Advanced Review

Transcriptional selectors, masters, and combinatorial codes: regulatory principles of neural subtype specification

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The broad range of tissue and cellular diversity of animals is generated to a large extent by the hierarchical deployment of sequence-specific transcription factors and co-factors (collectively referred to as TF's herein) during development. Our understanding of these developmental processes has been facilitated by the recognition that the activities of many TF's can be meaningfully described by a few functional categories that usefully convey a sense for how the TF's function, and also provides a sense for the regulatory organization of the developmental processes in which they participate. Here, we draw on examples from studies in *Caenorhabditis elegans*, *Drosophila melanogaster*, and vertebrates to discuss how the terms spatial selector, temporal selector, tissue/cell type selector, terminal selector and combinatorial code may be usefully applied to categorize the activities of TF's at critical steps of nervous system construction. While we believe that these functional categories are useful for understanding the organizational principles by which TF's direct nervous system construction, we however caution against the assumption that a TF's function can be solely or fully defined by any single functional category. Indeed, most TF's play diverse roles within different functional categories, and their roles can blur the lines we draw between these categories. Regardless, it is our belief that the concepts discussed here are helpful in clarifying the regulatory complexities of nervous system development, and hope they prove useful when interpreting mutant phenotypes, designing future experiments, and programming specific neuronal cell types for use in therapies.

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INTRODUCTION

The nervous system is by far the most diverse organ in most metazoans, comprising myriads of neural cell types that in part have defied systematic classification. Understanding the regulatory logic of TF deployment during nervous system development is therefore a challenging task. The enormous cellular diversity of the nervous system and lack of precise markers for many neural subtypes, together with a lack of genetic tools to comprehensively dissect the spatiotemporal function of many regulators, all combine to create confusion regarding the many different neural subtypes that are generated, and the function of TF's in their generation. In spite of this, investigators have identified certain underlying principles by which TF's generate cellular diversity from

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progenitor populations, which helps to understand the steps of nervous system construction. These break down into a number of functional categories, including selectors, master regulators, and combinatorial codes. In this review, we consider the uses of these terms when referring to the activities of TF's in neural determination and differentiation, and suggest an updated set of terms and their definitions.

Specifically, we consider the use of the terms spatial selector, temporal selector, tissue/cell type selector, terminal selector and combinatorial code, when considering TF function with respect to nervous system development (Box 1, Figure 1). In so doing, we

BOX 1

DEFINITIONS

Combinatorial Code. TF's mostly act in a combinatorial manner, meaning that the regulatory roles that any TF plays are mostly diversified by physical or genetic interaction with other TF's. Such combinatorial activity is fundamental to TF function; it increases the number of roles that a TF can play, it increases the sequence-specificity and -diversity of DNA-binding, and enhances the signal:noise ratio of gene regulation. The term combinatorial code has been used to refer to numerous activities that we define as follows: (1) Molecular definition; the combination of TF's that perform a specific gene regulatory function. (2) Cellular definition; the combination of TF's that is uniquely expressed by a specific cell type. (3) Developmental definition; the more restrictive usage of the term that describes the differences in the expression of a specific combination of TF's, by a group of cells, that instructively and predictably diversifies the fates of those cells. Such developmental combinatorial coding likely underlies most if not all differences in cell identity, but has only been rigorously demonstrated in a few cases. The manner in which the terminal and unique identities of neural subtypes is defined largely arises from the activity of combinatorial codes of TF's.

Spatial Selector refers to a TF playing a deterministic role to define the regional identity of a spatially defined developmental compartment of multipotent progenitors. A TF acting as a spatial selector defines the limits and developmental program of a spatial compartment along one of the three dimensional axes of the embryo, or of tissues therein (Figure 1(a)). Along a specific axis, 'neighboring' selectors often act cross-repressively to sharply discriminate intercompartmental boundaries and developmental potentials. Selectors established along different axes typically do not regulate one another's establishment, but they do functionally interact in combinatorial codes; different combinations of overlapping selectors encode unique developmental programs that increase the resolution and diversity of compartments. Experimentally, genetic loss of a spatial selector can result in (1) expansion of a neighboring spatial selector and its encoded developmental program into the missing selector's compartment, or (2) elimination of a compartment. Genetic gain-of-function can impose that spatial selector's developmental program, albeit with restrictions often based upon competition and cross-repressive activities with the local spatial selector.

Temporal Selector refers to a TF playing a deterministic role within a temporally defined compartment of multipotent progenitors (Figure 1(b)). A TF acting as a temporal selector defines the limits of compartments of developmental time in tissues or lineages. These temporal compartments confer differences in the developmental program of cells derived from each compartment. The ability of a progenitor cell to respond to any specific temporal selector has been termed the competence window for that temporal selector. Temporal transition from one temporal selector to another is mediated by cross-regulation and by lineage intrinsic (and extrinsic) factors, that ensure timely (and plastic) transitions. Experimentally, loss of temporal selector function can result in skipping of that developmental program or encroachment of a neighboring temporal selector's expression and developmental program. Prolongation of early temporal selector expression can extend its developmental program into a later temporal selector's time window, as long as the lineage is competent to respond.

Tissue/Cell Type Selector refers to a TF playing a deterministic role to commit progenitor cells to the generation of a specific tissue or cellular subclass (Figure 1(c)). These are alternately termed master regulators, lineage-specific regulators, tissue selectors, cell type selectors, or pioneer factors. The type selector differs from a spatial and temporal selector in that it is not constrained by (or confers) any spatial or temporal context (or information). A TF acting as a type selector triggers a cohesive/integrated (and often restrictive) genomic response to express TF's and effector genes that lead to the generation of a specific tissue or cellular subclass.

Many type selectors act within an interconnected network of TF's, which increases the robustness and selectivity of the genomic response. Experimentally, genetic loss of a type selector (or the selector network) results in a loss of the pertinent tissue or cell type. Genetic gain of a type selector (or the selector network) often reprograms progenitors or other terminal cell types into the pertinent cell type.

Terminal Selector refers to TF's that are activated around the time of the final mitosis or in early postmitotic cells to dictate expression of subtype-specific effector genes that define subtype identity and function. Terminal selectors fall into a number of categories: (1) Those that directly activate most effector genes unique to a specific terminal cell type fate. (2) Those that directly activate most effector genes that contribute to a functional subroutine, such as those required for neurotransmitter identity, an enhanced neurosecretory capacity, or a specific morphological or electrophysiological property. (3) We extend the prevailing definition of terminal selectors to also include those that directly activate specific effector genes that define a neuronal subtype. Experimentally, loss of a terminal selector results in a cell that fails to express effector genes associated with its identity. Gain of a terminal selector (or a combinatorial code of terminal selectors) into a postmitotic cell often dominantly activates its associated effector genes, leading to a gain of that specific identity or subroutine.

The principle distinction between a tissue/cell type selector and a terminal selector is that the type selector functions in progenitor cells to commit them to the generation of a broadly definable cell type (i.e., muscle or glia), while terminal selectors operate within the postmitotic cell (or differentiated cell) to determine aspects of final and unique terminal identity (e.g., the specific properties of a neuron or a glial subtype). We note that tissue/cell type selectors commit progenitors to a cell type fate and may subsequently also determine certain aspects of final and unique terminal identity, but we restrict the terminal selector definition to those TF's whose function is primarily restricted to the postmitotic or differentiated cell.

fully acknowledge that such categorization is hampered because of, (1) the vast diversity of neural types and subtypes, (2) our limited knowledge of the precise functions of many TF's during nervous system development, and (3) the technical limitations in our ability to address these issues with high resolution. Also, we are mindful that TF's can act in diverse roles in different cellular contexts, and that the lines between these functional categories can often be blurred. Nevertheless, we believe that classifying TF activities into a number of defined functional categories that convey a sense of their developmental role and mechanism of action, helps in clarifying the regulatory complexity of neural development.

