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Cellular diversity and developmental hierarchy in the planarian nervous system

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Our ability to dissect cell type diversity, development, and plasticity in the nervous system has been transformed by the recent surge of massive sequencing studies at the single-cell level. A large body of this work has focused primarily on organisms with nervous systems established early in development. Using planarian flatworms in which neurons are constantly respecified, replenished, and regenerated, we analyze several existing single-cell transcriptomic datasets and observe features in neuron identity, differentiation, maturation, and function that may provide the planarian nervous system with high levels of adaptability required to respond to various cues including injury. This analysis allows us to place many prior observations made by functional characterizations in a general framework and provide additional hypothesis and predictions to test in future investigations.

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Introduction

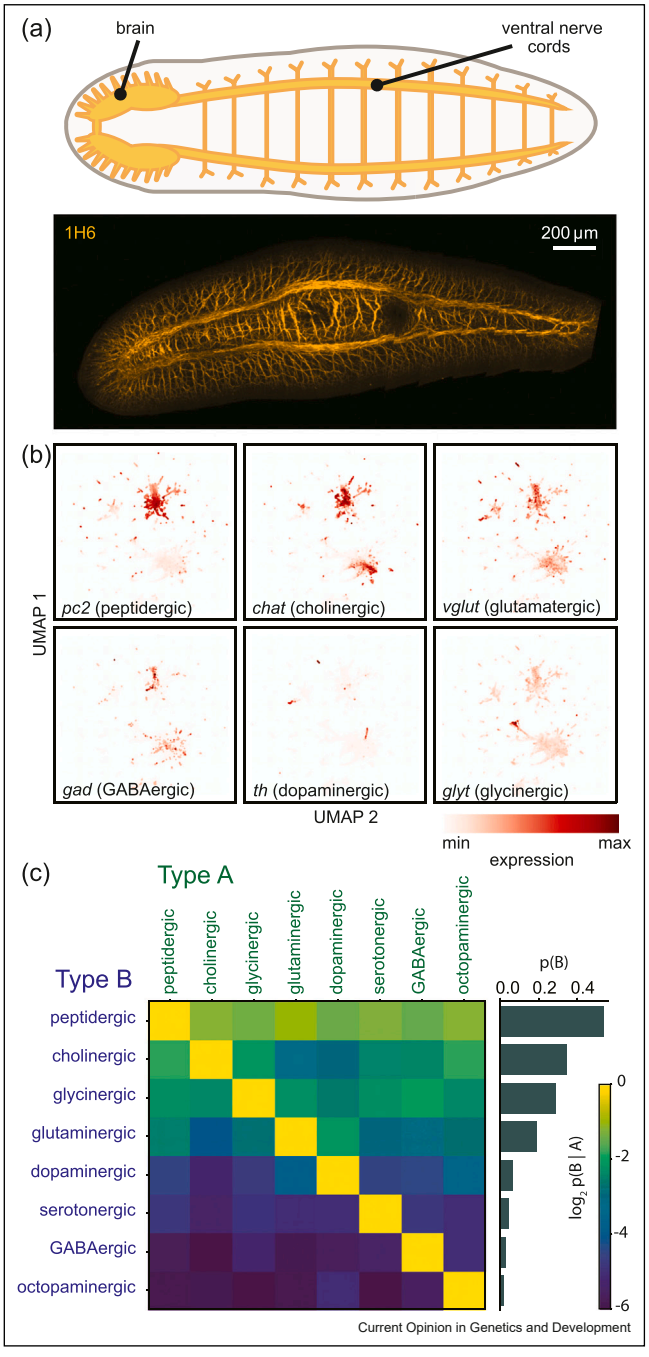
Understanding the development, function, and remodeling of the nervous system is an area of increasing interest. The planarian flatworm *Schmidtea mediterranea* is among the simplest animals possessing a central nervous system [1], which is composed of a bilobed brain and ventral nerve cords (Figure 1a). Planarians are masters of neural regeneration. Stem cells, historically called neoblasts, are broadly distributed throughout the body and can respecify, restructure, and regenerate the entire nervous system after essentially any injury [2–4]. Moreover, even in homeostatic conditions, the planarian nervous system is in a constant state of dynamic turnover, replacing old neurons with newly differentiated cells. The planarian brain also changes size by orders of magnitude to dynamically scale in proportion with animal size during growth and degrowth [5–7], which requires tight regulation of neural differentiation [8,9] and cell death [10,11]. When resizing, the ratio between neuronal types [5] and local geometric relations among neurons [12] are kept constant. This unique biology makes planarian a powerful system to study neuronal identity, diversity, and differentiation. Significant progress has been made through transcriptomics, RNAi screens, and *in situ* hybridization to examine gene expression and function. In the past few years, single-cell RNA sequencing (scRNAseq) and derived data analysis methods have provided new opportunities to dissect the planarian nervous system with cellular resolution [13–16]. Here, we both summarize what is known and integrate single-cell transcriptomic datasets with functional studies to extrapolate relationships between cellular diversity, fate decisions, and development in the planarian nervous system. While we focus on studies using *S. mediterranea*, our analysis also considers information collected from a related planarian species *Dugesia japonica* when the biology is expected to be comparable.

