Regenerative Adaptation to Electrochemical Perturbation in Planaria: A Molecular Analysis of Physiological Plasticity

HIGHLIGHTS

- Exposure to BaCl₂ causes the heads of Dugesia japonica to degenerate.
- Prolonged exposure to BaCl₂ results in regeneration of a BaCl₂-insensitive head.
- Ion channel expression is altered in the head to compensate for excitotoxic stress.
- TRPMa is upregulated in BaCl₂-treated animals; blocking TRPM prevents adaptation.
Regenerative Adaptation to Electrochemical Perturbation in Planaria: A Molecular Analysis of Physiological Plasticity

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SUMMARY
Anatomical homeostasis results from dynamic interactions between gene expression, physiology, and the external environment. Owing to its complexity, this cellular and organism-level phenotypic plasticity is still poorly understood. We establish planarian regeneration as a model for acquired tolerance to environments that alter endogenous physiology. Exposure to barium chloride (BaCl₂) results in a rapid degeneration of anterior tissue in *Dugesia japonica*. Remarkably, continued exposure to fresh solution of BaCl₂ results in regeneration of heads that are insensitive to BaCl₂. RNA-seq revealed transcriptional changes in BaCl₂-adapted heads that suggests a model of adaptation to excitotoxicity. Loss-of-function experiments confirmed several predictions: blockage of chloride and calcium channels allowed heads to survive initial BaCl₂ exposure, inducing adaptation without prior exposure, whereas blockade of TRPM channels reversed adaptation. Such highly adaptive plasticity may represent an attractive target for biomedical strategies in a wide range of applications beyond its immediate relevance to excitotoxicity preconditioning.

INTRODUCTION
A fundamental challenge faced by all living things is survival in an uncertain and changing environment. One of the fundamental goals of biology and medicine is to understand how physiology and morphology, whether at the cell or organism level, adjusts to stress—conditions that are not conducive to continued health and reproduction. Living tissues are confronted with continuous environmental challenge at all scales of size and organization, from exposure to agents that damage DNA and produce reactive oxygen species at the level of single cells to secreted factors and cell group activity that drive embryogenesis and repair/regeneration; to the physiological functions of immune, circulatory, and endocrine systems; and ultimately to the behavior of the entire animal. Thus, all components of an organism must continuously select from a very large space of possible transcriptional, protein-level, and physiological-level responses to adaptively maintain homeostasis along numerous parameters during its lifespan. Understanding this ubiquitous process is vital, with direct applications to reprotoxicology (the ability of embryos to regulate, or fail to regulate, their normal developmental sequence under diverse, potentially teratogenic influences), immunology, aging, and cancer. Harnessing this process is also a goal of regenerative medicine.

Major gaps remain in our understanding of the fundamental biology of computation in living media: how do cellular networks process real-time information toward near-optimal responses? On a population scale, recent work with bacterial persisters and yeast subjected to antibiotic and metabolic stress, respectively, reveals evolutionary aspects of epigenetic tolerance to stressors (Lopez Garcia de Lomana et al., 2017; Samani and Bell, 2016; Gonzalez and Bell, 2013; Kussell et al., 2005; Balaban et al., 2004; Tkachenko, 2018; Lambert and Kussell, 2014). However, experiments in *Drosophila* and other model systems reveal that, even within the lifetime of single individuals (i.e., not via selection), cells have the remarkable ability to execute appropriate responses to stressors (Soen et al., 2015; Elgart et al., 2015; Stern et al., 2012; Soen and Braun, 2000; Karin et al., 2016). This emerging body of work on cellular adaptation and plasticity (Stetina et al., 2015; Prymaczok et al., 2016) is complemented by similar robustness observed at the organ or even whole-organism scale (Pezzulo and Levin, 2015; Brunke and Hube, 2014; Sorek et al., 2013; Freddolino and Tavazoie, 2012).

One of the best examples of large-scale dynamic plasticity is regulative development, which is often able to adjust to drastic injury (Cooke, 1981; Tarkowski, 1961). One example can be seen in the processes that

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https://doi.org/10.1016/j.isci.2019.11.014
convert a tadpole face to that of a frog, which requires significant movements of the eyes and other organs to rearrange internal head structures into the correct species-specific outcome. It was recently shown that this is not a hardwired process in which the different components move in pre-determined paths (Vandenberg et al., 2012) and rely on a constant starting state to implement normal frog development. Tadpoles engineered to have scrambled anatomies (eyes, nostrils, and other structures in highly abnormal positions) still usually develop into normal frogs. Thus, the genome encodes a system with the ability to reach the required anatomical state from a range of diverse starting conditions, dynamically adapting to (evolutionarily) unexpected circumstances and readjusting the various tissues as needed to optimize subsequent survival.

Beyond development, metazoan survival throughout the lifespan requires a constant and active resistance to aging, wear and tear, and carcinogenic transformation (Rubin, 1985, 2006, 2007). Moreover, some species are capable of large-scale remodeling and pattern homeostasis throughout their lifespan. Planarians are a class of free-living flatworms that contains many highly regenerative members (Cebrìà et al., 2018; Saló et al., 2009). The freshwater planarian Dugesia japonica is one such species, able to restore an entire body (including the brain) from a mere fragment, scaling the growth with exquisite precision and ceasing further growth and remodeling when an allometrically correct body plan is complete (Levin et al., 2019; Sahu et al., 2017; Saló et al., 2009). Planarians possess bilateral symmetry, stem cell populations (Hill and Petersen, 2015; Tanaka and Reddien, 2011), and a true centralized brain with numerous complex behavioral responses including learning (Sarnat and Netsky, 1985; Corning and Freed, 1968). They appear to have even conquered the ultimate stressor—aging (Sahu et al., 2017; Petralia et al., 2014). Much progress has been made on the biochemical (Reddien, 2018; Owlim and Bartscherer, 2016) and physiological/bioelectrical (Lange and Steele, 1978; Chan et al., 2014; Durant et al., 2017; Sullivan et al., 2016; Beane et al., 2011, 2013) control of patterning during regeneration, making them an ideal model system in which one can investigate adaptive responses to novel stimuli that threaten homeostasis.

To establish a model for the study of adaptation at multiple levels of organization, we chose regeneration in D. japonica as a context to determine how cellular and body-wide plasticity must cooperate. Owing to the known dependence of planarian remodeling response on bioelectric events (Levin et al., 2019; Durant et al., 2017, Emmons-Bell et al., 2015; Beane et al., 2011, 2013; Oviedo et al., 2010), we challenged planaria with a novel stressor: the non-specific potassium channel blocker barium chloride (BaCl₂). The inability to pass potassium ions led to a severe head degeneration. Remarkably, however, the planaria soon regenerated a BaCl₂-tolerant head. We analyzed these BaCl₂-adapted heads using molecular and physiologic approaches, which revealed an example of transcriptional and physiological plasticity with significant implications for basic biology and neural injury repair.

RESULTS
When Exposed to BaCl₂, Planaria Heads Rapidly Degenerate and Subsequently Regenerate with Newly Acquired BaCl₂ Resistance

Given the ubiquitous importance of potassium channels in cell physiology, we first asked what would happen to planaria continuously exposed to broad K⁺ flux inhibitors. We cultured D. japonica flatworms in 1 mM barium chloride (BaCl₂), a powerful, widely used, universal potassium channel blocker (Jiang and Mackinnon, 2000; Latorre et al., 1997), which can also affect other divalent cation channels and has been extensively used in invertebrates (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Hanrahan et al., 1986). We observed rapid and complete degeneration of the entire head within 72 h (83% of observed worms showed degeneration, SD = 12% across all BaCl₂ treatments, Figure 1Ab), consistent with the importance of potassium currents in maintenance and physiology of this complex organ. Remarkably, however, when left to regenerate in fresh BaCl₂ solution, the worms regenerated normal heads that were insensitive to the toxic environmental conditions (Figures 1Ac and 1Ad). Although BaCl₂ could potentially form insoluble ions with sulfate and carbonate ions present in the Poland Spring water, we ruled out adaptation via reduction of BaCl₂ concentration in the media by making sure that media was frequently refreshed. Furthermore, degeneration of anterior tissues was never observed in control culture conditions (Figure 1Aa). Heads regenerated in BaCl₂ retained normal size and morphology (Figure 1Ad), and the BaCl₂-adapted worms exhibited motion that was not visually different from that of wild-type (WT) worms.

Degeneration involved tissue progressively bubbling off from the very anterior tip of the worm to the plane of the photoreceptors (roughly 1 mm of tissue), with apparent muscular contraction around the
Degeneration site that minimized internal tissue loss (Figure 1Ab). The process was complete after 72 h in BaCl₂ solution. Blastema formation was then initiated, and regenerative processes proceeded, although full regeneration took longer than normal (~4 weeks, as opposed to ~2 weeks, Figure 1Ac). After regeneration of the head, the worms were transferred into a fresh BaCl₂ solution and showed no signs of degeneration. We conclude that BaCl₂ exposure is acutely toxic to planarian anterior tissues but that the worms have the ability to produce new heads that are completely adapted to this novel, harsh condition.

Acquired Resistance to BaCl₂ Is Lost after 30 Days in Water

We then investigated whether the acquired adaptation to BaCl₂ was permanent or temporary. D. japonica worms were allowed to degenerate and regenerate heads in 1 mM BaCl₂ over the course of approximately 35 days (Figure 1Ba). They were then transferred to Poland Spring water for 30 days, with the water refreshed every 7 days (Figure 1Bb). They were then subjected to a second round of 1 mM BaCl₂ treatment. All worms displayed characteristic degeneration of anterior tissues after the second round of BaCl₂ treatment, revealing that the insensitivity to BaCl₂ is lost after 30 days in plain water (Figure 1Bc).