For simplicity, we restrict our scope to sequence-specific TF's and their co-factors. Thus, we do not touch upon the important roles played by chromatin state regulators loosely termed epigenetic regulators, and by regulatory RNA's. Also, we do not discuss the signaling pathways and morphogen gradients that are critical to establishing early axial patterns of TF expression within the embryo.[1] An extensive review of all TF's involved in nervous system development in worms, flies, and vertebrates is well beyond the scope of this review. Rather, we discuss salient examples from the various organisms that best illustrate the different functional categories described herein. Additionally, we do not extensively review all mechanisms of neural diversification, but instead refer the reader to other recent reviews on related topics, including binary cell decisions, such as mediated by Notch signaling,[2] and stochastic mechanisms of diversification.[3] We hope that the general principles discussed here may prove helpful for classifying neurons and glia based on developmental mechanisms, exploring, and interpreting mutant phenotypes in developmental studies, decoding TF functional logic, and also help guide efforts aimed at targeted programming of specific neural subtypes.

REGULATORY ACTIVITIES DURING NEURAL DEVELOPMENT

Spatial Selectors Define Compartments of the Overall Body Plan and Neuroectoderm

Spatial selectors map out compartments of unique developmental potential within the early developing organism. Garcia-Bellido coined the term 'selector factor' in light of *Drosophila* studies regarding the expression and mutant phenotypes of Engrailed and Hox genes.[4] Inherent to the original selector definition was the notion that a selector's expression delimits a spatially defined embryonic compartment and determines the developmental program of cells therein. In this way, selectors map out and define the building blocks of the embryonic body plan. The concept was

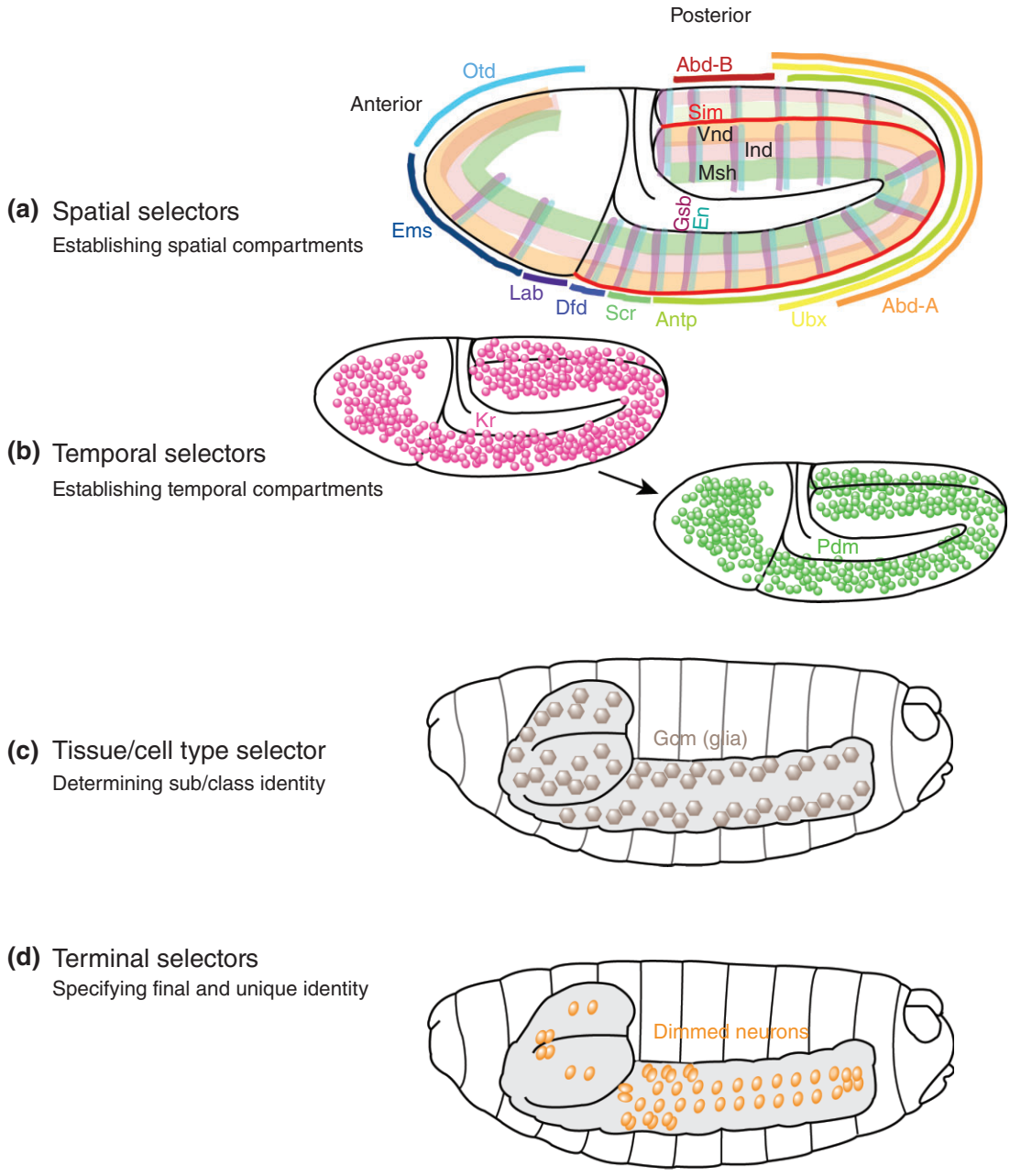

FIGURE 1 | Selector categories exemplified in the *Drosophila* embryo. (a) Spatial selector patterning of the neuroectoderm and delaminating neuroblasts. Shown are examples (rather than a complete map) of spatial selectors. These include spatial selectors along the entire A–P axis (anterior gap genes and Hox genes), whose expression pattern is represented by the bars alongside the embryo. These include gap genes Otd (Orthodenticle) and Ems (Empty spiracles), and the Hox genes Lab (Labial), Dfd (Deformed), Scr (Sex combs reduced), Antp (Antennapedia), Ubx (Ultrabithorax), Abd-A (Abdominal-A), and Abd-B (Abdominal-B). Also in the A–P axis, each segment is compartmentalized by segment polarity genes, as exemplified by the banding patterns of Gsb (magenta; Gooseberry) and En (blue; Engrailed). In the D–V axis, neuroectodermal and neuroblast compartments are mapped out by Vnd (ventral nervous system defective), Ind (intermediate nervous system defective), and Msh (muscle specific homeobox). The mesectoderm that forms the midline is determined by the spatial selector Sim (red band along midline; Simple minded). (b) Temporal selectors. During neuroblast proliferation, shifts in a temporal sequence of TF's occur over time that alter the developmental program of the lineage through time; such temporal selectors are depicted by the transition from Kr (pink; Kruppel) (pink) to Pdm (green; POU-homeodomain). (c) Tissue/cell type selector. During progenitor lineage progression, the type selector Gcm commits subsets of progenitors to a lateral glial cell fate. (d) Terminal selector. In postmitotic neurons, the terminal selector Dimmed activates a battery of genes that are together required for neuroendocrine identity and function for subsets of neurons.

inspired by intriguing phenotypes found in Hox gene and *engrailed* mutants, and expanded upon by Mann and Carrroll.[5] Loss of Hox gene expression results in homeotic transformations (replacement of certain body parts with a duplication of another body part) along the embryonic A–P axis[6] and loss of *engrailed* changes the morphology of the posterior region of the wing to an anterior region-like character.[7] Such phenotypes are caused by encroachment of a neighboring selector into the missing selector's compartment, predictably changing its developmental program. The mechanisms underlying this encroachment phenotype provide a useful criterion for defining spatial selectors. First, axial morphogenetic gradients broadly map out spatial selector expression along A–P and D–V axes (and P–D in appendages), on the scale of the whole embryo and also the scale of specific segments/tissues. Second, along any specific axis, cross-repression between neighboring spatial selectors (and/or their upstream regulators) sharpens the segregation of their expression and/or function to discrete compartments. This process scales the actual size of each compartment to a consistent fraction of the overall dimension of the axis; thus, the *Drosophila* wing is always compartmentalized appropriately, irrespective of the wing's size. Third, spatial selectors established along different axes generate a 3D Cartesian grid of combinatorial selector expression. Each combination of selectors subsequently determines a unique developmental program for the cells within each grid coordinate.[8,9]