Overlapping neurotransmitter usage in planarian neuronal populations

In 2018, Fincher et al. used scRNAseq to classify virtually all planarian cell types based on their transcriptomes, including ~12 000 neurons each with ~1 200 detected genes [13]. This dataset was supplemented with ~8 000 cells collected from the brain region to enrich for rare cell types. Because of homeostatic turnover, this 'neural atlas' also captures cells at various stages along the differentiation process.

In the original analysis [13], neural cells are divided into ~60 clusters, including neural progenitors (expressing a neoblast marker *smedwi-1*), ciliated (*rootletin*$^+$) and non-ciliated neuronal populations, *gpas*$^+$ neurons in the distal brain branches, and a large group of peptidergic neurons (expressing a neuropeptide preprocessing enzyme, prohormone convertase 2, *pc2* [17]) (Figure 1b). Importantly, these neuronal clusters do not correspond to distinct neurotransmitter usage, which is consistent with observations in other animals [18,19]. This challenges the conventional view, known as Dale's principle, which anticipates a one-to-one relationship between neurons and neurotransmitters [19], and motivates us to reanalyze the expression of canonical marker genes

Figure 1



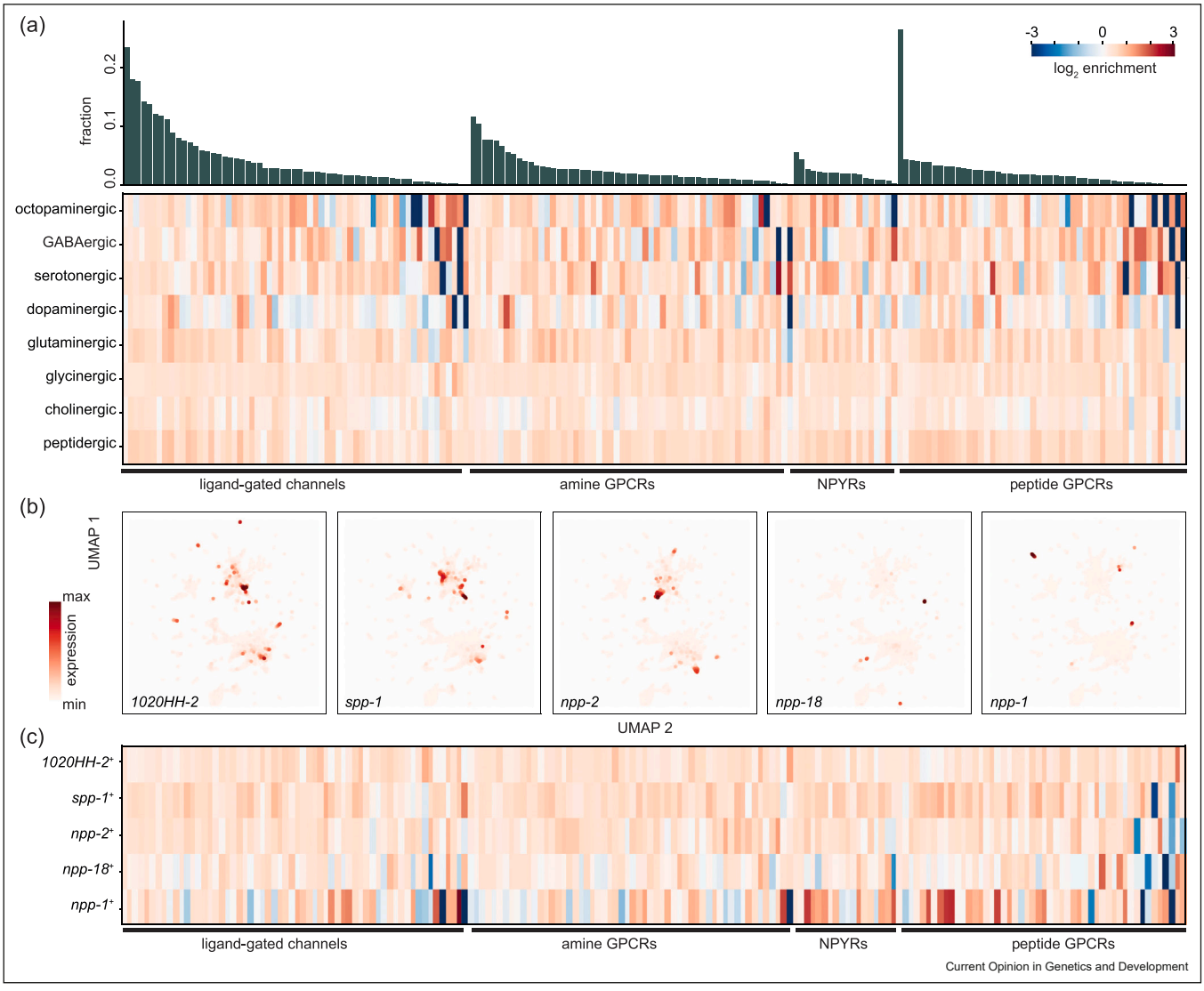
Overlapping neurotransmitter usage. **(a)** Top: schematic showing the planarian nervous system. Bottom: immunostaining of neural processes using 1H6 antibody [52]. **(b)** Expression of cell-type-specific markers, *smedwi-1*, *rootletin*, and *gpas*, along with genes associated with specific neurotransmitter pathways, *pc2*, *chat* (*choline acetyltransferase*), *vglut* (vesicular glutamate transporter), *gad* (*glutamic acid decarboxylase*), *th* (tyrosine hydroxylase), and *glyt* (glycine transporter), for peptidergic, cholinergic, glutamatergic, GABAergic, dopaminergic, and glycinergic neurons, respectively. scRNAseq data are analyzed using SAM [20]. **(c)** Left: within all cells that express neurotransmitter marker 