Transcriptionally Profiling the BaCl₂-Adapted Head

What is different about the new, BaCl₂-compatible heads when compared with the WT planarian head—what changes allow survival in BaCl₂ after the initial adaptation? We hypothesized that the conferred resistance to BaCl₂ treatment could be due to altered expression of ion channels or pumps that could compensate for, and buffer, the physiological perturbation. To test this hypothesis, we performed RNA sequencing (RNA-seq) and sub-network enrichment analysis (SNEA) on head samples from worms that were either treated with BaCl₂ and allowed to regenerate insensitive heads or kept in water following the BaCl₂ treatment. Table S1 provides all upregulated and downregulated transcripts identified. We report the total
number of reads generated for each sample as well as a table of the alignment percentage of the reads mapped to the reference in Tables S2 and S3, respectively. GO and SNEA data are provided in Tables S4 and S5, respectively. A subnetwork enrichment was also conducted to enhance the overall dataset and to provide additional information on processes affected in the BaCl2-resistant heads (Table 1).

We detected a number of transcripts that were up- and downregulated after BaCl2 exposure (Table S1, q-value<0.05, change > log2 = 3), and genes that were only identified in one condition but not the other were listed as highly changed in the appropriate table. Significantly upregulated genes included metalloendopeptidase, palmitoyl transferase, Slc17a1, NPYR-14, Rab-protein 8, SMAD6/7, and Slc2a1 (Table 3). Downregulated genes included Propionyl-CoA synthetase, CyclinA-like protein, fibropectin, Mariner Mos1 transposase, L-lactate dehydrogenase, TBC1 domain family member 9B, SPSB1, Slc38a2, C3H-zinc finger-containing protein 1, ybox protein 4-like protein, plastin 1, and innexin (Table 4). Interestingly, some ion channels and pumps were differentially expressed (Tables S1, 3, and 4) (p < 0.05). For example, Transient receptor potential ion channel Ma Dj-TRPMa (Fragment) was upregulated, appearing in the BaCl2-adapted heads but below detection in the wild-type, as well as an uncharacterized voltage-gated potassium channel, Slc17a-1, and NPYR14.

More broadly, downregulated functional sets in BaCl2-resistant worms included transmembrane potential, anion transport, and cell membrane depolarization, whereas processes such as innervation, long-term synaptic depression, and Na+ influx co-transport were increased in mean expression (Figure 2A and Tables 1 and S6). Many processes related to the immune system were upregulated, including leukocyte accumulation, leukocyte recruitment, monocyte recruitment, and T cell response (Table 1). Enriched gene networks were involved in both cellular structure and differentiation, as well as cell membrane depolarization confirming transcriptional rewiring of the bioelectric machinery (Table 1). We conclude that BaCl2-adapted heads exhibit transcriptional changes reflective of several subsystems’ adaptation to this novel bioelectrical condition and include proteins that could implement physiological homeostasis in response to BaCl2. Tables 1, S4, and S5 and Figure 2A all depict networks for cell processes that were altered in the BaCl2-adapted heads.

To ensure that the RNA-seq identified transcripts that were up- or downregulated in the BaCl2-adapted heads, we performed qPCR. qPCR for Dj-TRPMa using primers from (Inoue et al., 2014) showed a 2.4-fold increase in transcripts from BaCl2-adapted heads over WT (Figure 2Ba). qPCR for Slc2a1 showed a 1.3-fold increase in transcript abundance in BaCl2-adapted heads compared with WT (Figure 2Bb). Although a bit lower in magnitude than reported in the RNA-seq, both showed similar upregulation in BaCl2-adapted heads compared with WT. Thus, despite using a broad platyhelminthes transcriptome, we were able to identify transcripts that were indeed upregulated in BaCl2-adapted heads.

An Excitotoxicity-Based Model of BaCl2-Induced Neural Apoptosis and Adaptation

We next sought to understand the mechanism of BaCl2’s effect and the striking ability of planaria to overcome it with a newly regenerated structure. One plausible explanation for the head degeneration effect is induction of BaCl2-induced drop in resting membrane potential and subsequent neural excitotoxicity in the neuron-rich head of the planarian (Walter et al., 2001, Wright, 2004). Our model is based on the premise that an excitotoxic cascade is initiated by neuronal depolarization induced by the broad potassium channel blocker BaCl2. In other systems, strong depolarization activates V_memb-gated Ca^{2+} channels, which causes neurotransmitter exocytosis (McMahon and Nicholls, 1993; Sihra et al., 1993). As glutamate signals extracellularly via V_memb-depolarizing ionotropic receptors, this further increases cytosolic Ca^{2+}, leading to a positive feedback cycle (see “excitotoxic positive feedback” of Figure 2Cb). We also postulate that disruption of ion balance via BaCl2 should lead to the upregulation of channels and transporters that can help the tissues compensate by breaking positive feedback cycles.

The dynamics of our model under initial BaCl2 exposure and adapted state are shown in Figure 2C. The model makes several specific predictions, shown in Table 2. First, one of the immediate physiological effects of the BaCl2 should be a significant depolarization. Second, manipulation of several components (blockade of calcium channels and chloride channels, activation of dopamine signaling) should prevent the excitotoxicity storm and thus prevent head degeneration due to BaCl2 exposure. Third, blockade of TRPMa channels should counteract the adapted state and lead to head degradation even after adaptation to BaCl2. We thus tested each of these specific predictions in the BaCl2 degeneration and adaptation assay.
| Process                                | Gene Set Seed                  | # Of Measured Neighbors | Median Change | p Value |
|----------------------------------------|--------------------------------|-------------------------|---------------|---------|
| Neural/Voltage                         | Cell membrane depolarization   | 5                       | -1.86         | 0.037   |
|                                        | Cell-cell signaling            | 10                      | -1.29         | 0.036   |
|                                        | Anion transport                | 17                      | -1.17         | 0.049   |
|                                        | Transmembrane potential        | 85                      | -1.09         | 0.032   |
|                                        | Synapse structure              | 8                       | 1.05          | 0.019   |
|                                        | Long-term synaptic depression  | 50                      | 1.16          | 0.010   |
|                                        | Synaptogenesis                 | 83                      | 1.17          | 0.045   |
|                                        | Nervous system physiology      | 26                      | 1.24          | 0.047   |
|                                        | Neuronal plasticity            | 46                      | 1.24          | 0.013   |
|                                        | Transmission of nerve impulse  | 61                      | 1.28          | 0.012   |
|                                        | Regulation of action potential | 35                      | 1.42          | 0.005   |
|                                        | Innervation                    | 31                      | 1.88          | 0.003   |
|                                        | Neuronal guidance              | 5                       | 2.17          | 0.033   |
|                                        | Transmembrane signaling        | 8                       | 2.46          | 0.012   |
|                                        | Na+ influx co-transport        | 8                       | 2.99          | 0.002   |
|                                        | Neurotransmitter uptake        | 5                       | 3.73          | 0.019   |
| Immune                                 | T cell response                | 71                      | 1.04          | 0.041   |
|                                        | Monocyte recruitment           | 19                      | 1.42          | 0.027   |
|                                        | Leukocyte recruitment          | 25                      | 1.52          | 0.016   |
|                                        | Leukocyte migration            | 29                      | 1.58          | 0.043   |
|                                        | Leukocyte accumulation         | 12                      | 1.88          | 0.035   |
|                                        | Neutrophil adhesion            | 18                      | 1.88          | 0.042   |
| Other                                  | Melanocyte differentiation     | 7                       | -2.44         | 0.002   |
|                                        | Microtubule depolymerization   | 9                       | -2.26         | 0.011   |
|                                        | Stem cell maintenance          | 38                      | -1.20         | 0.043   |
|                                        | Stem cell proliferation        | 50                      | -1.15         | 0.005   |
|                                        | Melanogenesis                  | 21                      | 1.01          | 0.047   |
|                                        | Memory                         | 127                     | 1.05          | 0.036   |
|                                        | Endothelial cell function      | 54                      | 1.06          | 0.034   |
|                                        | Intracellular signaling cascade| 23                      | 1.24          | 0.004   |
|                                        | Endothelial cell development   | 8                       | 1.42          | 0.021   |
|                                        | Positive chemotaxis            | 12                      | 2.63          | 0.029   |

Table 1. Categories of Transcripts Altered by BaCl₂ Adaptation
Differentially expressed cell processes following degeneration and regeneration of a BaCl₂-insensitive head, focusing on processes related to membrane potential homeostasis and regeneration. The number of items in the pathway, as well as the median fold change of the network and the p value, is reported for each enriched process. All subnetworks are presented in Table S5.
Figure 2. Possible Mechanism of BaCl₂-induced Head Degradation Via Excitotoxicity, and Subsequent Adaptation

(A) Pathway Studio v10.0 was used to perform pathway analysis of RNA-seq data for (a) anion transport and (b) transmission of nerve impulse, two pathways critical to deprogression and regeneration of the planaria head. Tables S6A and S6B contain details of all of the components listed here.

(B) qPCR validation of two transcripts identified as upregulated in the RNA-seq—(a) Dj-TRPMa and (b) Slc2a1. Points represent levels for individual worms normalized to GAPDH. Line indicates median. Unpaired t tests were performed to assess significance, **p < .01, *p < .05, n = 3 for each condition.

(C) Proposed regulatory networks detailing (a) normal/un treated state, (b) the main excitotoxicity-related feedback induced by BaCl₂, and (c) proposed adaptations to BaCl₂ treatment. Red lines with flat endpoints show an inhibitory/downregulation relationship, whereas blue lines with circular endpoints show an activating/upregulatory relationship. Under normal conditions, K⁺ channels hyperpolarize the cell to regulate membrane excitability and therefore maintain an open state of CaV channels and upregulate glutamate signaling (a). Ba²⁺ is proposed to induce excitotoxicity in neurons by blocking K⁺ channels, leading to significant \( V_{mem} \) depolarization, which activates voltage-gated Ca²⁺ channels (CaV)
Calcium and Chloride Blockers Prevent BaCl₂-Induced Degeneration

The central component of our model is an excitatory storm and the attendant positive feedback loop involving ion channels and neurotransmitters (Figure 2C). Thus, it predicts that interrupting this loop (inducing neuroprotection) by targeting specific dopamine machinery (Vaarmann et al., 2013; Cepeda et al., 1998; Odaka et al., 2014), chloride channels (Hasbani et al., 1998; Rungta et al., 2015; Takeuchi et al., 2011; Chen et al., 1999), or L-type calcium channels (Szydlowska and Tymianski, 2010; Mark et al., 2001; Hasbani et al., 1998), should prevent degeneration.