We believe that one other class of embryonic compartment-defining TF's should be included as spatial selectors. These are the 'gap genes' which are defined by their mutant phenotype wherein a compartment (or body region) is eliminated, without expansion of a neighboring selector's developmental program.[10] We believe these should also be viewed as a subset of spatial selectors because they play a critical role in 3D patterning of the embryo and neuroectoderm. Indeed, the brain of flies and vertebrates are largely mapped out by such gap genes, that includes Orthodenticle (vertebrate *Otx1,2*) and Empty spiracles (vertebrate *Emx1,2*)[11,12] (Figure 1(a)). During *Drosophila* neurogenesis, Orthodenticle is predominantly expressed in the protocerebral brain neuromere (the most anterior of the three neuromeres) and its loss of function largely eliminates this neuromere due to loss of most neuroblasts. Empty spiracles is predominantly expressed in the deutocerebral and tritocerebral neuromeres, and its loss of function results in elimination of these structures.[13] In mouse, *Otx2* is expressed in the forebrain and midbrain region and *Otx1* is nested within this domain. Notably, *Otx2* nulls lack the rostral neuroectoderm that will become the forebrain, midbrain, and rostral hindbrain.[14,15] Also, mouse *Emx2* nulls are missing the dentate gyrus, as well as a reduced hippocampus and medial limbic cortex.[16,17] Remarkable conservation is seen between *Drosophila* and vertebrates in the relative expression and role of these TF's, as well demonstrated by cross-phylum rescue experiments.[11,18]

An overlapping spatial selector map for the *Drosophila* embryo and neuroectoderm has become well-defined, and shows remarkable conservation to that in vertebrates.[8,19] Along each axis, the spatial selectors are established by upstream regulatory events, that are not discussed here, and by cross-regulation[20–22] Within the neuroectoderm that gives rise to the ventral nerve cord (VNC), the whole A–P axis is patterned by Hox spatial selectors,[23,24] and this is overlaid by repeated intra-segmental A–P axes of 'segment polarity' spatial selectors including the TF's Engrailed (En), Invected (Inv), and Gooseberry (Gsb).[22] These A–P spatial selectors are additionally overlaid in the D-to-V axis by the spatial selector TF's (collectively known as columnar genes) muscle specific homeobox (Msh), intermediate nervous system defective (Ind), and ventral nervous system defective (Vnd), respectively.[25,26] Additionally, the VNC midline is specified by the spatial selector TF Single-minded (Sim)[27,28] (Figure 1(a)). Within this Cartesian grid of selectors, 30 lateral neuroblasts (NBs) delaminate from the neuroectoderm per VNC hemisegment, and form up to seven rows and six columns of NBs.[29] Thus, depending on its exact row/column position, each NB is endowed with a unique combination of A–P (segment polarity) and D–V (columnar) spatial selectors, that combinatorially encodes its individual identity and developmental program[30] (Figure 2(a)). Thus, NB 3–1 will generate a lineage of medial motor neurons and intersegmental interneurons, while NB7-3 generates a motor neuron, 2 serotonergic neurons and a corazonin-neuropeptidergic neuron.[36] The Hox A–P spatial selectors overlay this more broadly, altering the developmental program of specific NBs between segments.[24] For example, NB5-6 in thoracic segments has a larger lineage that that of NB5-6 in the abdomen, because the repression of posterior Hox genes in anterior segments allows for continued NB proliferation and the specification of unique late-born cells only in the thorax.[37]

Similarly, studies of the patterning of the mammalian and avian neural tube have provided a detailed picture of the establishment, cross-regulation and roles of combinatorially acting spatial selectors in generating distinct neural types in specific compartments.[38] Along the entire A–P axis, axial

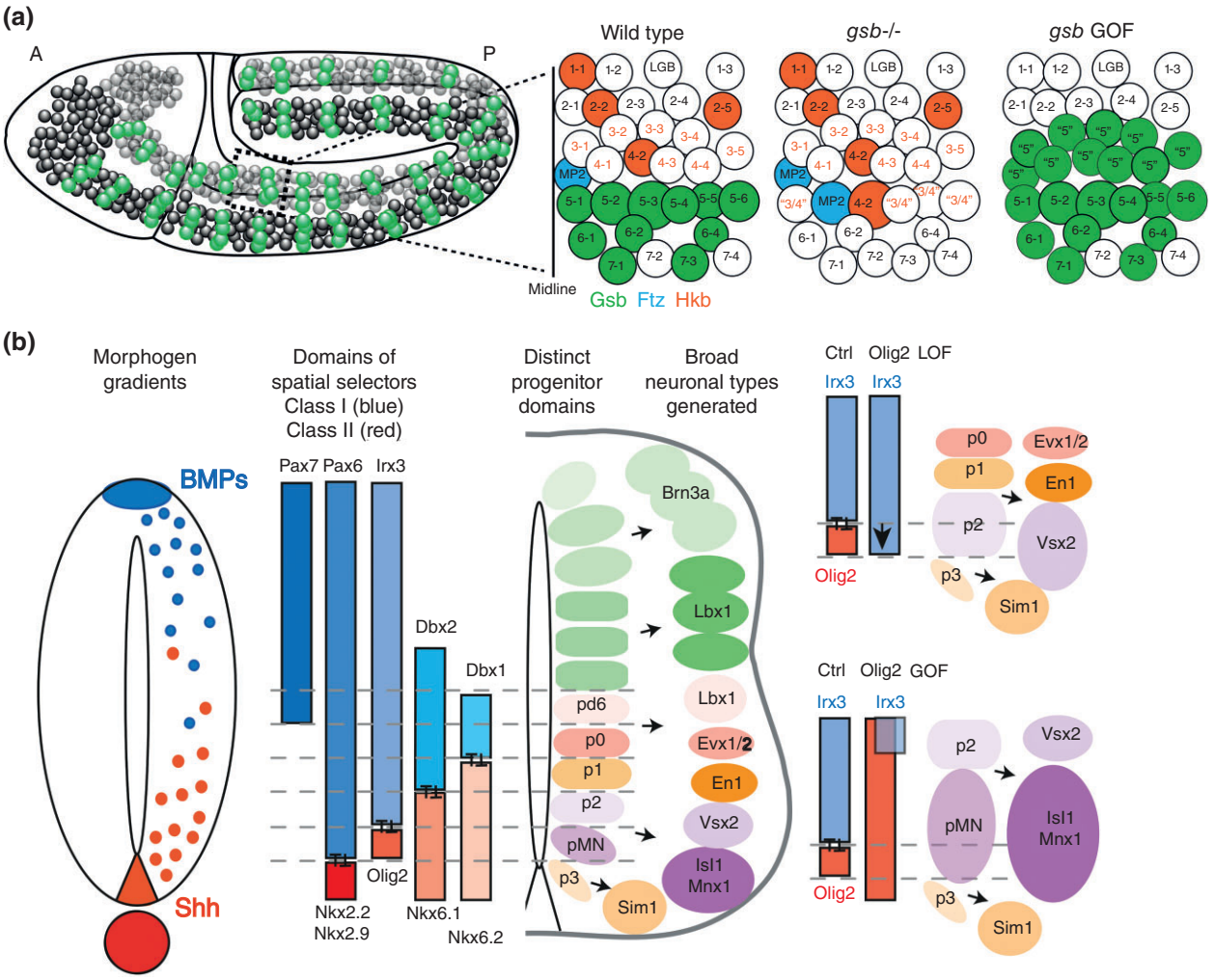

FIGURE 2 | Spatial selectors. (a and b) In the early *Drosophila* embryo, some 1200 NBs are formed, and are exposed to spatial selector information. (a; Right) The role of spatial selectors that determine intra-segmental A–P identity, exemplified here by expression of Gsb (green). In each hemisegment, Gsb is expressed by a subset of NBs, all in rows 5 and 6, and two in row 7. Ftz and Hkb mark other subsets (a few examples are shown here). In *gsb* mutants, row 5 identity is lost, and Ftz and Hkb become expressed by some row 5 NBs. Conversely, when *gsb* is misexpressed, row 3 and 4 NBs acquire row 5 identity (see[29] for more detail and references). (b) Generation of spatial selector compartments in the developing vertebrate neural tube. Morphogen gradients are established across neural tube neuroepithelial cells by Sonic hedgehog (SHH) from the notochord and floorplate, and Bone morphogenetic proteins (BMPs) from the roofplate. SHH establishes initially broad domains of expression of opposing Class I (blue; repressed by SHH) and Class II (red; activated by SHH) TF's. Class I and Class II TF's, whose threshold for repression or activation appose one another at a specific D–V step, are mutually antagonistic. This cross-repression results in sharp boundaries for their expression.[31,32] Across a number of antagonistic TF pairs, a set of six compartments (denoted p3 ventrally to pd6 more dorsally) of proliferating progenitors are generated, each with distinct combinations of TF's.[33] Each progenitor compartment then generates distinct sets of postmitotic neurons. For example, the pMN compartment generates motor neurons that initially all express *Isl1* and *Mnx1*. (b; Right panels) *Olig2* loss and gain-of-function tests, showing the ventral half neural tube. Upper panel shows *Olig1* and *Olig2* double mutants (*Olig2* LOF). In this mutant, the cross-repressive partner for *Olig2*, *Irx3*, expands ventrally into the pMN compartment. This reprograms the pMN compartment to a p2 compartment identity that generates an excess of *Vsx2*-expressing interneurons at the expense of motor neurons.[34] Lower panel shows *Olig2* gain-of-function (*Olig2* GOF). Motor neurons are generated more dorsally due in part to repression of *Irx3* within ventral regions, as well as loss of *En1* and *Emx1*-type neurons. Intriguingly, *Vsx2* expression is shifted more dorsally due to *Olig2*-mediated activation of *Lhx3*, which is required in motor neurons for their generation, but also in Vsx2 interneurons for their differentiation.[35]

