal area led to regeneration of abnormalities characterized by alterations in CNS patterning and polarity (conditions III-IX). These results suggest that VNC integrity and GJC play important role in regenerative patterning and polarity.

Since all animals regenerated tissues and developed blastemas, treatment with GJ inhibitor does not alter normal response to wound damage but rather instructively influences the identity of the newly formed tissue. Importantly only animals with disrupted VNC integrity and octanol treated led to novel phenotypes **(D)** characterized by multiple heads (white arrows) and pharynxes (gray arrows). Representative images of immunostaining with anti-synapsin antibody (green signal) over a pseudocolored red background are shown. In all cases original anterior end is to the top. Bars represent 200μm.

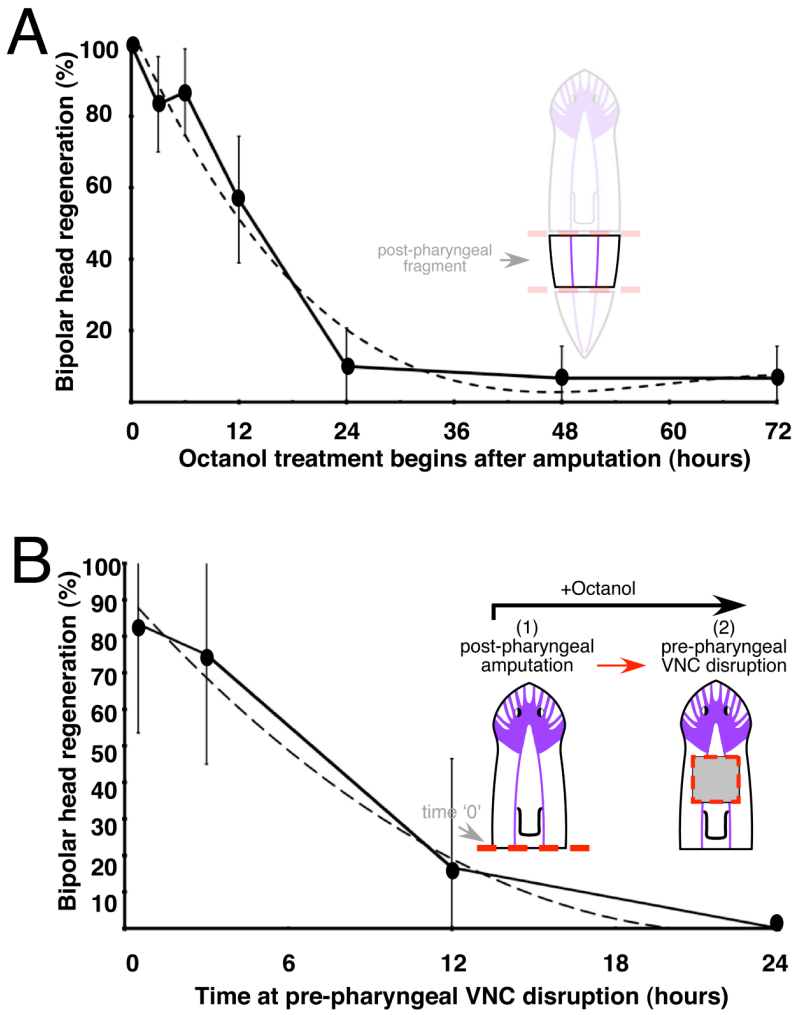

FIGURE 2. CNS-mediated inputs and GJC are required early during phases of regeneration

(A) The percentage of bipolar-head regeneration was recorded from post-pharyngeal fragments in which octanol treatment began at different time points post-amputation. The incidence of bipolar-head phenotype was sharply reduced when GJ blocker (octanol) treatment began a few hours post-amputation, suggesting that critical decisions to identify missing parts and set A/P polarity take place early (within first 3-6 hours) during regeneration. **(B)** Planarians were amputated at post-pharyngeal level, treated with octanol, and subjected to disruption of VNC at anterior areas (red dotted line in pre-pharyngeal area) at different time points after initial post-pharyngeal cut (time 0). Significant A/P duplications were observed when VNC contiguity was disrupted prior to 12 hours after amputation. Numbers in parenthesis indicate the sequence of amputations, being first post-pharyngeal and then VNC disruption at pre-pharyngeal level; all animals were treated with octanol. For each time point n≥ 10 animals. In all cases data are represented as mean +/− confidence intervals (95%) and curve fitting is shown with dotted line.