To test these predictions, we exposed worms to a combination of BaCl₂ and one of several blockers (previously characterized in planaria or other invertebrates), tracking the incidence of degeneration over 3 days and comparing with BaCl₂-only controls. All drugs had no effect in the absence of BaCl₂ (Figure S2). Since dopamine signaling is both a target of our model and one of the pathways identified by the RNA-seq as altered in the BaCl₂-adapted worms (Table S5), we used bromocriptine mesylate (0.5 μM) to activate monoaminergic signaling. Bromocriptine has been shown in mammals to be a dopamine agonist (Liberante et al., 2016; Oda et al., 2008; Parmar et al., 1984; Schneider et al., 1984; Via et al., 2010) but in D. japonica appears to have broader functions, agonizing dopaminergic signaling (Chan et al., 2014) and antagonizing serotonergic signaling (Chan et al., 2016). Previous work in D. japonica has shown that dopamine and serotonin oppose one another in head regeneration (Chan et al., 2014), indicating that bromocriptine targets general monoaminergic pathways.

We hypothesized that bromocriptine would modulate monoaminergic pathways, suppressing excitotoxic activity. We found that bromocriptine was effective at blocking degeneration with 74% of worms experiencing no degeneration until day 2 (Figures 4Ae and 4Af) and 50% of worms experiencing no degeneration up until day 22. Although this effect is noticeable, 38% of the worms did show head degeneration by day 22 and 9% of the worms died during this treatment. Most likely, this failure to protect the worms from degeneration is related to variability in individual worm response to the drug. NPPB (5 μM) and niflumic acid (1.25 μM) were used as calcium-activated chloride channel blockers. Both of these drugs have been shown to block Cl⁻ channels both in mammalian cells and in C. elegans (Bush et al., 2009; Schriever et al., 1999, White and Aylwin, 1990; Wu and Hamill, 1992). NPPB delayed degeneration in most worms (82%) until day 6, at which point all worms died, suggesting only a partial rescue of

Figure 2. Continued leading to sustained increases in cytosolic Ca²⁺, excess Ca²⁺-induced exocytosis of glutamate, and sustained activation of glutamate signaling, leading to further calcium entry to the cell (b, “excitotoxic positive feedback 1”). Ba²⁺-induced Vₘ depolarization may also open innexin hemichannels, leading to further glutamate release (b). Extracellular glutamate may activate glutamate-gated chloride channels and increased cytosolic Ca²⁺ may activate Ca²⁺-gated chloride channels, leading to chloride secretion from cells and further Vₘ depolarization (b, “excitotoxic positive feedback 2”). Sustained increases in cytosolic Ca²⁺ may initiate apoptosis via caspases, leading to neural degeneration (b). Adaptation to Ba²⁺-induced excitotoxicity is proposed to occur via a variety of changes to gene expression, including upregulation of TRPMa and NPY receptors, which break positive feedback loops supporting excitotoxicity (c).

See also Tables S1, S2–S4, S5, and S6.

Exposure to BaCl₂ Results in Depolarization of Anterior Tissues

Although the molecular and cell-level activity of BaCl₂ has been thoroughly characterized in in vitro preparations (Armstrong and Taylor, 1980; Hanrahan et al., 1986; Kurachi, 1986; Quayle et al., 1988), the effects of BaCl₂ exposure on physiological function and/or regeneration in vivo are largely unknown. Our excitotoxicity model predicts that BaCl₂ should initiate a significant depolarization of the head. To characterize the bioelectric state of the worm after exposure to the potassium channel blocker, we imaged D. japonica worms after 30 min in water (Figure 3Aa) and after 30 min in BaCl₂ (Figure 3Ab) using the voltage reporter dye DiBAC₄(3), which grows brighter with increasing depolarization. We have previously demonstrated that DiBAC₄(3) is a reliable indicator of depolarization in D. japonica (Oviedo et al., 2008; Beane et al., 2011, 2013, Durant et al., 2017, 2019). Figure S1 shows that depolarization with 100 nM of the ionophore valinomycin in combination with 15 mM K⁺-gluconate can be visualized in D. japonica fragments within an hour of treatment. To compare relative depolarization while minimizing confounding factors, Control and BaCl₂-treated worms were mounted and imaged together on the same PIC. After just 30 min in the BaCl₂ solution, worms displayed a significant depolarization of anterior tissues (Figure 3Ab, quantified in 3B), as expected from a K⁺ channel blocker and predicted by our model. This depolarization was most appreciable in the dye DiBAC₄(3), which grows brighter with increasing depolarization. We have previously demonstrated that treated worms were mounted and imaged together on the same PIC. After just 30 min in the BaCl₂ solution, worms displayed a significant depolarization of anterior tissues (Figure 3Ab, quantified in 3B), as expected from a K⁺ channel blocker and predicted by our model. This depolarization was most appreciable in the dye DiBAC₄(3), which grows brighter with increasing depolarization.
the excitotoxic effect (Figures 4Ag and 4Ah). Niflumic acid, on the other hand, was able to prevent head degeneration or death up until day 2 in 92% of the treated planaria but did occasionally induce ectopic eyes (1.9%) (Figures 4Ai and 4Aj). Finally, nicardipine hydrochloride (2.5 μM) was used to block L-type calcium channels (Beane et al., 2011; Hockerman et al., 1997; Mendonca-Silva et al., 2006; Nogi et al., 2009). Nicardipine induced a delay in degeneration in 78% of worms through 2 days of treatment (Figures 4Ak and 4Al). Taken as a whole, exposure to these channel activity modifiers similarly prevented BaCl2-mediated head degeneration (Figure 4A, quantified in 4Am) and mostly also prevented BaCl2-induced death in the planaria, suggesting that these drugs directly acted on the mechanism by which BaCl2 is toxic. We conclude that, as predicted by our model, drugs predicted to break the positive feedback loop of excitotoxicity efficiently suppress the BaCl2-induced head depgression.

### Blocking Adaptation

Conversely, we next asked whether our model also suggested efficacious methods of counteracting the BaCl2-adapted state. A transcript identified at significant levels only in the BaCl2-adapted heads

| Intervention | Reasoning | Experiment |
|--------------|-----------|------------|
| Dopamine signaling agonists (e.g., quinpirole, bromocriptine, cabergoline) | Agonists to dopamine signaling have shown mitigated excitotoxicity (Vaarmann et al., 2013; Cepeda et al., 1998; Odaka et al., 2014) | Dopamine agonist and serotonin 5-HT2-like receptor antagonist bromocriptine significantly delayed head degeneration in the majority of worms for the duration of the test (35 days) compared with control, which showed full degeneration within 24 h (Figures 4Ae and 4Af) |
| Cl− channel inhibitors | Previous studies have shown decreased neural death during excitotoxic cascades in the presence of chloride channel antagonists (Inoue et al., 2007; Chen et al., 1998; Rungta et al., 2015; Liang et al., 2007) | Chloride channel blockers NPPB and NFA significantly delayed head degeneration upon exposure to BaCl2. 12% of NPPB- and 17% of NFA-treated worms showed some degeneration by day 3 compared with 100% of controls (Figures 4Ag–4Aj) |
| Calcium channel antagonists | Excitotoxicity is mediated by high intracellular Ca2+ levels, a component of which arises from neuronal voltage-gated Ca2+ channels (Lai et al., 2014; Szydlowska and Tymianski, 2010). And calcium channel antagonists can prevent neuronal death (Prentice et al., 2015) | L-type calcium channel blocker Nicardipine delayed degeneration in 69% of worms (31% showed some degeneration) through 3 days of treatment compared with 100% degeneration in controls (Figures 4Ak and 4Al) |
| TRPM antagonists | TRP channels show complex involvement in excitotoxicity (Szydlowska and Tymianski, 2010; Zheng and Phelan, 2014; Aarts and Tymianski, 2005; Bengtson et al., 2004; Li et al., 2012). We found that a TRPM channel is significantly upregulated in Ba2+-adapted regenerated heads (Table 3A), suggesting TRPM inhibition may sabotage the mechanism by which planaria adapt to BaCl2 | TRPM antagonist AMTB leads to rapid (less than 24 h) head degeneration in BaCl2-adapted worms, whereas adapted worms maintained in BaCl2 did not degenerate (Figure 4B) |

**Table 2. Summary of Targets Predicted from BaCl2-Induced Excitotoxicity Hypothesis and the Results of Experimental Interventions**
**DISCUSSION**

Planarian Regeneration Can Compensate for Dramatic Physiological Perturbations

Habituation to extreme physiological stressors has been known since the classic experiments of Jollos, who showed that lineages of paramecia exposed to toxins or extreme heat gained resistance that persisted for hundreds of generations (Jollos, 1933, 1934). However, very little information is available on the mechanism of such plasticity taking place in single organisms (i.e., not due to multi-generational selection) (Elgart et al., 2015). Here we established a model for studying the interplay of physiological and transcriptional plasticity on a short timescale. The planarian head degeneration model also has the advantage that it reveals a multi-scale phenomenon: the cellular stress must be coupled to organism-wide patterning networks, as the animal has to rebuild a complex new structure that will maintain its anatomical integrity while the cells within are forced to significantly alter their normal physiological function.