1, the fraction of cells also expressing marker 2. Right: fraction of cells expressing each marker in the neural atlas. In addition to the cell populations and their respective marker genes shown in (b), *tbh* (*tyramine β-hydroxylase*) and *sert* (serotonin transporter) are markers for octopaminergic and serotonergic neurons, respectively. All gene contig numbers are provided in Supplementary Table 1.

associated with each neurotransmitter using self-assembling manifolds (SAM) algorithm [20], a method with higher sensitivity in separating cell types (Figure 1b). These neurotransmitter genes are indeed enriched in different clusters, but there is still significant overlap in their expression.

This overlap is not only at the cluster level. We also detect significant coexpression of neurotransmitter marker genes in individual cells, even though the frequency of dropout in scRNAseq is expected to be high (Figure 1c). In particular, ~60% of peptidergic neurons ($pc2^{+}$) appear to use at least one other small-molecule neurotransmitter. Similarly, the most abundant non-peptidergic population, cholinergic neurons (expressing choline acetyltransferase, *chat*) have ~45% cells expressing *pc2*. Even in small populations with highly restrictive spatial distributions, that is, GABAergic neurons (expressing glutamic acid decarboxylase, *gad*) and octopaminergic neurons (expressing tyramine β-hydroxylase, *tbh*) [9], 40% cells coexpress *pc2*, while > 50% cells express genes associated with other small-molecule neurotransmitters, including glutamate and acetylcholine. Rather than a result of promiscuous regulation, communication by multiple transmitters from a single neuron can be functionally important in driving synergistic signals [21,22] and is far more ubiquitous than previously thought [23]. Additionally, cotransmission offers circuit flexibility as different stimulation frequencies can release different neurotransmitters and the signaling range of neuropeptides and small-molecule neurotransmitters varies significantly [24].