signaling gradients direct the deployment of partially overlapping, cross-repressive *Hox* genes along the neural tube[39] that determine regional progenitor developmental potential. For example, *Hoxa6/Hoxc6* act at forelimb levels and *Hoxa10/Hoxc10/Hoxd10* at hindlimb levels to generate appropriate types of motor neurons at each level.[40–42] The D–V axis is compartmentalized by the responsiveness of TF's to

opposing gradients of bone morphogenetic protein and wingless ligands (BMPs and Wnts; dorsal source) and sonic hedgehog (*Shh*; ventral source). This establishes approximate domains of expression for those TF's, which is then sharpened by a series of binary cross-repressive TF interactions that strictly compartmentalizes the expression for each TF[31] (Figure 2(b)). These TF's act as spatial selectors because they define the developmental program of a spatial compartment and are established across an axis in part by mutual interaction.

The best defined example is patterning of the ventral half neural tube by SHH, secreted from the notochord and floorplate.[43] It represses the so-called Class I set of TF's and activates Class II TF's, at progressively lower thresholds from V-to-D. Opposing Class I and Class II factors, whose boundaries of expression coincide around a D–V step, then mutually antagonize one another's expression.[32] Thus, in V-to-D order these steps comprise (Class I vs. Class II TF's); *Pax6* vs. *Nkx2.2*, *Irx3* vs. *Olig2*, *Dbx2* vs. *Nkx6.1*, and *Dbx1* vs. *Nkx6.2*.[44] This establishes six progenitor compartments, each defined by a combinatorial TF code of spatial selectors. Subsequently, specific neural cell types are generated from each progenitor compartment. Loss of one of these spatial selectors results in the expansion of its opposing regulator, and shifts the developmental program of the compartment. Thus, loss of *Nkx6.1* and *Nkx6.2* allows ventral expansion of their respective repressive partners, *Dbx2* and *Dbx1*, to re-specify progenitor cells from motor neuron or V2 interneuron fate, into progenitor cells that now differentiate into V1 and V0 interneurons[45] (Figure 2(b)).

Finally, it is worth noting the extent to which there is conservation between *Drosophila* and vertebrates in axial patterning activities and in the spatial selector TF's that pattern the embryo and neuroectoderm, as fully reviewed elsewhere.[19,46]

Temporal Selectors Define Temporal Compartments Within Progenitor Lineages

Proliferating neural progenitors go through a sequence of changes in developmental potential that stereotypically generate distinct cell types at different time-points.[47–49] Several mechanisms have been identified that generate this fourth dimensional axis of information; however, the clearest example of TF's acting in this temporal axis stems from studies in the *Drosophila* CNS. Here, sequential expression of a series of TF's temporally compartmentalizes distinct developmental potentials within single NB lineages, thereby generating different neural types at each timepoint.[49] Temporal TF cascades of various compositions have been identified in all regions of the *Drosophila* CNS, and during both embryonic and larval stages.[49–52] We suggest that these TF's be termed temporal selectors: (1) They are sequentially and transiently deployed throughout progenitor proliferation to compartmentalize time. (2) They segregate the developmental program of cells arising from each temporal compartment. (3) They exhibit cross-regulatory relationships (directly and indirectly) that limit or coordinate co-expression and/or co-functionality.

Temporal selectors within the embryonic *Drosophila* VNC NBs are the best understood. Delaminated multipotent *Drosophila* NBs undergo a series of asymmetric divisions that generates an NB and a ganglion mother cell (GMC) that either divides to generate two neurons and/or glia (type I proliferation mode),[53] or directly differentiates into a neuron (type 0 proliferation mode).[54] As a VNC NB lineage proceeds, it sequentially expresses the TF's, Hunchback (Hb) > Kruppel (Kr) > POU-homeodomain factors Nubbin and Pdm2 (Pdm) > Castor (Cas) > Grainy head (Grh)[55,56] (Figure 3(a)). In most cases, the GMC and neurons from each NB division retain the temporal TF as a birth date marker. Importantly, the role of the temporal TF's is not necessarily to instructively determine a specific cellular fate, but rather to mark a change in temporal identity that commits the GMC and neurons to a difference in fate. This allows for the use of the same temporal TF cascade by many different lineages to generate their own unique diversity of lineage-specific neuronal subtypes.[49] Regardless, although this temporal cascade is common to many VNC lineages in *Drosophila*, temporal cascades comprising other TF's have been identified in the larval *Drosophila* brain[49,50,52] (Figure 3(b)). Thus, numerous different temporal cascades may be operational in different regions of the developing nervous system.[57]

Somewhat akin to spatial selectors, elimination of a temporal selector can result in; (1) precocious expression of the next TF and developmental program in sequence, such as in cases of precocious Kr expression in *Hb* mutants, (2) prolonged expression of an earlier temporal TF, such as for Pdm prolongation in *cas* mutants, or (3) skipping of a selector's developmental program, as in certain lineages in *Hb* and *Kr* mutants.[56,58] These phenotypes arise due to cross-regulatory interactions that exist between temporal TF's. First, gain-of-function studies show that TF's activate the next TF in the cascade and repress the next plus one TF. Also, overexpression of early acting TF's dominates over later-activated TF's to expand early born fates at the expense of later-born fates,[56] within a specific competence window.[59]