As a model system, we chose planaria because of their remarkable ability to regulate anatomy despite drastic injury (Saló et al., 2009; Owlarn and Bartscherer, 2016), targeting the function of K⁺ channels, as these are required for a wide range of processes at the level of cell behavior (Urrego et al., 2014; Pardo and Stuhmer, 2014) and body-wide patterning (Adams et al., 2016; Dahal et al., 2012; Simons et al., 2015; Masotti et al., 2015). Bioelectric processes underlie behavioral plasticity in the brain, suggesting

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*(Table 3)* was TRPM-a—a member of a family of channels that sense chemical, mechanical, and osmotic signals (opening with cell swelling) and convey signals to the genome via increased intracellular Ca²⁺. Thus, we next blocked TRPM via the well-characterized TRPM blocker AMTB (Lashinger et al., 2008, Yapa et al., 2018), which has previously been shown (and confirmed by RNAi) to be effective in inhibiting TRPM-a in planaria (Inoue et al., 2014). Although exposure to 100 μM AMTB alone did not induce any head degeneration or death (Figure 4Bb), BaCl₂-adapted planaria began degenerating their heads after just an hour and a half of exposure to AMTB (24 h time point shown in Figure 4Bd). By 24 h of exposure, 98% of heads had fully degenerated, whereas worms that were maintained in BaCl₂ showed no degeneration (Figure 4Bc). This rate of degeneration is equivalent to the controls that had never experienced BaCl₂ adaptation (Figures 1Aa, 4Aa, and 4Ab). Furthermore, in the presence of both BaCl₂ and AMTB, 62% of the planaria died within 48 h, suggesting that the mechanism by which they adapted to the BaCl₂ could not overcome both BaCl₂ and AMTB. Thus, we conclude that, consistent with the known mechanisms of excitotoxicity, BaCl₂ adaptation can be erased by TRPM blockade.

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**Figure 3. Visualization of Relative Membrane Potential in WT and BaCl₂-treated D. japonica Flatworms**

(A) Voltage-sensitive dye was used to determine pattern of resting potentials in planaria. White arrowheads indicate the anterior of the worm. Images are pseudocolored to allow for ease of visualization of depolarization patterns, but worms were imaged in the same frame so as not to confound data after pseudocoloring, and all image analysis was done using raw un-colored images. (a) Untreated *D. japonica* flatworm imaged with DiBAC₄(3) dye. (b) *D. japonica* flatworm imaged with DiBAC₄(3) dye after 30 min in BaCl₂. Scale bars, 0.5 mm.

(B) Quantification of average pixel intensities in untreated and BaCl₂-treated worms. Bars represent mean ± SD. Welch’s unpaired t test, ***p = 0.00002.

See also Figure S1.
the hypothesis that ionic mechanisms may be of considerable interest in understanding different kinds of adaptation (Pezzulo and Levin, 2015). Interestingly, recent work in Drosophila mutants revealed the importance of regulation of excitability to buffer against environmental changes (Kim et al., 2017).

**Figure 4. Targeting Ion Channels Allows Modulation of Degeneration and Adaptation**

(A) A variety of drugs targeting ion channels were used to test our excitotoxicity hypothesis. (a and b) D. japonica worm before treatment (a) and after 2 days in water (b). (c and d) Planaria treated with 1 mM BaCl₂ for 0 h (c) show no phenotype, but after 48 h, the head deprogresses (d). (e and f) Planaria in dopamine agonist bromocriptine (0.5 μM) and 1 mM BaCl₂ solution for 0 (e) and 2 days (f). Bromocriptine is able to prevent head degeneration upon exposure to BaCl₂ in 74% of worms (f). (g) Exposure to BaCl₂ and calcium-activated chloride channel blocker NPPB (5 μM) has no effect at the time of treatment, but within 2 days (h) NPPB has prevented head degeneration in 84% of worms. (i and j) Calcium-activated chloride channel blocker Niflumic acid (1.24 μM) exposure in combination with BaCl₂ has no effect at 0 days (i) but prevents head degeneration in 92% of worms within 2 days (j). Scale bars, 0.5 mm. (k) Prevalence of head degeneration phenotype with each of the drug treatments listed in (e–l). The overwhelming majority of worms with head degeneration in the BaCl₂-treated worms are replaced with the majority of worms not experiencing head degeneration when treated with ion channel modulators.

(B) Resensitization of worms to BaCl₂. BaCl₂-adapted D. japonica worms in (a) water, (b) water with 100 μM AMTB hydrochloride, or (c) water with BaCl₂ do not induce head degeneration in BaCl₂-adapted worms. (d) However, treatment with AMTB (100 μM) in addition to further treatment with BaCl₂ resulted in full head degeneration within 2 days. Scale bars, 0.5 mm. (e) Prevalence of head degeneration phenotypes in BaCl₂-adapted, BaCl₂-treated planaria with or without AMTB treatment, as shown in (c) and (d). Treatment with AMTB resulted in a near-complete change from normal heads to fully degenerated heads.

See also Figure S2.
Upon exposure to BaCl₂, a potent non-specific K⁺ channel blocker, planaria experience a striking degradation of the entire head. Remarkably, they are then able to produce a new head that is insensitive to the BaCl₂ (Figure 1A). We characterized this example of large-scale adaptation to a drastic physiological challenge by regenerative processes.

Extended Time in Water Results in a Loss of BaCl₂ Resistance

The acquired resistance to BaCl₂ is not permanent. When BaCl₂-insensitive worms are placed in water for 30 days, they lose their resistance to BaCl₂, and upon a second exposure to BaCl₂ will undergo degeneration and regeneration of anterior tissues again (Figure 1B). Interestingly, 30 days is approximately the time required for cellular turnover in planaria (Pelletier and Sánchez Alvarado, 2007); it is not known yet whether the BaCl₂ adaptation and de-adaptation is in some way tied to the temporal profile of neoblast activity and somatic cell turnover. Taken together, this temporal profile of adjustment to novel stimuli, and its return to normal, highlights the ability of living systems to adaptively, flexibly integrate information from the internal and external environments of the organism via the interplay between physiological information and genetic programs. The fact that BaCl₂ adaptation spontaneously reverses suggests that the adapted state is not a stable attractor in the transcriptional landscape (Huang et al., 2005; Sullivan et al., 2016); instead, it is compatible with a dynamic monitoring system that adjusts to novel stressors but can also detect their cessation. Future development of transgenic strains of planaria expressing novel physiological fluorescent

Table 3. Summary of Transcripts Upregulated in Heads Pre-Conditioned by BaCl₂ Exposure

| Protein Name                              | TCONS     | Gene Name | Log Fold Change | p Value | q Value |
|-------------------------------------------|-----------|-----------|-----------------|---------|---------|
| Metalloendopeptidase (EC 3.4.24.-)        | TCONS_00013146 | NA       | –               | p < 0.001 | 0.004   |
| Voltage-gated potassium channel           | TCONS_00008343 | NA       | –               | p < 0.001 | 0.020   |
| Palmitoyltransferase (EC 2.3.1.225)       | TCONS_00010738 | CLF_106085 | –               | p < 0.001 | 0.022   |
| GCR085                                    | TCONS_00007438 | gr085   | –               | 0.001   | 0.027   |
| Transient receptor potential ion channel Ma DJ| TCONS_00000400 | NA       | –               | 0.001   | 0.030   |
| Slc17a-1 (Vesicular glutamate transporter) | TCONS_00006778 | vglut slc17a-1 | –            | 0.001   | 0.030   |
| NPYR-14 (Neuropeptide Y receptor)         | TCONS_00002461 | npyr-14 | –               | 0.001   | 0.031   |
| Neuroglian                                | TCONS_00013039 | EGR_00,245 | –              | 0.001   | 0.031   |
| Ubiquitin thioesterase (EC 3.4.19.12)     | TCONS_00004623 | NA       | –               | 0.001   | 0.040   |
| Slc2a-1 (Glut1, glucose transporter)       | TCONS_00003196 | slc2a-1  | 3.11838         | 0.001   | 0.030   |
| SMED-SMAD6/7-2                            | TCONS_00000433 | NA       | 2.31825         | 0.002   | 0.050   |
| Clone ZZD1463 mRNA sequence (Rab-protein 8)| TCONS_00005887 | Rab8     | 2.12996         | p < 0.001 | 0.004   |
| Aquaporin                                 | TCONS_00007316 | aquaporin | 2.01898         | p < 0.001 | 0.004   |

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reporters will be invaluable in tracing the exact temporal profiles of signaling cascades during this process in vivo.

**BaCl$_2$ Adaptation Involves a Unique Transcriptional Signature**

To investigate the mechanism of head degeneration and subsequent BaCl$_2$ tolerance, we analyzed transcriptomes comparing WT heads with BaCl$_2$-adapted heads. Importantly, we analyzed completely regenerated heads, so that the profiles would reflect not mechanisms involved in head regeneration per se, but differences in mature normal and BaCl$_2$-insensitive heads. RNA-seq analysis revealed a number of transcriptional differences that distinguish WT (BaCl$_2$-sensitive) heads from BaCl$_2$-adapted heads (Tables 3 and S1). Unfortunately, no gain-of-function technology exists in planaria that can be used to misexpress transcripts; however, we exploited the druggable nature of some of the key targets to individually modulate both the degeneration and adaptation phases (Figure 4). It must be noted that important changes can occur at the level of physiology, not transcription, and it is likely that additional mechanisms of plasticity that are invisible to RNA-seq remain to be characterized in future work.

**Transcriptional Rewiring of Bioelectric Networks**

One of the most salient aspects of the RNA-seq dataset was the identification of changes in genes involved in regulating bioelectric state (Table 1). Overall, 1.98% of the transcripts identified by RNA-seq in planaria were affected by BaCl$_2$ exposure (q < 0.05, >2-fold change). A number of channels and pumps are altered in the transition from BaCl$_2$ sensitivity to adaptation, including calcium-sensitive transporters, solute transporters, voltage-gated channels, and others (Tables S1, 1, and 3). Interestingly, many of these channels are transporters, suggesting that the physiological buffering of a depolarizing treatment is dependent on employing alternative means of transporting ions into and out of anterior cells and tissues. Newly developed platforms for bioelectric simulation will enable construction and analysis of quantitative physiological models linking post-translational ion flow dynamics (channel opening/closing) to transcriptional regulation and may explain how the observed changes in the electrogenic mRNA profile can compensate for BaCl$_2$ exposure (Pietak and Levin, 2016; Cervera et al., 2015, 2016a, 2016b). Future functional analysis will also investigate physiological repercussions of detected transcriptional changes.