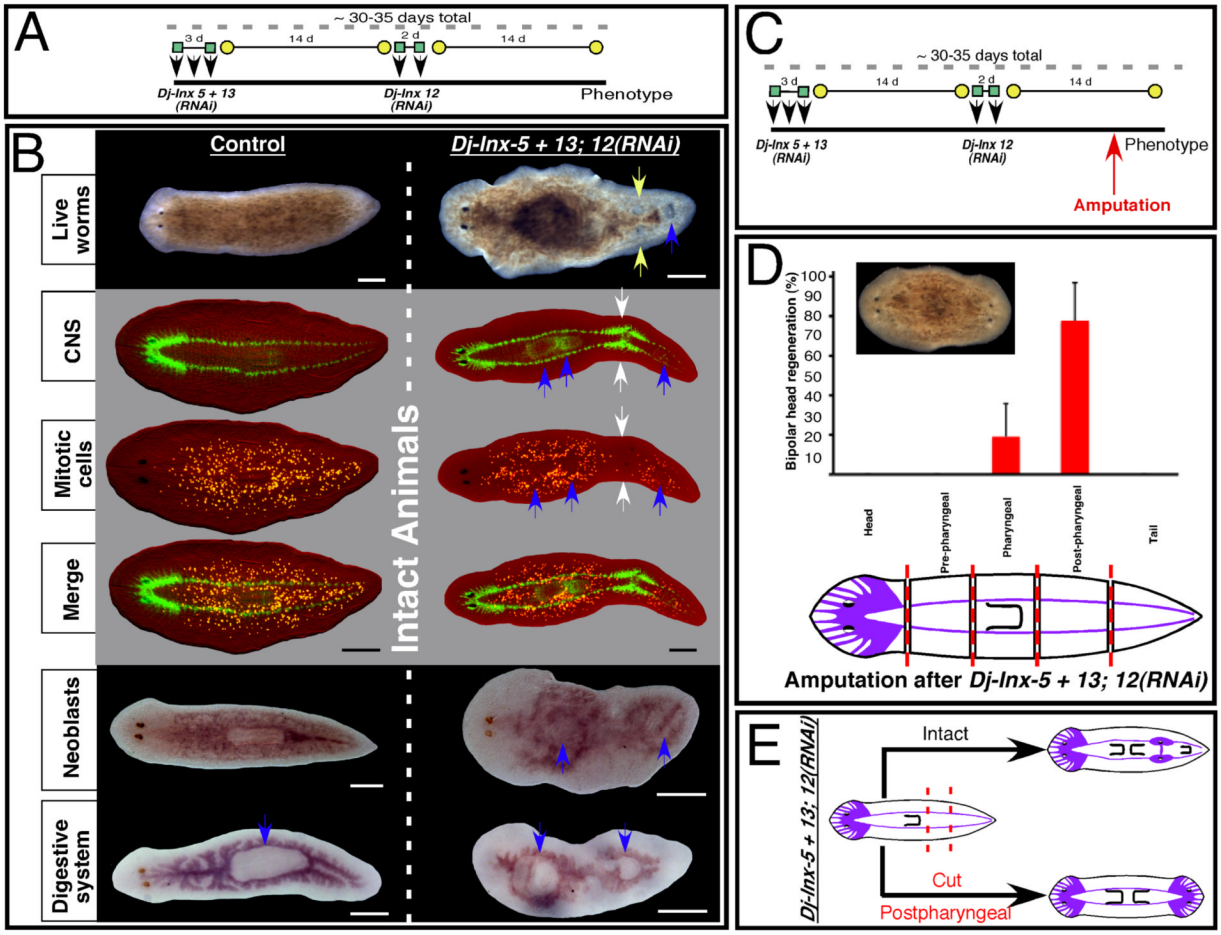

FIGURE 3. Loss-of-function by RNAi of three innexin genes led to A/P polarity phenotype during homeostasis and regeneration

(A) Microinjection schedule of double-stranded RNA (dsRNA) in intact planarians. A total of five injections (arrows between green squares) were performed over 19 days. Injecting a mix (1:1) of *Dj-Inx-5 + 13* dsRNA for 3 consecutive days and 2 injections of *Dj-Inx-12* ds-RNA 2 weeks after first injections led to strong and reproducible phenotypes ~14 days after last injection. Control animals were always run in parallel and injected with water under same schedule. **(B)** Representative phenotypes following 30-35 days since first injection. Conditions: control (water injected) and *Dj-Inx-5 + 13, −12(RNAi)*. Control animals (left column) did not display any behavioral or anatomical abnormalities as assayed by markers of CNS (anti-synapsin antibody), mitotic cells (anti-H3P antibody), proliferative neoblasts (*Dj-Inx-11* antisense probe), and digestive system (*Dj-Inx-7* antisense probe). In contrast, animals subjected to *Dj-Inx-5 + 13, −12(RNAi)* displayed obvious behavioral and morphological changes (right column). Random contractions, ectopic photoreceptor pigmentation (yellow arrow), and extra pharynxes (blue arrows) were evident, especially in post-pharyngeal areas. Ectopic pharynxes, brain tissue (white arrows) and striking alterations in the distribution of neoblasts and the digestive system were observed. Remarkably, the orientation of ectopic brain and pharynx were sometimes reversed with respect to the original A/P axis, and the distribution of neoblasts and digestive system adopted anatomical changes consistent with the new structures developed (e.g.: no mitotic activity surrounding brain and pharynx and formation of primary intestinal branch in post pharyngeal area followed by posterior bifurcation around ectopic pharynx). Bar is 500 μm. **(C)** Microinjection dsRNA schedule for worms amputated

(red arrow) after ~25-28 days of first injection. **(D)** To analyze the roles of *Dj-Inx-5 + 13, −12 (RNAi)* during regeneration, injected animals were amputated at different levels along the A/P axis producing 5 fragments. Pharyngeal and post-pharyngeal fragments regenerated animals with polarity problems (inset representative picture). Data are represented as mean +/− confidence intervals (95%) and red dotted line indicates plane of amputation. **(E)** Summary illustration of phenotypes obtained in intact and regenerating of *Dj-Inx-5 + 13, −12(RNAi)* animals. In both cases, polarity problems and ectopic organs appeared (as after pharmacological inhibition of GJs). The original anterior ends are shown to the left.