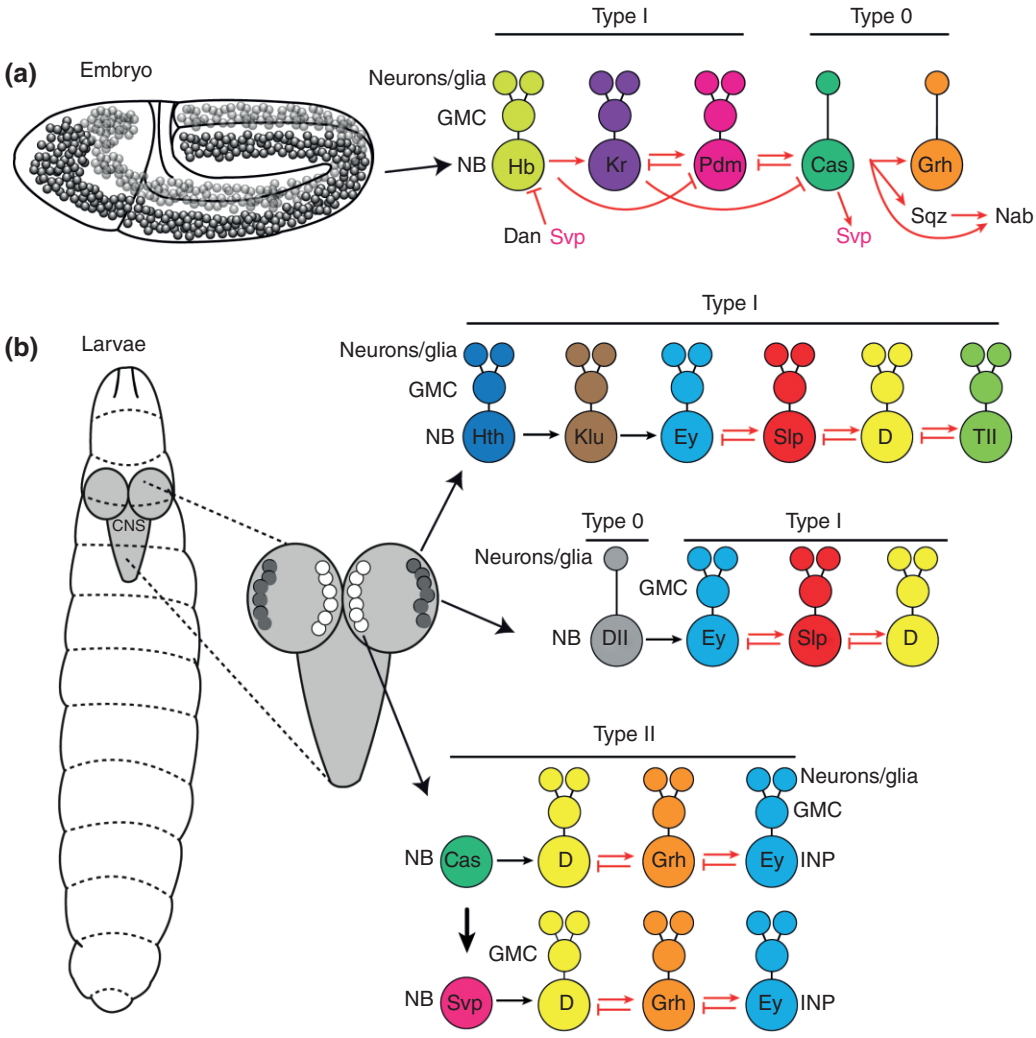

FIGURE 3 | Temporal selectors. (a) In the *Drosophila* embryo, most if not all NBs undergo a stereotyped sequential progression of temporal selector expression, from Hb (Hunchback), to Kr (Kruppel) to Pdm (POU-homeodomain), to Cas (Castor) and to Grh (Grainy head). Note that the expression of each temporal selector may persist through several NB divisions and may also overlap (not shown here). In each case, the GMC (ganglion mother cell) and neurons/glia arising from each NB is marked by the temporal selector expressed from its parental NB. The temporal selectors are cross-regulatory (shown by activation and repression arrows), but additional switching factors participate in the transition between temporal selectors. These include Dan (distal antenna) and Svp (Seven up) switching factors which ensure timely downregulation of Hb. A subset of lineages (as shown here from NB5-6T) re-express Svp and/or express the subtemporal TF's Sqz and Nab, which act to subdivide larger temporal windows, such as the Cas window in NB5-6T. The majority of embryonic NB lineages start off in the Type I proliferation mode wherein one GMC is generated that generates two daughter cells, but most lineages then switch to Type 0 mode, wherein no GMC is generated. (b) In larvae, post-embryonic neuroblast lineages show greater diversity in lineage progression and temporal selector cascades. Studies have identified several alternate temporal selector cascades, which involve mostly different TF's than those observed in the embryo. (top) In the optic lobe, two different cascades have been identified, controlling temporal progression in different parts of the lobe. (bottom) In the central brain, a specialized subset of NBs exist, Type II NBs, which generate a proliferative daughter cell; the intermediate neural progenitor (INP). Intriguingly, the NB and INPs express distinct temporal cascades. Neighboring temporal selectors are often co-expressed during the transition from one to the next (not depicted). Black arrows refer to temporal flow, while red arrows refer to regulatory interactions (see main text for details).

Second, additional layers of regulatory control of transition points also exist; the Hb to Kr switch is regulated by so-called switching TF's Seven up, Distal antenna and Distal antenna related[60,61] (Figure 3(a)).

While the temporal gene cascade explains how NBs generate distinct cell types over time, the cellular diversity observed in many lineages exceeds the obvious coding capacity of the TF cascade. To this end, several additional mechanisms explain how the temporal cascade may be further diversified beyond temporal TF number in any cascade. First, temporal selectors can transiently overlap with a neighboring

temporal selector to form combinatorial temporal windows.[62] Second, one of the switching TF's, Seven up, has been shown to be transiently expressed at two stages of NB lineage progression, acting to promote distinct cell fates at both stages.[63] Finally, in cases where a temporal selector persists for numerous NB divisions, these broader temporal windows may be subdivided by the action of so-called subtemporal genes, which are activated by temporal selectors, but act subsequently to diversify the outcome of that temporal selector's activity.[64] The existence of overlap in temporal selector expression (single and combinatorial coding), 'double-action' of temporal selectors (early and late), and subtemporal TF's, all combine to greatly diversify temporal coding in progenitors (Figure 3(a)). The end result of these regulatory mechanisms may well be that every daughter of a neuroblast has a unique fate based on temporal and subtemporal selector genes, although in the vast number of cases the responsible TF's have not been identified.

The functional outcome of temporal selector activity results in NB lineage-specific traits that presumably reflect an integration of information provided by temporal and spatial selectors. A clear example is that of thoracic NB5-6T-specific generation of Ap1-4 neurons. The overlap of Antp (spatial) and Castor (temporal) after the sixth NB division leads to an additional six rounds of NB division that generates the Ap1-4 neurons via coordinate activation of Collier. While Antp and Castor overlap in many NBs, the activation of Collier and the subsequent activation of a TF cascade that specifies the Ap1-4 neurons is unique to six NBs out of the ~700 in the VNC. This unique NB5-6 state is due to the combined action of a number of spatial selectors, i.e., Hox genes and Hox co-factors,[37] and presumably segment polarity and columnar genes. Few such examples exist to show how spatial and temporal selectors interact genetically, thus an important goal for future work would be to determine how temporal and spatial TF's combine to generate cell type diversity at the *cis*-regulatory level of downstream genes.

In vertebrates, the emergence of diverse neural subtypes from single lineages or spatial compartments is evident in many regions, but best described in the retina and cortex. In these regions, lineage-tracing shows that single lineages generate distinct neural subtypes dependent on birth date.[65–67] Clonally cultured retinal or cortical progenitors show that switches in neural type generation are intrinsic to cortical progenitors,[68] but are not so well intrinsically coordinated in retinal progenitors.[69] However, clear temporal TF cascades have not yet been identified within vertebrates. Regardless, in both of these regions, the Hb homolog Ikaros is required for the determination of early progenitor fates. Akin to the role of Hb in *Drosophila*, loss of *Ikaros* reduces the number of early-born neural fates, and maintained *Ikaros* expression extends the generation of early-born fates at the expense of later-born fates within a designated competence window.[70,71] Another clue to the conservation of temporal selector mechanisms is the role for Seven up and its vertebrate ortholog *Coup-TF I/II* in switching and subtemporal roles during lineage progression.[60,63,72] As the variety of temporal codes in different *Drosophila* neural lineages and the complexities of mammalian temporal coding become more fully described, it will be interesting to see if such mechanistic conservation represents the tip of the iceberg or just a few fortuitous but rare examples.

Tissue/Cell Type Selectors

The term master regulator was coined by Susumu Ohno to define TF's that he postulated must hierarchically coordinate gene deployment throughout development.[73] Studies thereafter found 'master regulator' to be a powerful descriptor for TF's that were necessary and sufficient for cell type determination and/or differentiation. A useful definition for a master regulator is one that acts in progenitors to trigger a discrete, cohesive/integrated (and often exclusive) genomic response that commits the progenitors to a specific tissue or cell type fate. Many of these master regulators are pioneer factors; referring to factors that can engage their targets even on nucleosomal (closed) DNA, and that can act at early stages of cellular reprogramming to transcriptionally engage silenced genes.[74] We suggest that a suitable term for this type of TF activity is tissue/cell type selector, as used by Mann and Carroll.[5] Loss of tissue/cell type selector function prevents formation of the specific tissue or cell type. Conversely, misexpression of a tissue/cell type selector into other cell types can predictably reprogram the cell's fate. This is a useful criterion given recent advances in cellular reprogramming, although most type selectors are limited in the range of cell types that they can predictably reprogram.