Most small-molecule neurotransmitters act via ionotropic or metabotropic receptors, while dopamine and neuropeptides act through metabotropic receptors. Recent work in *C. elegans* shows that individual neuronal types express unique combinations of neurotransmitters coupled with specific receptors [25]. To examine whether similar combinatorial coding is present in planarians, we compile a list of 184 putative receptors, including G-protein-coupled receptors (GPCRs) that have been cataloged previously [26] and homologs of conserved ligand-gated ion channels, but did not observe clear patterns in neurotransmitter-receptor associations (Figure 2a). Peptidergic neurons also express ionotropic receptors, and inversely, other neuronal populations express a variety of peptide-binding GPCRs. This phenomenon is also observed within neuronal subtypes. For example, peptidergic neurons include nonoverlapping populations each expressing a specific neuropeptide

Figure 2



Expression of putative neurotransmitter receptors in neuronal populations. **(a)** Expression of receptors in neurons expressing specific neurotransmitter markers. Enrichment is defined by the fraction of cells expressing a specific receptor within a neuronal population normalized by the fraction of all cells expressing any neurotransmitter markers that also express the same receptor. **(b)** Examples showing the expression of neuropeptide precursors in distinct clusters. **(c)** Enrichment of receptors in peptidergic neuronal subtypes. All gene contig numbers are provided in Supplementary Table 1.

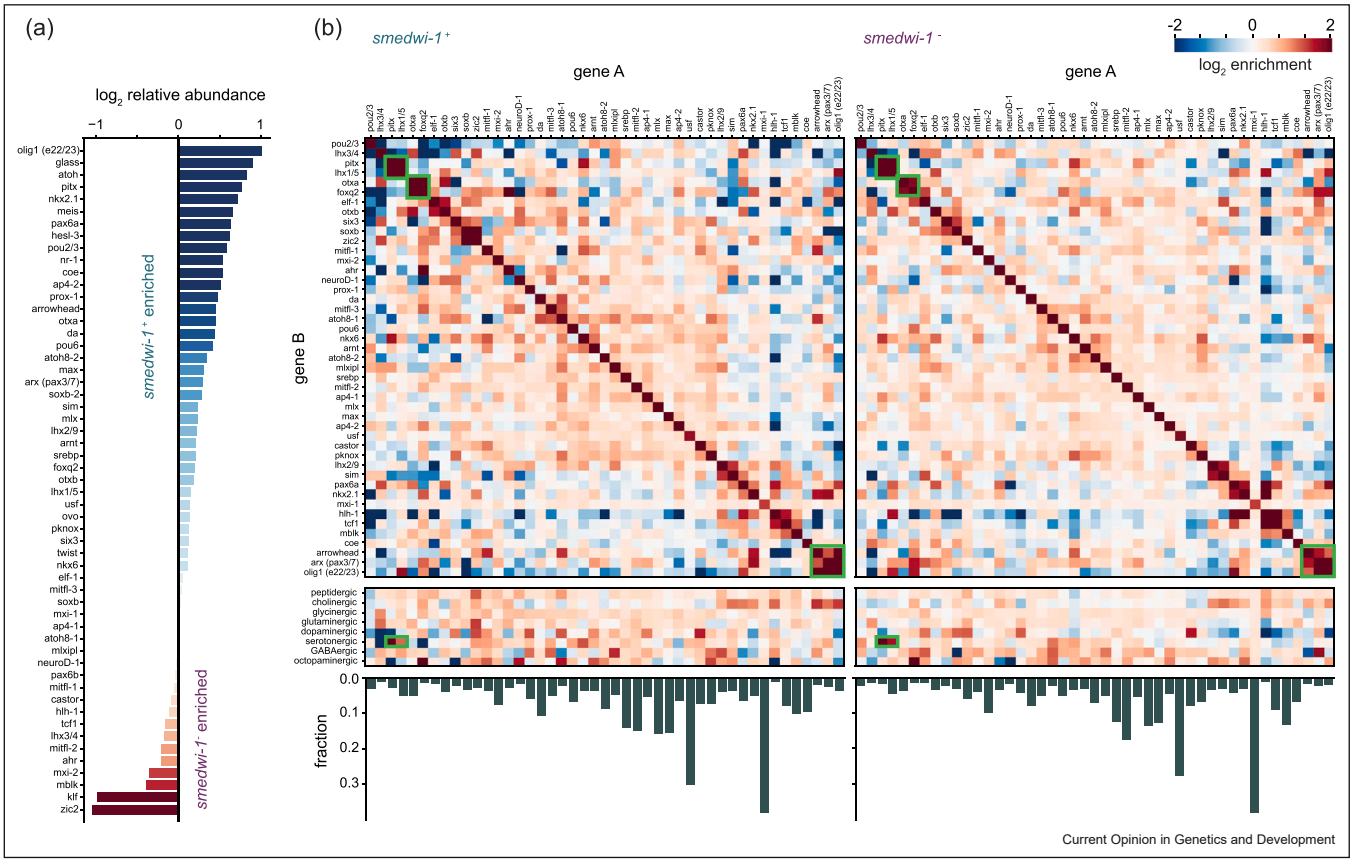
(Figure 2b) [12,17]. Each of these subtypes also express a diverse set of receptors (Figure 2c). Together, our observations imply that planarian neurons can receive input from multiple types of incoming signals and communicate through parallel information channels [27].