**An Excitotoxicity Model of Degeneration and Adaptation**

To understand this striking phenomenon, we formulated and tested a model based on excitotoxicity (Mark et al., 2001; Szydlowska and Tymianski, 2010). We propose that depolarized V$_{mem}$ resulting from Ba$^{2+}$ block of K$^+$ channels (Walter et al., 2001), as well as the Ba$^{2+}$ ion’s ability to substitute for Ca$^{2+}$ in a variety of processes (Condrescu et al., 1997, Dingledine et al., 1992, Zhou et al., 2012, Ni et al., 2014), induces glutamate-mediated excitotoxicity (Figure 2C). It was hypothesized that Ba$^{2+}$ induces a strong depolarization of V$_{mem}$ by blocking K$^+$ channels (Wright, 2004; Walter et al., 2001), which in turn activates voltage-gated Ca$^{2+}$ channels, leading to increases in both intracellular Ca$^{2+}$ and potentially Ba$^{2+}$ (Mark et al., 2001; Szydlowska and Tymianski, 2010). Increased Ca$^{2+}$ and Ba$^{2+}$ then induce glutamate exocytosis, which may signal at ionotropic glutamate receptors to further depolarize V$_{mem}$ and increase cytosolic Ca$^{2+}$ and Ba$^{2+}$ in positive feedback (Mcmahon and Nicholls, 1993, Shiha et al., 1993; Mark et al., 2001; Lau and Tymianski, 2010), leading to an excitotoxic cascade and neural death via Ca$^{2+}$ activation of apoptosis (Zhang and Bhavnani, 2005; Mcmahon and Nicholls, 1993). One of the key predictions of our model is that a strong anterior depolarization should be present early in the process, which was indeed observed (Figure 3).

Remarkably, the organism is able to alter its transcriptional profile to regenerate new heads that are resistant to BaCl$_2$. Neuroprotective changes in BaCl$_2$-adapted heads include increased expression of neuropeptide Y receptors and dramatically decreased expression of innexins (invertebrate gap junctions), both of which have been previously shown to inhibit glutamate-induced excitotoxicity. Increased NPY signaling has been found to modulate excitotoxicity (Greber et al., 1994; Grundemar et al., 1991a, 1991b; Whittaker et al., 1999; Silva et al., 2005) and may act through inhibition of Ca$^{2+}$ channels (Grundemar et al., 1991a, Mccullough et al., 1998). Past work in numerous systems has shown a strong neuroprotective effect of gap junction blockade in excitotoxic cascades, where gap junction inhibition has shown significant reduction in neural death (Wang et al., 2010, 2012; Galinsky et al., 2017; Takeuchi et al., 2011; Thompson, 2015; Belousov et al., 2017). Other changes include increased expression of a TRPM channel, increased aquaporin channels, and increased levels of a voltage-gated K$^+$ channel, which appear to convey neuroprotective adaptations to the regenerated heads. Decreases in glutamine transporter Slc38a2 (which increases neural glutamate via the glutamine-glutamate cycle [Bak et al., 2006]) were also found. These findings
reveal the transcription-level acquisition of tolerance to a significant toxic challenge by planarian regeneration. Additional adaptive changes, at the protein or physiological levels, may also have occurred and will be studied in subsequent work.

**Ion Channels’ Roles in Degeneration and Adaptation**

Our model implicated specific classes of targets in the degeneration and adaptation process. Indeed, we found that blockade of calcium and chloride channels were able to counteract the toxic effects of BaCl₂ exposure (Figures 4Ag–4Al). Likewise, monoaminergic activation was also able to prevent degeneration (Figures 4Ae and 4Af). These results demonstrate that an adaptive stress response (BaCl₂ insensitivity) can be artificially induced without prior exposure to the actual stressor. Importantly, our model also enabled identification of a simple intervention to reverse the adapted state—TRPMa channel blockers rapidly induced BaCl₂-sensitivity to BaCl₂-adapted heads (Figure 4B), revealing the induction of TRPMa after BaCl₂ exposure as a key functional step in the observed regenerative plasticity. Although any pharmacological reagent may have additional targets, the use of several diverse channel drugs to cleanly abrogate a very specific process, and improve tissue health and integrity (i.e., not simply cause toxicity), supports the predictive value of our model. Any future models of this process can likewise be evaluated by their ability to identify reagents that enable modulation of the plasticity process. The use of small molecule drugs to target these kinds of processes in vivo is an important complement to genetic manipulation studies, as it may allow therapeutic applications that do not require gene therapy. Targeting both phases of the adaptive response (the initial toxicity and the resulting adapted state), as shown in our data, represents an important strategy in applications.

**Conclusion**

Planaria are champions of plasticity and robustness, not only repairing their anatomy after amputation in normal conditions, but also apparently able to adjust their physiology to survive and regenerate under significant physiological perturbation. This occurs within an individual’s lifetime, not via population selection, revealing the ability of tissues to activate appropriate responses to relieve physiological stressors. Using this and similar tractable models will allow a better understanding of the dynamics of biological circuits, not only hardwired activity emergent from genetically encoded features, but also flexible adaptation to unpredictable stimuli. The study of the mechanisms and algorithms that enable this property blurs the line between developmental genetics, adult physiology, and the kind of plasticity studied in neuroscience. Interestingly, our NGS analysis implicated targets belonging to the Memory/Neuroplasticity categories (Table 1), suggesting the hypothesis, which we will test in future work, that regenerative and physiological plasticity are related to (and perhaps early forms of) cognitive plasticity in higher animals (Baluska and Levin, 2016).

We chose BaCl₂ for two reasons. First, because as a very broad K⁺ channel blocker, it would challenge tissues with a stress that could not easily be overcome through simple redundancy among potassium channel family members. Second, because to our knowledge, exposure to significant quantities of BaCl₂ is not something planaria encounter in the wild. Thus, it is plausible that plasticity to BaCl₂ toxicity is not a genetic response that has been specifically selected for; rather than a hardwired response to a common feature of the planarian environment over evolutionary time, it is likely that the remarkable adaptation we observe is an example of a more general and still poorly understood feature of biology: robustness in the face of novel stresses. The algorithms and mechanisms by which living systems match transcriptional and physiological responses to environmental challenges represent a key intellectual challenge for the coming decade, with significant implications both for biomedicine and for the design of highly resilient systems in engineering.

Understanding cellular adaptation to stress may have implications in addition to shedding light on evolvability and regulative morphogenesis. Although other species may not exhibit the convenient head degeneration phenotype, ionic disbalance and stress response is a universal biological and biomedical phenomenon. Moreover, preconditioning (exposure to preinjury stressors to achieve induction of tolerance) (Yokobori et al., 2013) is an important topic in the biomedicine of brain injury and epilepsy (Blondeau et al., 2000; Fern et al., 2014), where ion channels are already beginning to be viewed as therapeutic targets for neuroprotection (Skaper, 2011). Future work on the modulation of regeneration via developmental pharmacology will exploit novel biomaterials, computational models, and ion channel drugs to transition these advances toward biomedical applications (Herrera-Rincon et al., 2018; Churchill et al., 2018; Pai et al., 2018).
| Protein Name                             | TCONS       | Gene Name          | Log Fold Change | p Value   | q Value |
|-----------------------------------------|-------------|--------------------|-----------------|-----------|---------|
| Tubulin alpha chain (fragment)          | TCONS_00000293 | TBA3 TR27993      | –               | p < 0.001 | 0.004   |
| Marvel containing potential lipid       | TCONS_00001520 | EgrG_000088200    | –               | p < 0.001 | 0.004   |
| GRG041                                  | TCONS_00002574 | gco41             | –               | p < 0.001 | 0.004   |
| Propionyl-CoA synthetase                | TCONS_00010061 | CLF_100045        | –               | p < 0.001 | 0.004   |
| Propionyl-CoA synthetase                | TCONS_00010062 | CLF_100045        | –               | p < 0.001 | 0.004   |
| cAMP-dependent protein kinase regulatory subunit | TCONS_00014767 | PRKAR2A           | –               | p < 0.001 | 0.004   |
| CyclinA-like protein (fragment)         | TCONS_00014792 | NA                | –               | p < 0.001 | 0.004   |
| Tubulin alpha chain                     | TCONS_00000293 | NA                | –               | p < 0.001 | 0.004   |
| Uncharacterized protein C1orf177 homolog | TCONS_00015160 | CA177 TR105569    | –               | p < 0.001 | 0.004   |
| Fibronectin type 3 and ankyrin repeat domains protein 1 (FANK1) | TCONS_00015183 | CLF_102920        | –               | p < 0.001 | 0.004   |
| Mariner Mos1 transposase                | TCONS_00015672 | HmnN_000702600    | –               | p < 0.001 | 0.004   |
| Tubulin alpha chain                     | TCONS_00015804 | NA                | –               | p < 0.001 | 0.004   |
| L-lactate dehydrogenase (EC 1.1.1.27)   | TCONS_00014875 | NA                | –               | p < 0.001 | 0.007   |
| TBC1 domain family member 9B            | TCONS_00012203 | CLF_111893        | –               | 0.001     | 0.031   |
| SPSB                                    | TCONS_00002395 | Spsb              | –9.764          | p < 0.001 | 0.004   |
| SPSB                                    | TCONS_00015862 | Spsb              | –6.74196        | p < 0.001 | 0.004   |
| Slc38a-2 (Na+-coupled neutral amino acid transporter) | TCONS_00008866 | slc38a-2         | –5.10772        | p < 0.001 | 0.004   |
| Tubulin alpha chain                     | TCONS_00000243 | MS3_11,213        | –5.00986        | p < 0.001 | 0.004   |
| Tubulin beta chain                      | TCONS_00015047 | TR83001           | –4.80567        | p < 0.001 | 0.043   |
| Tubulin beta chain                      | TCONS_00015847 | NA                | –4.02875        | p < 0.001 | 0.004   |
| Tubulin alpha chain                     | TCONS_00014885 | NA                | –3.9266         | p < 0.001 | 0.004   |
| C3H-zinc finger-containing protein 1    | TCONS_00015349 | NA                | –3.87679        | p < 0.001 | 0.004   |
| Phosphotransferase (EC 2.7.1.1)         | TCONS_00015828 | slc25a-4         | –3.86765        | p < 0.001 | 0.004   |
| Y box protein 4-like protein            | TCONS_00000137 | NA                | –3.65495        | p < 0.001 | 0.004   |
| Protein FAM154A                         | TCONS_00015465 | EmuJ_000818000    | –3.60524        | p < 0.001 | 0.004   |
| Plastin-1                               | TCONS_00015023 | CLF_110637        | –3.56204        | p < 0.001 | 0.016   |
| Innexin                                 | TCONS_00005338 | inx               | –3.54459        | p < 0.001 | 0.004   |
| Tubulin beta chain                      | TCONS_00000701 | NA                | –3.41177        | p < 0.001 | 0.004   |
| Tubulin beta-2C chain                   | TCONS_00000158 | NA                | –3.37944        | p < 0.001 | 0.004   |
| SJCHGC04177 protein                     | TCONS_00015105 | NA                | –3.37057        | p < 0.001 | 0.004   |
| Tubulin alpha chain (fragment)          | TCONS_00015786 | CLF_103359        | –3.25329        | p < 0.001 | 0.004   |
| Tubulin beta chain (fragment)           | TCONS_00002700 | bt3              | –3.05291        | p < 0.001 | 0.004   |
| Expressed conserved protein             | TCONS_00014926 | EmuJ_001095400    | –2.98249        | p < 0.001 | 0.004   |