The template for our modern understanding of a tissue/cell type selector function was established in a gain-of-function study when the TF *MyoD* was shown to convert cultured primary fibroblasts, pigment cells, neural cells, and adipose and liver (but not all cell types) into skeletal muscle cells.[75,76] Today, *MyoD* is known to operate within an interconnected myogenic type selector network with *Myf5* and *Mrf4*. This network commits progenitors to a myogenic fate and, with *MyoD*, ushers muscle through its

differentiation.[77] Partial redundancy in this network means that an overt absence of skeletal muscle (albeit with numerous defects) only occurs in the absence of all of *MyoD*, *Myf5* and *Mrf4*.[78–80] Numerous other type selector networks have been identified, including the embryonic stem cell network of *Oct4*, *Sox2*, *Nanog*, *Klf4* and *Esrrb*[81,82] which are necessary for stem cell fate and are together sufficient to reprogram mature somatic cells to an induced pluripotential stem cell (iPS) fate.[83–85] In the reprogramming field, TF's with a more restricted potential to reprogram cells, i.e., from a fibroblast to a neural stem cell are often referred to as lineage-restricted. We do not however feel that this nomenclature is a good match for actual *in vivo* conditions, because tissues are made up by many different types of lineages, and any one lineage may, even at a late developmental stage, contain widely different cell types.

Also well-characterized is the retinal determination network in *Drosophila*. This tissue-type selector network is initially triggered by Twin of Eyeless (Toy) and then comprises a heavily-interconnected network of Eyeless (Eye), Sine Oculis (So), Eyes Absent (Ea) and Dachshund (Dac). The essential role of members of this network is evidenced by a lack of eye formation in loss-of-function mutants, and the sufficiency and interconnectivity of the members is readily observed in the ability of misexpression of these TF's in imaginal disks to activate (most of) the rest of the network and generate ectopic eye tissues.[86,87] Such tissue/cell type selector networks sit at the top of a transcriptional hierarchy to commit progenitors to a specific tissue or cell type fate. However, frequently, they also trigger coherent batteries of effector genes in terminally differentiated cells.[78,87] This can be viewed as ushering of cells from progenitor commitment through to final differentiation of type-specific fate, and typically utilizes feedforward TF function to carry it out, as will be discussed below.

In the developing nervous system, there is no TF with a *bona fide* tissue/cell type selector function that is necessary and sufficient for a 'generic' pan-neural fate in any organism. In fact, the only identified *bona fide* tissue/cell type selector in the nervous system is Glia cells missing (Gcm, also Glide) in *Drosophila*,[88] which commits neuroglioblast progenitors to a glial fate[89–91] (Figure 1(c)). Gcm is necessary and sufficient for glia commitment; in *gcm* mutants neuroglioblast lineages only generate neurons, and conversely, Gcm misexpression generates an excess of glial cells at the expense of neurons (Figure 4(c)). Indeed, in line with the notion of Gcm is a cell type selector, Gcm misexpression triggers gliogenesis in the mesoderm.[95] Gcm triggers a glial-specific pathway that is instructive for gliogenesis. Critically, Gcm activates Repo (Reversed polarity) expression around the time of the final mitosis. Repo is a glial-specific TF required for glial differentiation and maintenance, and as such may be considered to function as a terminal selector.[96–98] In addition, Gcm also represses the neuronal fate through activation of Tramtrack, which cooperates with Repo to block neuronal differentiation.[99,100] Thus, Gcm triggers a coherent glial-commitment genomic program that is also restrictive in that it blocks an alternate neuronal pathway.

Neurons are a highly distinctive cell type, and therefore it is perhaps surprising that no single neuron type selector has been identified. Instead, there appear to be TF's that act in subsets of neuronal progenitors to commit them to the generation of specific neuronal subclasses. While this does not fall neatly into the definition of a tissue/cell type selector, for a broadly definable cell type like muscle or glia, these TF's are essential and often sufficient for commitment of progenitors to a particular type of neuron. We believe that an increasing number of such examples will emerge in the future, and we predict that it will be biologically relevant to consider 'neuronal type' selectors as a primary organizing principle of neuronal commitment in progenitors. Indeed, the diversity of the nervous system may prove to require a variety of neuronal type selectors rather than being able to rely on a single pan-neuronal type selector. At this time, however, there is insufficient evidence to suggest that such a core mechanism lies at the heart of neuronal type commitment.

With these considerations in mind, we discuss the role of so-called proneural basic helix-loop-helix (bHLH) TF's that play essential deterministic roles in many instances of neural stem cell and neuronal type commitment.[101] In *Drosophila*, genes of the Achaete–Scute (AS-C) complex become restricted to a single cell within an ectodermal cell equivalence group through interaction with Notch signaling, and are necessary and sufficient for this cell to become a NB[102,103] (Figure 4(a)). While critical for NB formation throughout the fly, different proneural genes function in NBs at different body regions; the AS-C genes, *achaete*, *scute*, and *lethal of scute* mostly overlap in NBs of the CNS, whereas sensory organ precursors (SOPs) for certain peripheral neurons alternately express bHLH TF's Atonal or Amos.[104–107] Such subclass and compartmental specificity is a hallmark of proneural function and carries with it important implications for proneural function; the function of proneural genes within their specific progenitor populations often cannot be substituted by other proneural genes[108–110] (Figure 4(a)). This