Complex and dynamic coexpression of neural transcription factors

Neoblasts are the only mitotically active cells within the planarian and the source of all differentiated cell types including neurons [2–4]. A fundamental question in neurogenesis is how fate decisions are made in stem cells to produce the complete diversity of neuronal cell types. Several studies have identified putative neural progenitors that are in transition between neoblasts and neural fates based on the coexpression of stem-cell and neural markers [9,28–30]. A potential reservoir of these progenitors is located in the ventral–medial region between the brain lobes. Within this region, decreased hedgehog (Hh) signaling reduces production of neural progenitors, whereas increasing the signaling activity leads to accumulation of neural progenitors [9].

Several transcription factors (TFs), including two homeobox factors *nkx2.1* and *arx*, are expressed in both

Figure 3



Coexpression of neural TFs. **(a)** Abundance of TF-expressing cells in *smedwi-1*$^{+}$ neural progenitors relative to that in *smedwi-1*$^{-}$ differentiated neurons. **(b)** Top: enrichment defined by the fraction of cells expressing TF B within cells expressing TF A, normalized by the total fraction of TF B-expressing cells. Calculation was made using cells expressing at least one of the listed TFs. Bottom: enrichment of neurotransmitter markers within TF-expressing populations. Green boxes highlight TF groups mentioned within the text. Genes are ordered based on hierarchical clustering of their associations with other TFs. All gene contig numbers are provided in Supplementary Table 1.

neural progenitors and differentiated neurons. RNAi-mediated knockdown of these TFs results in reduction of multiple neuronal populations. Specifically, *arx* is required for the maintenance of cholinergic and octopaminergic neurons in the ventral–medial region; *nkx2.1* is essential for GABAergic and octopaminergic neurons [9]. Similarly, *pitx* and a LIM-homeodomain factor, *lhx1/5-1*, are necessary for specifying serotonergic neurons, while *lhx1/5-1* also functions cooperatively with *nkx2.1* to specify ventral–medial GABAergic neurons [31]. This phenomenon is not restricted to the ventral–medial region; production of GABAergic, cholinergic, and peptidergic neurons in the dorsal–lateral region is regulated by a TF, *tcf1* [32]. Additionally, several *soxb* homologs and members of bHLH family (e.g. *hesl-3*, *coe*, and *sim*) are expressed in progenitors and loss-of-function studies find various defects in neural development [28,30,33,34]. Although the exact neuronal populations under the regulation of these TFs remain to be more thoroughly characterized, they likely also affect multiple populations.

Strikingly, this set of TFs, including *arx*, *nkx2.1*, and *lhx* paralogs, is highly conserved and forms a coregulatory network required for neurogenesis in vertebrate telencephalon [35]. More recently, the same TF combination, together with *soxb2* paralogs, is found to be expressed in the proliferation zone of annelid mushroom bodies [36]. This high level of conservation implies that these TFs may function as a gene-regulatory kernel conserved across bilateria.

To dissect the interactions between neural TFs, we compile a list of 55 TFs that have been studied in the context of neural regeneration [28,30,32,33,37] and quantify their expression in the scRNAseq dataset. Their relative abundance in neural progenitors (*smedwi-1*$^{+}$) and differentiated neurons (*smedwi-1*$^{-}$) suggests that they may function at various stages of differentiation (Figure 3a). *Olig1*, a conserved master regulator of neural specification [38,39], *pitx* and *nkx2.1* are among the top that are enriched in progenitors, whereas *mblk* [33],

zic2, and a *klf* homolog are enriched in differentiated neurons.