Table 4. Summary of Transcripts Downregulated in Heads Pre-Conditioned by BaCl2 Exposure

(Continued on next page)
Limitations of the Study

One of the limitations of the study is that assays are not yet available in planaria to directly observe the physiological effects of all of the reagents used. Most of the pharmacological tools we applied have been used in planaria, and all have been utilized in other invertebrates, but NPPB and Niflumic acid have not heretofore been characterized in planaria. Likewise, there is currently no working technology for introducing foreign genes into planaria, preventing the use of molecular-genetic (dominant negative and fluorescent reporter) tools to directly observe the dynamics of the dopamine pathway under our various conditions. Furthermore, it is possible that some of the genes listed in Tables 3 and 4 as expressed in one condition but not detected in the other may reflect technical or statistical failures to detect transcript. Finally, it will be important in future work to conduct a large-scale RNAi effort targeting all of the genes identified by our RNA-seq to determine whether additional mechanisms of tissue adaptation to stress are also critical beyond those implicated in the specific model we propose.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.11.014.

ACKNOWLEDGMENTS

We thank the members of the Levin lab and Nicolas Roleau for useful discussions and Hans Gonzembach and the diligent undergraduates including Hannah Stowe, Quynh Anh Phan, Si Kun Wang, Sara E. Mitchell, Tien Hoang, John Fernandez, and Carolyn H. Nguyen who have helped us tend to our worm colony. We also thank members of the Agata lab for providing us with the Dj-TRPMa primer sequences. We gratefully acknowledge support by an Allen Discovery Center award from the Paul G. Allen Frontiers Group (No. 12171), the Templeton World Charity Foundation (No. TWCF0089/AB55), and the Barton Family Foundation. Research was sponsored by the Defense Advanced Research Projects Agency (DARPA) under Cooperative Agreement Number HR0011-18-2-0022. The content of the information does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred. Approved for public release; distribution is unlimited.

AUTHOR CONTRIBUTIONS

M.L. and M.E.-B. conceived and planned the study. M.E.-B., F.D., A.T., D.D., and J.M. performed planarian experiments and analyzed data. K.M., A.T., and D.D. performed molecular biology (QPCR validation). A.P.
built and analyzed the excitotoxicity model. C.J.M. and A.K. analyzed RNA-seq data. M.E.-B, A.K., A.P., and M.L. wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: May 21, 2019
Revised: October 1, 2019
Accepted: November 5, 2019
Published: December 20, 2019

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Supplemental Information

Regenerative Adaptation to Electrochemical Perturbation in Planaria: A Molecular Analysis of Physiological Plasticity

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Supplemental Information

Supplemental Figures

Figure S1. Valinomycin and potassium gluconate-induced depolarization can be visualized with DiBAC₄(3). Related to Figure 3. Pretail fragments (fragments that contain the trunk region of the worm posterior to the pharynx and anterior to the tail) of Dugesia japonica were cut and placed into vehicle (A) or 100nM Valinomycin + 15mM potassium gluconate (K⁺-gluc) (B) for 1 hour, then mounted and imaged. Both Valinomycin and K⁺-gluc were used to optimize depolarization. Images were taken of ventral surface of fragment and were far-field and dark-field corrected. Images are pseudocolored to allow for ease of visualization of depolarization patterns, but worms were imaged in the same frame so as not to confound data after pseudocoloring, and all image analysis was done using raw images. Scale bars 0.5mm.
Figure S2. Drug treatment alone does not affect planaria, Related to Figure 4. (A) Exposure to Bromocriptine (a,b), NPPB (c,d), Niflumic acid (e,f), or Nicardipine (g,h) for 2 days had no observable effect on D. japonica morphology.
Supplemental Tables

Table S2. Data statistics for RNA-seq, Related to Figure 2 and Tables 1 and 3. The 40 bp single end reads generated by Illumina HiSeq 2500 were quality checked and processed to remove the low quality bases and the adapter contamination. The table below lists the data generated for the individual samples.

| Sample name      | Chemistry | Barcode sequences | Total number of reads generated Post-processing (in millions) |
|------------------|-----------|-------------------|-------------------------------------------------------------|
| WildType Head 2  | 40*1      | TS13-AGTCAA       | 33.67                                                       |
| WildType Head 1  | 40*1      | TS12-CTTGT        | 39.18                                                       |
| BaCl2 Head 2     | 40*1      | TS11-GGCTAC       | 36.72                                                       |
| BaCl2 Head 1     | 40*1      | TS10-AGCTTT       | 34.64                                                       |

Table S3. Alignment statistics for RNA-seq experiments, Related to Figure 2 and Tables 1 and 3. As the gene feature file for *Dugesia japonica* is not available, the reads were aligned to the *Dugesia japonica* (complete CDS regions) taken from NCBI (https://www.ncbi.nlm.nih.gov/nuccore/?term=Dugesia%20japonica) which consists of 138,026 nucleotide sequences, downloaded from the NCBI database using Tophat. The table below shows the alignment percentage of the reads mapped to the reference.

| Sample name      | No of reads (in millions) | Alignment percentage |
|------------------|---------------------------|----------------------|
| WildType Head 2  | 33.67                     | 92.60%               |
| WildType Head 1  | 39.18                     | 94.30%               |
| BaCl2 Head 2     | 36.72                     | 93.90%               |
| BaCl2 Head 1     | 34.64                     | 96.20%               |
Table S4. Gene Ontology Analysis, Related to Figure 2 and Tables 1 and 3. Most significant gene ontology categories affected at the transcript level with BaCl$_2$. All data for gene ontology can be found in Table S5.