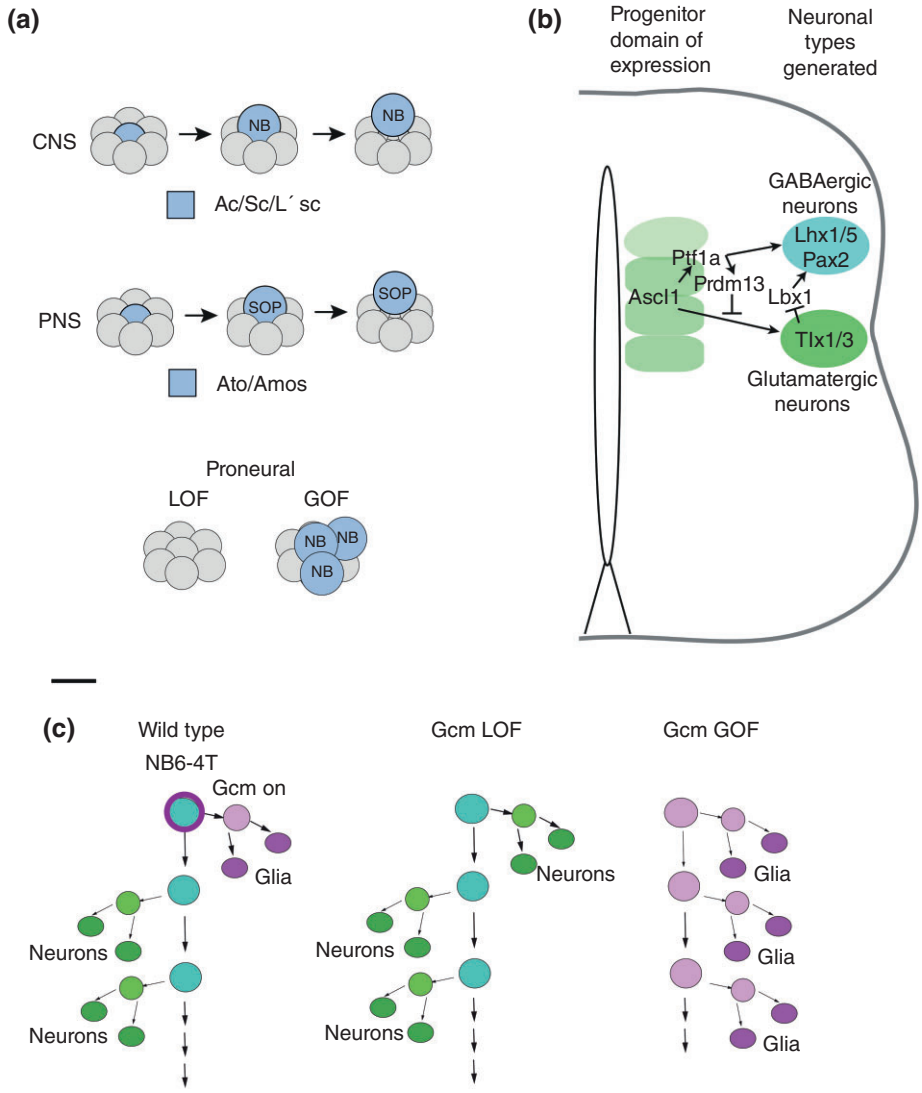

FIGURE 4 | Tissue/cell type selectors. (a) In *Drosophila*, during the process of Notch-mediated lateral inhibition, NBs (in CNS) and SOPs (in PNS) are selected from an equivalence group of cells. Critical for their neural progenitor identity is the expression of proneural TF's, which establish many features of early NBs and SOPs. However, studies reveal that different proneural members are expressed by different progenitors, with a general theme of Ac, Sc, and L'sc in the CNS, and Ato and Amos in the PNS. (b) In vertebrates, the proneural gene *Ascl1* is expressed in a number of dorsal progenitor domains. *Ascl1* activates *Tlx1* and *Tlx3* in postmitotic neurons dIL_B and dI5, and the *Tlx* TF's have a direct role as terminal selectors in activating their glutamatergic neurotransmitter identity. The expression of *Tlx1* and *Tlx3* further represses an alternate GABAergic fate by repressing *Pax2*. *Ascl1* also activates *Ptf1a* expression after a Notch-mediated delay. *Ptf1a* serves a dual function. It activates *Prdm13*, which interferes with *Ascl1* transcriptional activity to repress its function. It also activates *Lhx1/5* and *Pax2* among other TF's to promote the generation of dIL_A and dI4 GABAergic neurons. Thus, *Ascl1* establishes an opposing loop to establish glutamatargic and GABAergic fates for postmitotic neurons.[92,93] (c) Gcm is a cell type selector for glial cell fate. In thoracic segments, the neuroblast NB6-4T generates glial cells and neurons. NB6-4 initially expresses cytoplasmic Gcm (purple ring), but after the first NB division the resulting ganglion mother cell (GMC) increases Gcm expression, which becomes nuclear and drives glial cell generation in the progeny of that cell. In *gcm* mutants, no glial cells are generated by NB6-4T. When *gcm* is overexpressed in the lineage, glial cells are generated at the expense of neurons.[94]

highlights their context specificity and selectivity of action.[110] For this reason, it has been satisfying to see that local spatial selectors and tissue-specific type selectors play key roles in selecting an appropriate proneural gene to generate the correct neuronal type for each compartment or tissue; for example, axial selectors in the neuroectoderm or developing notum select AC-S gene expression[111,112] whereas Eyeless in the forming eye selects Atonal.[113,114]

Vertebrate analysis has found conserved and divergent functions for proneural genes.[115] A role for vertebrate proneurals in neuro(glio)blast specification

from ectodermal tissue, such as in *Drosophila*, is rare; although notable exceptions include *Neurogenin 1* and *2* (*Neurog1*, *2*; Atonal family) function in specific cranial placode regions, where they commit ectodermal cells to become neuronal progenitors of the cranial ganglia.[116,117] Instead, vertebrate proneural genes mostly act during progenitor proliferation to control Notch signaling, progenitor cycling, and neuronal type commitment.[101] Roles played by *Neurog2* and *Ascl1* factors provide good examples of the variety of functions that proneural genes play in promoting neuronal type generation in vertebrates. *Ascl1* (AS-C family; formerly *Mash1*) mutant mice exhibit severe loss of neurons and appropriate subtypes in the ventral telencephalon, autonomic ganglia, olfactory sensory epithelia, and subsets of dorsal neural tube interneurons.[118,119] Also, *Neurog1* and *Neurog2* are essential for neurogenesis of cranial and dorsal root sensory ganglia and neural tube motor neurons.[117,120–123] Elegant gene swapping studies have shown that *Ascl1* and *Neurog2* have mostly context-specific functions in these neuronal populations. Swapping *Ascl1* and *Neurog2* into one another's respective genomic loci demonstrated that *Ascl1* dominantly imposes its neuronal type specificity in the dorsal telencephalon and neural tube, which could be viewed from the perspective of a sufficiency for generation of this specific neuronal type, in this specific context. In contrast, *Neurog2* can replace *Ascl1* function in the ventral telencephalon but fails to fully recapitulate full mature differentiation of sympathetic neurons, and fails to rescue *Ascl1* mutants in the neural tube.[121] Thus, these factors act in a highly context-specific manner, at times fulfilling necessary and sufficient roles for neuronal subtype specification, but in other contexts acting in a necessary but not sufficient role, and for certain neuronal subtypes these different proneural genes can compensate for one another.

A role for the bHLH factor, *Ascl1*, has also been found in the feedforward diversification of two neuronal identities in the dorsal neural tube of vertebrates[92,93] (Figure 4(b)). These insightful studies show how a proneural gene sets in motion a transcriptional cascade that generates two distinct neuronal types. *Ascl1* becomes expressed in proliferating pD3-pD5 dorsal neural tube progenitors. Initially, *Ascl1* induces a large set of TF's and general neuronal genes[92] as well as *Tlx1/3*, which acts as a terminal selector to directly activate a glutamatergic gene battery.[122] Thereafter, *Ascl1*+ progenitors switch to *Ptf1a* activation, which feedforwards via *Prdm13* to repress *Ascl1* activity and block glutamatergic differentiation,[93] while simultaneously promoting GABAergic terminal fate via TF activation and direct activation of a GABAergic gene battery.[92] Such feedforward opposing loops are becoming recognized as a common mechanism to diversify neuronal subtypes from a common progenitor pool (see also Ref 62).

Terminal Selectors in the Determination of Unique Neural Subtypes and Subroutines

The final differentiation of cells into unique neural terminal identities requires the activation of subtype-specific repertoires of effector genes that define final and unique properties, e.g., neurotransmitter identity, electrophysiological properties and axon/dendrite morphology. The final differentiation step could conceivably mainly involve the maintained or redeployed activities of spatial, temporal, and tissue-type selectors. Indeed, TF's that act in these capacities in progenitors are known in certain cases to be maintained or redeployed in certain terminally differentiating neuronal subtypes. In such cases, the spatial, temporal, or tissue/cell type selector can directly contribute to terminal differentiation, typically in a feedforward manner.[78,87,124,125] This attests to the multiple roles that any one TF may play throughout development, and also to the context specificity of their activities. However, rather than such selectors always having such a dual role, a multitude of studies instead point to the roles of a separate functional category of TF's, expressed and acting at postmitotic stages of a lineage to differentiate the final and unique subtype identities of neurons.

Pioneering work for understanding the roles of such postmitotically active TF's stems from the identification of *Caenorhabditis elegans* MEC-3 and UNC-86, LIM-HD, and POU-HD TF's, respectively, in specifying touch receptor neuron identity.[126,127] Similarly, studies in *Drosophila*, revealed that the LIM-HD TF's Apterous and Islet were postmitotically and selectively expressed by small subsets of neurons, and controlled many aspects of their unique identities, such as their specific axon pathfinding and neurotransmitter identity.[128–131] More recently, characterization of Islet and Lim3 DNA-binding in *Drosophila* motor neurons confirms that they coordinately regulate a wide variety of effector genes that confer morphological and electrophysiological properties.[132] In vertebrates, studies of the related *Isl-1* gene also pointed to a critical postmitotic role in somatic motor neuron specification,[133] including a cooperative role for islet-1 and Lhx3 in defining their cholinergic identity.[134] Intriguingly, studies in *Drosophila*, zebrafish and mouse revealed that LIM-HD TF's are expressed in a combinatorial manner in subsets of motor neurons, and that

their combinatorial action dictates motor neuron subtype identity, as first revealed by differential axon pathfinding.[135–138] Such late, postmitotic-acting and neuronal subtype-specific TF's, with a clear role in terminal cell fate differentiation, were originally denoted 'cell fate determinants' or 'late acting regulators.' We believe that these TF's should more appropriately be denoted terminal selectors; a term proposed by Hobert and colleagues[139] (Box 1).

The terminal selector definition originally referred to TF's that activate the expression of most effector genes that together define the unique identity of a neuron,[140,141] and more recently to those TF's that coregulate a battery of effector genes that together define an important functional subroutine for the neuron. This latter definition satisfyingly describes a widespread phenomenon seen in all phyla (see below). One other aspect of the original terminal selector definition holds that the TF(s) be required throughout the life of the cell, in order to maintain expression of the effector genes that they first activated.[140,142] However, while a common observation, we believe that this definition unnecessarily excludes many TF's that play critical roles in activating the expression of effector genes that are important for subtype-specific neuronal identity and function. Most notably, axon/dendrite morphology is often established early in development and the TF's and axon/dendrite guidance effector genes need not necessarily be maintained for life. We favor including such TF's within the terminal selector definition if they fulfill certain experimental criteria. First, that the terminal selector TF(s) is relatively selectively expressed in specific neural subtypes. Second, that that the terminal selector TF(s) activate effector genes important for neural morphology or function. Third, that loss-of-function for the terminal selector TF(s) severely reduces or eliminates those effector genes. Fourth, that gain-of-function for the terminal selector TF(s) would result in the activation of those effector genes in other cells (albeit with context-dependent restrictions, as are observed with selector function in all categories).