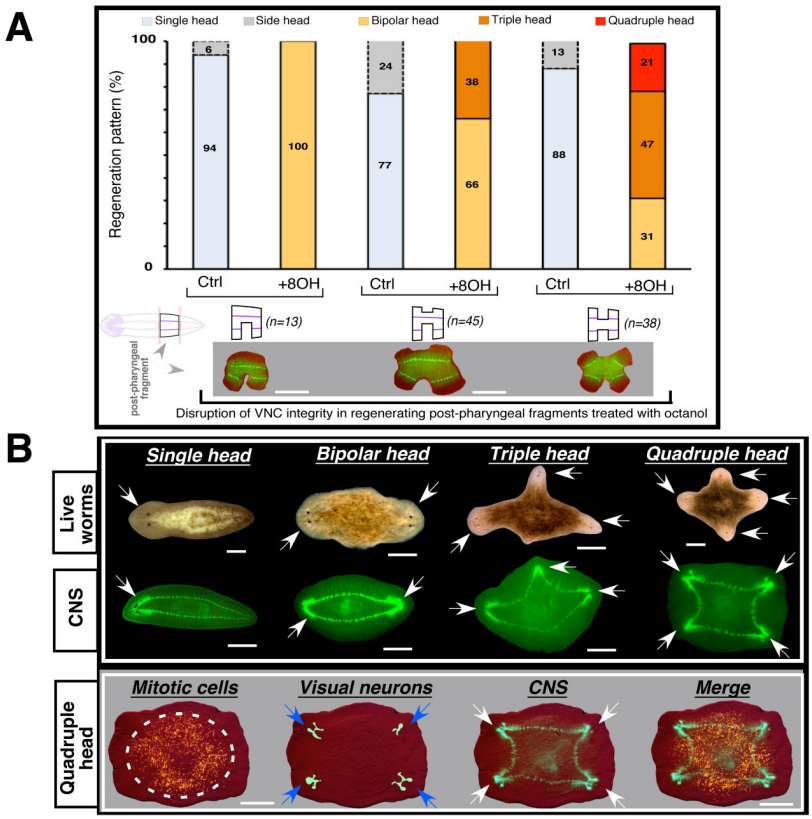

FIGURE 4. GJ-mediated and neural information prevents ectopic and permanent axes formation in lateral and posterior-facing wounds

(A) Post-pharyngeal fragments were cut laterally (once or twice simultaneously) in the presence of octanol or not (control, untreated). In all cases (n>90) treated animals developed A/P polarity problems; when both lateral sides were simultaneously cut (schematics and synaptotagmin immunostaining are shown under graph), the fragment produced bipolar or triple heads. Additionally, lateral amputations disrupting the VNC led some animals (~20%, n=38) to produce anterior structures in all 4 blastemas. Note that untreated (control) animals mostly regenerated single headed worms; in some cases where head duplication was observed, this never affected the polarity of the animal. **(B)** Representative images of animals with single, bipolar, triple, and quadruple heads (white arrows). Triple immunostaining (CNS, mitotic cells, and visual neurons) in quadruple-headed planarians revealed that additional brains were linked by common VNCs (n>10 each). Further, immunostaining in with anti-H3P antibody (recognizes mitotic cells) indicated that mitotic cells were restricted to the new pre-pharyngeal area of each new axis. Visual neurons connecting photoreceptors with brain (assayed by anti-VC-1 antibody) also differentiated within each new brain. Bars are 500 μm.