| Go Term Theme       | Category                                           | Frequency | PAGE Z-Score | PAGE Raw p-value | FDR       |
|---------------------|----------------------------------------------------|-----------|--------------|------------------|-----------|
| Biological Process  | translation [go:0006412]                          | 96        | 6.602042     | 4.06E-11         | 5.72E-09  |
|                     | intracellular protein transport [go:0006886]       | 53        | 2.867622     | 0.004136         | 0.291566  |
|                     | cell cycle [go:0007049]                           | 17        | -2.63838     | 0.00833          | 0.391526  |
|                     | tricarboxylic acid cycle [go:0006099]             | 21        | 2.35727      | 0.01841          | 0.439486  |
|                     | transmembrane receptor protein tyrosine kinase signaling pathway | 5        | 2.270731     | 0.023163         | 0.439486  |
|                     | pyrimidine nucleotide biosynthetic process [go:0006221] | 5         | 2.248818     | 0.024524         | 0.439486  |
|                     | protein glycosylation [go:0006486]                 | 29        | 2.239905     | 0.025097         | 0.439486  |
|                     | dna recombination [go:0006310]                     | 8         | -2.21806     | 0.026551         | 0.439486  |
|                     | de novo' pyrimidine nucleobase biosynthetic process [go:0006207] | 6        | 2.196554     | 0.028052         | 0.439486  |
|                     | dna integration [go:0015074]                       | 20        | -2.14806     | 0.031709         | 0.447092  |
|                     | malate metabolic process [go:0006108]              | 6         | -2.03848     | 0.041502         | 0.492306  |
|                     | vesicle-mediated transport [go:0016192]            | 35        | 1.986827     | 0.046942         | 0.492306  |
| Molecular Function  | structural constituent of cytoskeleton [go:0005200] | 89        | -7.40681     | 1.29E-13         | 2.69E-11  |
|                     | structural constituent of ribosome [go:003735]     | 100       | 6.812948     | 9.56E-12         | 9.94E-10  |
|                     | acyl-coa dehydrogenase activity [go:0003995]       | 9         | 3.133764     | 0.001726         | 0.119655  |
|                     | nad-dependent histone deacetylase activity (h3-k14 specific) | 7         | 3.026156     | 0.002477         | 0.128796  |
|                     | nad+ kinase activity [go:0003951]                  | 7         | 2.825761     | 0.004717         | 0.196221  |
| Process                                          | Count | Log2 Fold Change | Adjusted P-Value | FDR          |
|-------------------------------------------------|-------|-----------------|------------------|--------------|
| Proton-transporting ATP synthase activity, rotational mechanism | 7     | 2.604398        | 0.009204         | 0.275972    |
| Protein tyrosine phosphatase activity [go:004725] | 38    | -2.54166        | 0.011033         | 0.275972    |
| Nucleotidyltransferase activity [go:0016779]     | 12    | 2.513792        | 0.011944         | 0.275972    |
| Microtubule binding [go:0008017]                 | 39    | -2.45754        | 0.013989         | 0.275972    |
| Mannosyl-oligosaccharide binding activity        | 8     | 2.449566        | 0.014303         | 0.275972    |
| Extracellular matrix structural constituent [go:0005201] | 16    | -2.43919        | 0.01472          | 0.275972    |
| Flavin adenine dinucleotide binding [go:0050660] | 27    | 2.410711        | 0.015921         | 0.275972    |
| Histone-lysine N-methyltransferase activity [go:0018024] | 14    | 2.330139        | 0.019799         | 0.297096    |
| Inorganic anion exchanger activity [go:0004542]  | 7     | 2.326406        | 0.019997         | 0.297096    |
| Carboxypeptidase activity [go:0004180]          | 7     | 2.091671        | 0.036468         | 0.425427    |
| Peptidyl-dipeptidase activity [go:0008241]      | 7     | 2.091671        | 0.036468         | 0.425427    |
| Chromatin binding [go:0003682]                   | 9     | 2.068059        | 0.038634         | 0.425427    |
| Ubiquitin-protein transferase activity [go:0004842] | 48    | 2.020134        | 0.043369         | 0.425427    |
| Phospholipid binding [go:0005543]                | 6     | 1.997395        | 0.045782         | 0.425427    |
| Carbohydrate binding [go:0030246]                | 16    | 1.970462        | 0.048785         | 0.425427    |
| Cellular Component                              |       |                 |                  |              |
| Ribosome [go:0005840]                            | 83    | 5.158659        | 2.49E-07         | 1.77E-05    |
| Small ribosomal subunit [go:0015935]             | 9     | 3.061898        | 0.002199         | 0.078078    |
| Cytosol [go:0005829]                             | 11    | 2.920361        | 0.003496         | 0.082745    |
| Nucleolus [go:0005730]                           | 21    | -2.30831        | 0.020982         | 0.346204    |
| Large ribosomal subunit [go:0015934]             | 10    | 2.206271        | 0.027365         | 0.346204    |
Table S6. Lists of genes depicted in Figure 2A and B, Related to Figure 2. (A) Full list of altered anion transport-related genes shown in Figure 2A. (B) Full list of altered transmission of nerve impulse-related genes shown in Figure 2C.

### Table 6A

| Name      | Probe Value | Description                                                                 |
|-----------|-------------|-----------------------------------------------------------------------------|
| ABCA1     | 1.0018      | ATP-binding cassette, sub-family A (ABC1), member 1                           |
| SLC12A2   | 0.3578      | solute carrier family 12 (sodium/potassium/chloride transporters), member 2 |
| SLC26A3   | -2.0998     | solute carrier family 26 (anion exchanger), member 3                         |
| SLC4A3    | 0.0388      | solute carrier family 4, anion exchanger, member 3                           |
| SLC26A4   | -1.201      | solute carrier family 26, member 4                                           |
| SLC17A1   | 10.9346     | solute carrier family 17 (organic anion transporter), member 1               |
| SLC22A11  | 3.5889      | solute carrier family 22 (organic anion/urate transporter), member 11        |
| ABCC3     | 1.3558      | ATP-binding cassette, sub-family C (CFTR/MRP), member 3                       |
| SLCO1A2   | -1.2222     | solute carrier organic anion transporter family, member 1A2                 |
| ABCC2     | -0.4388     | ATP-binding cassette, sub-family C (CFTR/MRP), member 2                       |
| ABCC1     | -0.896      | ATP-binding cassette, sub-family C (CFTR/MRP), member 1                       |
| SLC22A6   | -1.1533     | solute carrier family 22 (organic anion transporter), member 6               |
| ABCB1     | -0.896      | ATP-binding cassette, sub-family B (MDR/TAP), member 1                       |
| CAT       | 0.4148      | catalase                                                                     |
| UCP1      | -0.2243     | uncoupling protein 1 (mitochondrial, proton carrier)                         |
| TSPO      | 0.6788      | translocator protein (18kDa)                                                 |
| m_Slco1a1 | -1.2222     | solute carrier organic anion transporter family, member 1A1                  |

### Table 6B

| Name      | Probe Value | Description                                                                 |
|-----------|-------------|-----------------------------------------------------------------------------|
| CTNNB1    | -3.0765     | catenin (cadherin-associated protein), beta 1, 88kDa                       |
| SLC8A1    | 1.8271      | solute carrier family 8 (sodium/calcium exchanger), member 1               |
| Gene      | Value  | Description                                                                 |
|-----------|--------|-----------------------------------------------------------------------------|
| SLC12A2   | 0.3578 | solute carrier family 12 (sodium/potassium/chloride transporters), member 2|
| SLC4A3    | 0.0388 | solute carrier family 4, anion exchanger, member 3                           |
| GRIA2     | 0.3083 | glutamate receptor, ionotropic, AMPA 2                                       |
| GRIK2     | 2.546  | glutamate receptor, ionotropic, kainate 2                                    |
| TRPC3     | 0.5344 | transient receptor potential cation channel, subfamily C, member 3           |
| ASIC5     | 2.4432 | acid-sensing (proton-gated) ion channel family member 5                       |
| P2RX4     | 0.8279 | purinergic receptor P2X, ligand-gated ion channel, 4                          |
| CACNA2D1  | 1.3416 | calcium channel, voltage-dependent, alpha 2/delta subunit 1                  |
| KCNA3     | 0.8759 | potassium voltage-gated channel, shaker-related subfamily, member 3          |
| KCNC2     | -1.0571| potassium voltage-gated channel, Shaw-related subfamily, member 2            |
| KCNH2     | 0.7843 | potassium voltage-gated channel, subfamily H (eag-related), member 2         |
| CAT       | 0.4148 | catalase                                                                     |
| FAAH      | -0.8367| fatty acid amide hydrolase                                                   |
| GLUL      | -1.415 | glutamate-ammonia ligase                                                     |
| NPR1      | 0.6392 | natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A) |
| DAO       | 1.1035 | D-amino-acid oxidase                                                         |
| ERG       | 1.5128 | v-ets erythroblastosis virus E26 oncogene homolog (avian)                   |
| FOS       | -0.2607| FBJ murine osteosarcoma viral oncogene homolog                               |
| JUN       | 0.3939 | jun proto-oncogene                                                           |
| REST      | -0.1284| RE1-silencing transcription factor                                            |
| EGFR      | 1.2985 | epidermal growth factor receptor                                             |
| MEN1      | -1.3847| multiple endocrine neoplasia I                                               |
| MARK2     | -1.9312| MAP/microtubule affinity-regulating kinase 2                                 |
| UBC       | 0.2766 | ubiquitin C                                                                  |
| RANBP2    | -0.375 | RAN binding protein 2                                                        |
| CAV1      | -1.0436| caveolin 1, caveolae protein, 22kDa                                           |
| SNAP25    | -0.228 | synaptosomal-associated protein, 25kDa                                        |
| ANK2      | 1.1996 | ankyrin 2, neuronal                                                          |
| KIF5B     | -0.1274| kinesin family member 5B                                                     |
| PANX1     | -3.5446| pannexin 1                                                                   |
| AGRN      | -1.2874| agrin                                                                        |
| GFAP      | 1.8981 | glial fibrillary acidic protein                                               |
| NALCN     | 0.8476 | sodium leak channel, non-selective                                            |
| INS       | 1.5798 | insulin                                                                      |
| NTN1      | 1.616  | netrin 1                                                                     |
| TNF       | -0.25  | tumor necrosis factor                                                        |
| CDC42     | -0.2927| cell division cycle 42 (GTP binding protein)                                 |
| RAC1      | 0.0752 | ras-related C3 botulin toxin substrate 1 (rho family, small GTP binding protein Rac1) |
| Gene | Value | Description |
|------|-------|-------------|
| RAP1B | 0.6267 | RAP1B, member of RAS oncogene family |
| RASGRF1 | -0.9149 | Ras protein-specific guanine nucleotide-releasing factor 1 |
| MYLK | 1.7125 | myosin light chain kinase |
| CASP3 | -0.7907 | caspase 3, apoptosis-related cysteine peptidase |
| GPER1 | 0.3777 | G protein-coupled estrogen receptor 1 |
| NPY1R | 85.4924 | neuropeptide Y receptor Y1 |
| CXCR4 | 85.4924 | chemokine (C-X-C motif) receptor 4 |
| MMP9 | 3.4586 | matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase) |
| CDK5 | -0.2833 | cyclin-dependent kinase 5 |
| BRSK2 | -0.8513 | BR serine/threonine kinase 2 |
| RAB8A | 2.13 | RAB8A, member RAS oncogene family |
| RAB10 | 0.0681 | RAB10, member RAS oncogene family |
| APC | 1.0329 | adenomatous polyposis coli |
| PICK1 | 1.3926 | protein interacting with PRKCA 1 |
| ACE | 0.9108 | angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 |
| PRMT1 | -0.1001 | protein arginine methyltransferase 1 |
| DBI | -0.085 | diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein) |
| TSPO | 0.6788 | translocator protein (18kDa) |
| MSRA | -1.1203 | methionine sulfoxide reductase A |
| PITPNA | -0.3698 | phosphatidylinositol transfer protein, alpha |
| GRIK1 | -3.1039 | glutamate receptor, ionotropic, kainate 1 |
**Transparent Methods**

**Planarian care**

A clonal strain of *Dugesia japonica* was maintained at 13 °C in Poland Spring water with weekly feedings of liver paste and twice-weekly water changes, as in (Oviedo et al., 2008a). Worms were maintained at 13°C for the duration of the experiment in order to prevent spontaneous fissioning, which occurs at a greater rate at higher temperatures and which would interfere with interpretation of results regarding timing of regeneration (Oviedo et al., 2008a). Due to experimental temperature being 13°C, worms were taken from a cold-adapted 13 °C colony which is continuously maintained at that temperature. Worms were starved for at least one week prior to the beginning of each experiment.