We also observe pairwise enrichments between TFs quantified by their coexpression in individual cells (Figure 3b). There is strong overlap between *pitx* and *lhx1/5*, which are both associated with serotonergic neurons. This interaction is supported by the loss of serotoninergic neurons following the knockdown of either of these factors [31]. Similarly, *foxq2* and *otxa* appear to be coregulated. They have been shown to operate sequentially in specifying neurons in the planarian eye [40], though they may be co-opted to regulate other neural lineages as well. Other coexpressed groups include *arrowhead*, *arx*, and *olig1*, with *arrowhead* and *arx* reported to both be expressed specifically at the ventral–medial boundary of the brain [9,33]. The coenrichments also appear dynamic during differentiation and generally less specific in differentiated neurons (Figure 3b).

One key role of neural TFs, as observed in other organisms, is to function as 'terminal selectors' in regulons to collectively control neural fate decisions and activate terminal differentiation gene batteries (e.g. receptors, enzymes, and synaptic proteins) in specific neuronal populations [41]. However, except for a few TFs, such as *pitx* and *lhx1/5-1*, many TFs only have weak associations with others and low specificity to individual neuronal populations (Figure 3b). The lack of clear association between cell populations and TF groups is inconsistent with the notion of terminal selectors, but may instead suggest a phenotypic convergence whereby similar functional neurons can be specified through multiple developmental pathways [18]. This interpretation is indeed supported by the observations that knockdown of single TFs often leads to partial loss of multiple neuronal populations [9,32]. Finally, the dynamic change in TF coexpression between immature and developed neurons suggests that initial specification, fate diversification, and maintenance may require different, but overlapping sets of TFs.

Adaptive programs of neural development

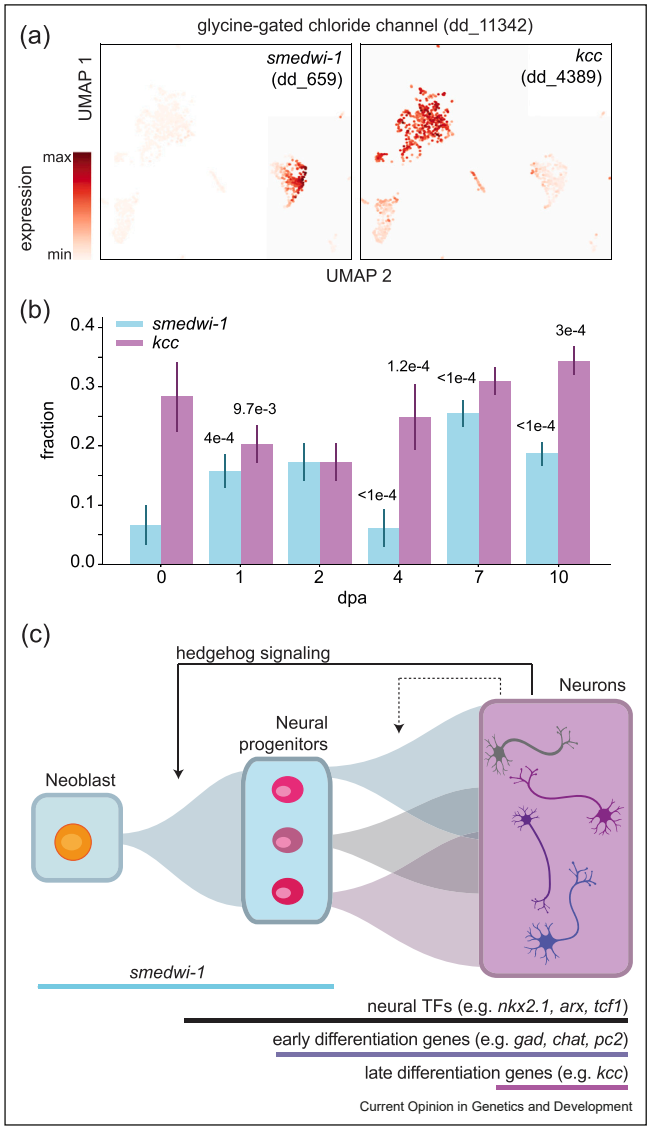
Many organisms develop their nervous system only once with minimal cellular turnover throughout their adult life. In contrast, planarians face variable developmental 'start points' after injury and undergo constant homeostatic turnover even in uninjured tissues. This necessitates adaptive developmental programs that can respond to changes in existing neural structures and cell types.