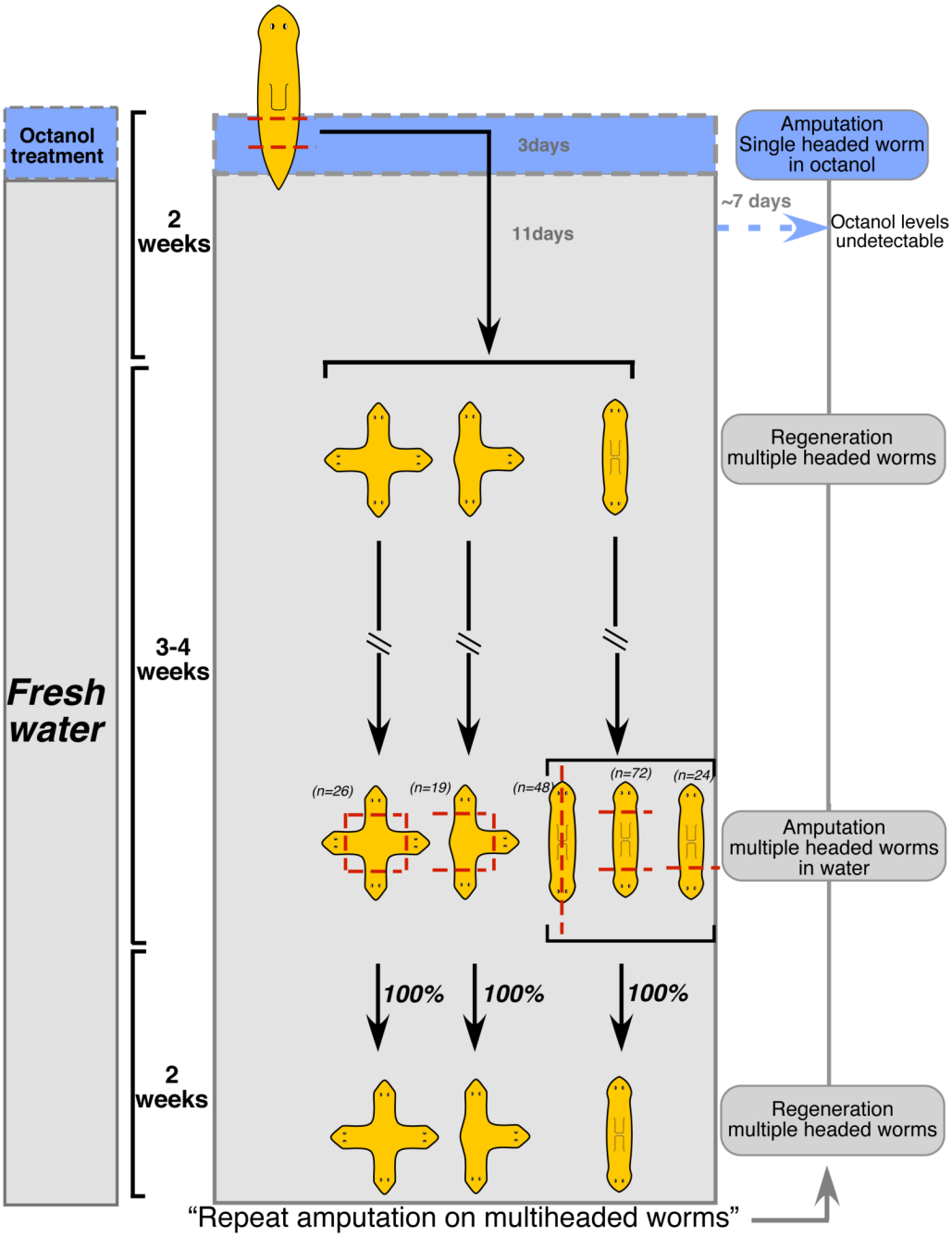

FIGURE 5. Morphogenetic changes persist for several rounds of regeneration in absence of GJ inhibitor

Schematic illustrating that short treatment with octanol (3 days, blue area) is able to induce morphogenetic changes that persist for weeks, and across multiple rounds of regeneration. Multi-headed worms were obtained from post-pharyngeal regenerating fragments amputated in presence of octanol. The period of time in which animals were in fresh water is depicted with gray background. About one month after first amputation, multiheaded worms were amputated in different planes (transversally or longitudinally, dotted lines) along the A/P axis in plain water (in absence of octanol). Transverse amputations produced single or multiple simultaneous decapitations and longitudinal cuts divided bipolar worms in two mirror images

fragments. In all cases, the ectopic heads were regenerated reconstituting multiple headed worms; from longitudinal cuts, two double-headed worms were formed. Total number of decapitations are represented within parentheses. This procedure was repeated two more times and morphogenetic changes persisted (not shown).