**BaCl₂ Adaptation and Loss of Adaptation**

Whole worms were maintained at 13 °C in untreated tissue culture plates containing either Poland Spring water or 1 mM BaCl₂ (MP Biomedicals Inc) in Poland Spring water (BaCl₂ solution). Both water and BaCl₂ solutions were replaced once a week. A maximum of 30 worms were kept in each plate (diameter 100 mm), which contained approximately 50 mL of solution. Progression of response to BaCl₂ was assessed by regular examination of animals using a stereoscope. Worms that had fully regenerated in refreshed BaCl₂ for at least three weeks and were indistinguishable in appearance from untreated worms were designated “BaCl₂-adapted worms.”

To assess the persistence of the BaCl₂ adaptation, BaCl₂-adapted worms were removed from the 1mM BaCl₂ solution and maintained in untreated tissue culture plates at 13 °C in Poland Spring water for 30 days before being placed again in 1mM BaCl₂ solution.

**Drug treatments: BaCl₂ and ion channel blockers**

In order to test the effects of particular channels on BaCl₂ adaptation, planaria were exposed to 1mM BaCl₂ in the presence of drugs that modify channels. In each case, planaria were exposed to either BaCl₂+ treatment solution (Poland Spring water with 1 mM BaCl₂ and the respective drug) as well as 3 control solutions. Control solutions were Poland Spring water plus matching concentration of DMSO, drug control solution (Poland Spring water plus the drug (dissolved in DMSO)), or BaCl₂-control solution (Poland Spring water, 1mM BaCl, and matching DMSO concentration). DMSO concentration never exceeded .1% as higher concentrations have been shown to interfere with normal planarian function (Pagan et al., 2006, Stevens et al., 2015, Yuan et al., 2012). Worms were maintained in tissue culture treated dishes at 13 °C. Multiple concentrations were tested for each drug, and the lowest effective concentration was used for each.

Bromocriptine mesylate at a final concentration of 0.5 μM (Tocris #0427), dissolved in Poland Spring from a stock dissolved in DMSO (final DMSO concentration: 0.0075%) was used to block monoamines. Inhibition of calcium activated chloride channels was performed using 5.0 μM 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, Cayman Chemical #17292) or 1.25 μM niflumic acid (Tocris #4112) in Poland Spring from a stock dissolved in DMSO (final DMSO concentration of 0.000726%, respectively). Inhibition of calcium channels was performed using 2.5 μM nicardipine hydrochloride (Sigma-Aldrich #N7510) in Poland Spring from a stock dissolved in DMSO (final concentration of DMSO 0.0025%). Inhibition of TRPM was achieved using 100 μM AMTB hydrochloride (Tocris #3989) in Poland Spring from a stock dissolved in DMSO (final concentration of DMSO 0.1%). For AMTB treatments, media was refreshed once a week, all other solutions were refreshed every two days.
Visualization of relative membrane potentials with DiBAC$_4$(3) dye imaging

DiBAC$_4$(3) (bis-(1,3-dibutylbarbituric acid)-trimethine oxanol) (Invitrogen) was used as previously described (Oviedo et al., 2008b). Briefly, a stock solution (1.9 mM) was diluted 1:1000 (1.9 µM) in Poland Spring water or 1 mM BaCl$_2$ solution, and worms were soaked in the DiBAC$_4$(3) solution for >30 minutes before imaging. Worms were then immobilized in 2% low-melting point agarose, using custom-fabricated Planarian Immobilization Chips as per Dexter and colleagues (Dexter et al., 2014). Images of the ventral side of immobilized planaria were captured with the Nikon AZ100 Stereomicroscope using epifluorescence optics and NIS-Elements imaging software. Treatment and control animals were captured within the same frame and care was taken to ensure that planaria did not move during image acquisition. No data points or image features were removed from our analysis. To quantify differences in relative depolarization, average pixel intensity of the head region, defined as the anterior 1/6$^{th}$ of the worm, was quantified with ImageJ, after flat-field correction. This treatment avoided confounding the analysis by including fluorescence due to background and slime.

RNA extraction and RNA-seq

Total RNA was extracted from wild type and BaCl$_2$-adapted $D.$ japonica heads. Two biological replicates were collected for each treatment, with 25 worms pooled per replicate. Worms were kept in 1 mM BaCl$_2$ for 35 days, when BaCl$_2$-insensitive heads had fully regenerated. Heads (as defined by the anterior 1/6$^{th}$ of the worm) were then amputated and RNA was extracted using Trizol (Ambion/ThermoFisher) as per the manufacturer’s instructions. RNA was pelleted via isopropanol extraction and then suspended in 80% ethanol. Following DNase-treatment, the concentration of RNA was crudely quantified via NanoDrop™, and samples were stored at -80°C. Samples were sent to the Whitehead Institute Genome Technology Core for sequencing. Quality control for RNA was conducted using an Agilent 2100 Bioanalyzer. While RIN scores cannot be used with planarian species due to an absence of 28s rRNA peaks (Kim, 2019, Liu and Rink, 2018), we confirmed quality of RNA prior to library prep with the sequencing core. Library prep was performed on high quality RNA using the TruSeq PolyA kit (Illumina) as per manufacturer’s protocol. All sequencing libraries were then quantified, pooled and sequenced at single-end 40 base-pair using the Illumina HiSeq 2500. Following sequencing, data processing was done using the standard Illumina pipeline and Fastq files were generated for data processing and assembly.

Data Processing and Assembly

Raw Illumina files were processed by Genotypic Technology Ltd. (Bangalore, India) for read mapping and the identification of differentially expressed transcripts. Raw data were downloaded and processed (Adapter, B-block and low-quality base filtering) using Genotypic's proprietary Perl script for removing adapter and low-quality base trimming. RNA-Seq reads were then mapped using the ultra-high-throughput short read aligner Bowtie (Langmead, 2010) and reference alignment was conducted with TopHat-2.0.13 (Trapnell et al., 2009), a fast splice junction mapper for RNA-Seq reads. Processed data were inputted into TopHat and default parameters for directional libraries were used. Cufflinks-2.2.1 was used to assemble transcripts and to identify transcripts that were differentially expressed using default settings and -G option to facilitate read mapping. Biological replicates were combined using Cuffmerge and then analyzed using CuffDiff (2.2.1) for replicate samples by modeling the variance in fragment counts across replicates as a function of the mean fragment count. Each replicated condition was first used to build a model. Models were generated from each biological replicate and were averaged to provide a single global model for all conditions in the experiment. Cuffdiff was then used...
to determine significant changes in transcript levels. Data and alignment statistics for the Illumina runs are presented in Tables S2 and S3.

Expression level estimation was reported as fragments per kilobase of transcript sequence per million mapped fragments (FPKM) value together with confidence intervals. The false discovery rate or FDR-adjusted p-value of the test statistic (q-value) was also generated. An in-house pipeline was developed to automate the abovementioned mapping and assembly process. All transcripts were annotated for Gene Ontology (GO) by performing a blast alignment. The 138,026 transcript sequences of *D. japonica* were subjected to blast against the 385,255 sequences of Platyhelminthes database extracted from Uniprot as a reference. Table S1 contains all processed transcript data, including fold change and p-value.

Pathway Studio 10.0 (Elsevier) and ResNet 11.0 were used for sub-network enrichment analysis (SNEA) of cell processes. The option of “best p value, highest magnitude fold change” in Pathway Studio was used for duplicated probes. Transcripts were successfully mapped using Name and Alias. SNEA was performed to identify gene networks that were significantly different in the treatment samples compared to control. A Kolmogorov–Smirnov test with 1000 permutations was conducted to determine whether specific networks were preferentially regulated compared to the background reference probability distribution. Networks were constructed based on common regulators of expression and regulators of specific cell processes. The enrichment p-value for a gene seed was set at p< 0.05. Additional details on the use of SNEA can be found in (Langlois and Martyniuk, 2013).

**qPCR**

1 µg total RNA extracted from the anterior 1/6th of *Dugesia japonica* was DNase treated and used as a template to synthesize cDNA (iScript gDNA Clear cDNA Synthesis Kit, BioRad, USA). PCR primers for Dj-TRPMa were obtained from the Agata lab (Inoue et al., 2014). Slc2a1 primers (F: 5’-TTGAACGATTCCGACCGCA-3’, R: 5’-GGGTTTGCTTTGGGACTAGGA-3’) were designed based on the upregulated sequences identified in the RNAseq analysis and were validated using melt curve analysis. GAPDH (Takano et al., 2007) was used as the reference gene. For qPCR, each 10 µL reaction was run in duplicate and contained: 5 µL of 2x PowerUp SYBR Green Master Mix (Applied Biosystems, USA), 0.5 µL of 10 µM of forward and reverse gene-specific primers, and 1.33 µL of diluted (1:20 for Slc2a1, 1:5 for Dj-TRPMa) cDNA template. qPCR was performed using the Step One Plus Real Time System (Applied Biosystems, USA) according to manufacturer’s instructions. Relative expression was analyzed using the Pfaffl method (Pfaffl, 2001).

**Data and Software Availability**

RNA-Seq unprocessed data has been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and is accessible through GEO Series accession number (GSE98084).
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