Evidence for adaptive feedback between existing and differentiating neurons is found at multiple stages of differentiation. At early stages, Hh signaling from differentiated neurons regulates progenitor production in the ventral–medial space [9]. The specification of optic neurons is controlled by self-organizing cues produced from both differentiating and differentiated cells [42,43]. Dopaminergic neurons can regenerate after selective chemical ablation, while the turnover of other neuronal types is not affected, suggesting that the loss of specific neuronal population can be detected to induce differentiation on demand [44]. Finally, peptidergic neuronal subtypes form defined spatial patterns with tightly regulated distances between homotypic neurons, suggesting that the diversification of neuronal subtypes likely responds to local cues at late stages of differentiation [12].

Even after the initial fate decisions, neurons need to undergo functional maturation. The timing of maturation and the feedback received from other neurons during this time may have significant influence on the fully matured neurons. As neuronal maturation is mostly uncharacterized in planarians, it remains an important question whether this process is also under adaptive regulation. To search for potential molecular markers to distinguish mature and immature neurons, we examined the expression of a highly conserved potassium-chloride channel (*kcc*) which is not expressed until late neural development in a variety of animals ranging from worms to humans [45,46]. Lack of *kcc* reverses chloride gradients across plasma membranes, changing the action of neurotransmitters such as GABA and glycine on ligand-gated chloride channels from inhibitory to excitatory [45,46]. The excitatory action by GABA can trigger transient 'giant depolarizing potentials' in neonatal mammalian brains that help neurons refine their synaptic connectivity and efficacy [47]. Correct timing of *kcc* expression is also important for development of dendritic branching and spine morphology [48].

We find that *kcc* is expressed in a maturation-dependent manner in planarian neurons. Within several neuronal populations expressing ligand-gated chloride channels, a subset of cells express a *kcc* homolog, which is excluded from the *smedwi-1*$^{+}$progenitor population (Figure 4a). This suggests that like other organisms, *kcc* is not expressed until late neuronal maturation. This trend is present not just in homeostatic turnover, but throughout regeneration. From a recent scRNAseq dataset containing cells collected throughout the time course of regeneration [49], we subset neurons expressing ligand-gated chloride channels. Within this population, we observe clear regeneration-dependent dynamics (Figure 4b). The fraction of *smedwi-1*$^{+}$ cells increases at known stages of cell proliferation during regeneration [8,50], while *kcc*$^{+}$ fraction drops in the first three days before recovering. Based on these dynamic features, we propose that *kcc* expression provides a readout to measure the kinetics of neuronal maturation and to investigate how the maturation process is regulated upon injury and/or during homeostasis, which is an important future research avenue.

Figure 4



kcc is a putative mature neuron marker. **(a)** Subclustering of neurons expressing a glycine-gated chloride channel (dd_Smed_v4_11342_0_1) shows that *smedwi-1* (dd_Smed_v4_659_0_1) and *kcc* (dd_Smed_v4_4389_0_1) are expressed in distinct clusters. This expression pattern is observed in all neuronal populations that express any of the ligand-gated chloride-channel homologs. **(b)** Fractions of ligand-gated chloride channel expressing neurons that also express either *smedwi-1* (blue) or *kcc* (magenta) at different time points in regeneration. Error bars: 95% confidence intervals calculated using a binomial distribution. p-values: significant ($p < 0.05$, Welch's t-test) changes compared with the previous time point. **(c)** A model for neural differentiation in the planarian nervous system. Dashed line: potential feedback lacking experimental data.

Conclusions

In the current model summarized in Figure 4c, the myriad of neuronal types are specified from neoblasts by a set of neural TFs and differentiated through neural progenitors. The output of neurogenesis needs to respond to injury, and therefore, is likely regulated by adaptive mechanisms to provide sufficient flexibility in development as required for regeneration. Although scRNAseq is powerful at uncovering cell types and states, it provides limited information about gene regulation. Understanding cis-regulation should be key to fully dissect the gene regulatory network that controls fate decisions in neural differentiation. In addition, lineage tracing tools are needed to convert the current static pictures into dynamic differentiation trajectories. With recent progress in planarian transgenics [51], these studies may become feasible in the near future. Finally, how the diverse cell types form neural circuits and control behaviors is largely unknown in planarians. Quantitative measurement of behaviors coupled with systematic perturbation of neural cell types and their communication can help to bridge this gap in our knowledge. Access to calcium reporters would allow direct observation of neural activity even during regeneration. With emerging technologies, we are now stand in an exciting position to connect scales from genes and synapses to circuits and behaviors in this dynamic and regenerative neural architecture.

Conflict of interest statement

None.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gde.2022.101960.

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