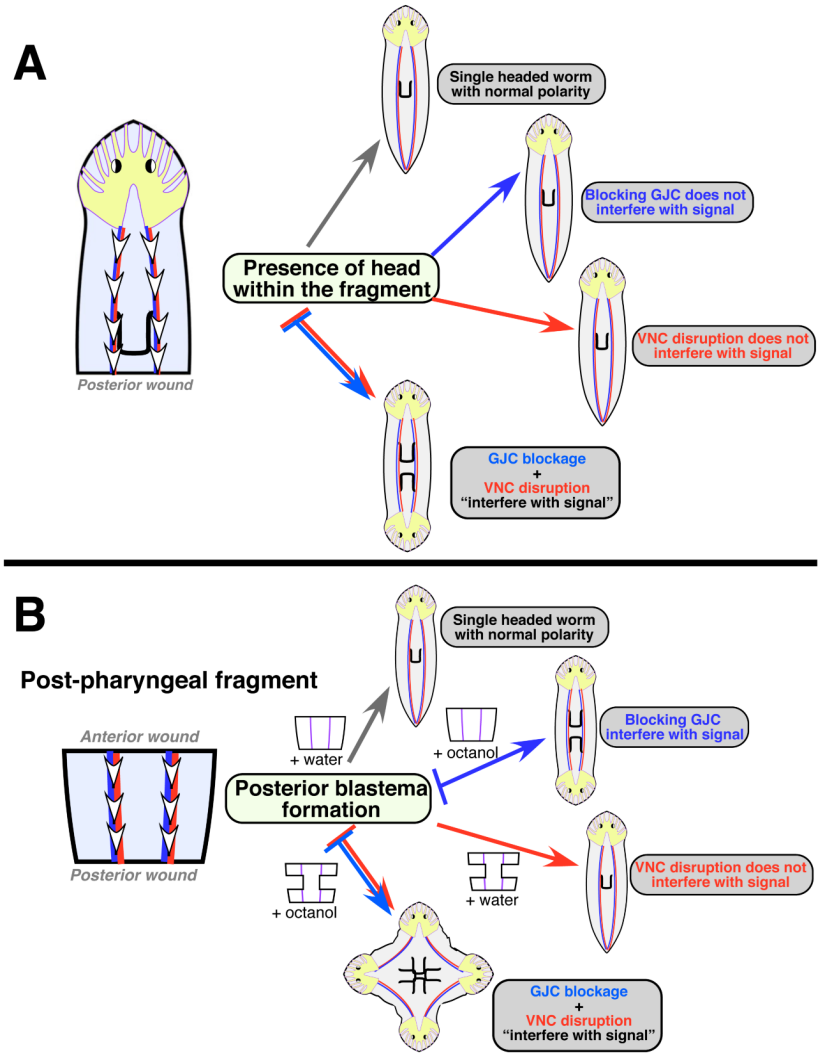

FIGURE 6. Schematic model of GJ-mediated and neural signals during regeneration

(A) Post-pharyngeal amputation generates a regenerating worm with pre-existing head and a posterior wound. Our data suggest that long-range signals –informing about the presence of a head within the fragment-travel from anterior (brain in yellow) to the posterior wound (white arrowheads) through two pathways: ventral nerve (VNC, red line) and GJ-mediated signals associated with VNC (blue line) and parenchymatic cells (light blue background). In all, untreated animals or animals exposed to GJ blocker (octanol) and animals with VNC disruption regeneration of the missing posterior area is recreated without polarity problems. However, in animals where both octanol treatment and VNC disruption take place, signals coming from anterior areas are disrupted, leading to abnormalities in polarity (bipolar animals). **(B)** Blastema fate determination in regenerating post-pharyngeal fragments with anterior and posterior-facing wounds. Instructive signals from anterior wound travel to posterior wound through the same pathways as in "A" but in this case the information carried is to inform about the anterior blastema formation that instruct neoblasts to form a posterior blastema. In animals exposed to octanol this information is altered and animals regenerate bipolar heads. In the case of VNC disruption alone most animals regenerate without problems. If both VNC are disrupted and GJ are inhibited, animals regenerate four heads. Specific cuts for VNC disruption are represented for each case.