The terminal selector term was originally defined and elegantly illustrated by the role of combinatorially acting TTX-3 and CEH-10 in defining the unique AIY neuron identity in *C. elegans*.[139] These TF's act as a combinatorial code (and strictly cooperatively) at a specific 16 bp *cis*-regulatory motif present in the regulatory regions of most effector genes that are unique to AIY-specific identity, but not effector genes that control pan-neuronal identity or morphology.[139] Similar roles have been described for TF's in other *C. elegans* neurons.[126,127,142] Perhaps the most extreme example of a TF acting as a terminal selector is CHE-1, which appears to singularly define and activate most effector genes that define the identity of ASE gustatory neurons in *C. elegans*.[143]

This regulatory scheme, the differentiation of final and unique cell fate by one or two TF's acting at most effector genes that uniquely define a neuron subtype's entire identity, appears somewhat common (although not universal) in *C. elegans*, but no examples have been found in more complex metazoans such as *Drosophila* and mouse. This is likely related to the ratio between the number of neurons and the number of TF's. In *C. elegans* there are 302 neurons and 959 somatic cells (in the hermaphrodite),[144] in comparison to ~1000 TF's.[145] In contrast, *Drosophila* has ~150,000 neurons and ~723 TF's,[146] while mouse has about 70 million neurons and ~1500 TF's.[147,148] Such an increase in the ratio of neuron number and subtypes to TF's inherently necessitates increased combinatorial coding. Indeed, recent work from the *C. elegans* system itself demonstrates the combinatorial coding of TF's acting as terminal selectors, even in a system that does not necessarily require it. For example, TTX-3 acts with CEH-10 to directly activate the majority of AIY-defining effector genes, including its cholinergic battery.[139] However, in other neurons, TTX-3 acts with an unidentified TF to activate an AIA neuron effector gene battery, including its cholinergic battery. In NSM neurons, TTX-3 acts with UNC-86 to activate an effector gene battery including a serotonergic neurotransmitter battery. Comparatively, UNC-86 acts with CFI-1 to activate a cholinergic gene battery in IL2 sensory neurons and the URA motor neurons.[149] Thus, even in *C. elegans*, there is evidence of extensive combinatorial coding of TF's that act in the capacity of terminal selectors.

In *Drosophila*, examples of TF's acting as terminal selectors for a battery of genes that fall into numerous functions are best illustrated by roles for Islet, Lim3 and Even-skipped. In their distinct motor neuron pools, Even-skipped and Islet/Lim3 coordinately direct expression of batteries of effector genes for axon pathfinding (such as *unc-5, beaten path, Fas2, Neuroglian, robo2, and robo3*) (reviewed in Ref 150) and for electrophysiological properties.[132,151,152] In the future, it will be interesting to determine the extent to which the *cis*-regulatory use of 'selector motifs' by terminal selectors, as well demonstrated in *C. elegans*,[140] is also observed in *Drosophila* and vertebrates.

In a few rare cases, a single TF may act as a terminal selector for a specific subroutine in most neurons in which it is expressed. The best resolved of these are for the postmitotic TF's DAF-19 (in *C.*

elegans) and Dimmed (in *Drosophila*). Dimmed is exclusively expressed in the majority of neurosecretory neurons of the *Drosophila* nervous system. It is necessary and sufficient for direct activation of a coherent gene set that scales up the neurosecretory capacity of the neuron including the dense core vesicle biogenic machinery and neuropeptide processing enzymes.[153–155] A similar neuroendocrine terminal selector has not been identified in other species, although the Dimmed ortholog, *Bhlha15*, plays critical roles in the differentiation of an excocrine cellular phenotype in many tissues in vertebrates.[156] DAF-19 is exclusively expressed in all *C. elegans* ciliated sensory neurons, and it directly activates expression of a battery of effector genes that are required for cilia formation and function without affecting other cell-specific or pan-neuronal features of those neurons.[157,158] As stated above, the founding definition for a terminal selector did not include subtype-specific morphological features. However, DAF-19 stands out in this regard due to its clear function in all ciliated neurons to direct expression of genes required for ciliated structure, and we propose that such TF's be referred to as terminal selectors.

Perhaps the most easily defined and best-understood example of terminal selectors acting to govern a subroutine is neurotransmitter identity, which requires co-expression of a coherent set of biosynthetic enzymes, vesicular transport proteins and synaptic reuptake transporters.[142,159] These are best described in *C. elegans* and vertebrates, yet are poorly defined in *Drosophila*. Analysis of how neurotransmitter gene sets are coordinately regulated has highlighted the role of terminal selectors acting via subroutines. An important observation has been that different TF's (or combinations thereof) can act as the terminal selector for a specific neurotransmitter subroutine in different neurons. For example, in *C. elegans*, different TF combinations activate the same cholinergic gene battery in different neurons (see below). This has important consequences for our understanding of terminal selectors, as it indicates that while certain TF's act as the terminal selector for a specific subroutine in many neurons, this is not always the case. Thus, we should consider TF's as acting in the capacity of a terminal selector in each specific neuronal subtype context, and should not view a TF as being a terminal selector for a specific subroutine in all neurons, unless this is proven. We provide examples of these below.

Cholinergic gene battery: The cholinergic gene battery includes vesicular acetylcholine transporter (*VAchT*), choline acetyltransferase (*ChAT*), high affinity choline transporter (such as *Slc5A7*), and ATP-citrate lyase (*Acly*). In *C. elegans*, this gene battery is regulated by distinct sets of TF's in different cholinergic neurons; by UNC-3 in a cholinergic subset of motor neurons,[160] by TTX-3 and CEH-10 acting cooperatively in AIY neurons in which these TF's also act as terminal selectors for the neuron's entire unique gene expression profile,[139] and by TTX-3 with an unknown TF in AIA neurons.[149] The cholinergic genes each have separable *cis*-regulatory motifs that respond to these TF codes in their respective neurons. In vertebrates, *Isl1* is expressed in most cholinergic neurons. Recently, ChIP-seq, loss- and gain-of-function genetics and reporter analysis showed that *Isl1* is a direct regulator of a cholinergic battery of genes. Intriguingly, *Isl1* utilizes cooperatively acting LIM-HD regulators to induce these genes in different neurons; *Lhx3* in spinal motor neurons and *Lhx8* in forebrain cholinergic neuronal types,[134] and perhaps also with *Phox2a* in cranial motor neurons.[161] Perhaps surprisingly, *Isl1/Lhx3* and *Isl1/Lhx8* complexes cannot substitute for one another within their respective neurons, indicating that the cooperation of *Lhx3* or *Lhx8* is not interchangeable between the two populations.[134] Such studies provide certain lessons regarding TF function with regard to such subroutines. These TF's all perform other functions unrelated to cholinergic phenotype in the neuron, and also play distinct functions in other neurons.[133,149,162] Further, any specific subroutine may be directed by different TF's in distinct neurons, and only in some cases the same TF's. In *Drosophila*, no TF's have been described as acting in the capacity of a cholinergic terminal selector. The *acj6* gene (*abnormal chemosensory jump 6*) is required for normal levels of ChAT in olfactory neurons, but its role is only partial and its gain-of-function ability to increase expression of a reporter for the cholinergic locus (comprising ChAT and VAChT) is very small.[163]

Dopaminergic gene battery: The dopamine gene battery tyrosine hydroxylase (*TH*), aromatic L-amino acid decarboxylase (*Aadc*) also known as dopa decarboxylase, vesicular monoamine transporter 2 (*VMAT2*), and dopamine transporter (*DAT*). A combination of AST-1, CEH-43 and redundantly-acting CEH-20/CEH-40 activates a dopaminergic synthesis and transport gene battery in all *C. elegans* neurons as a terminal selector code.[164] Vertebrates have a number of different dopaminergic neuronal cell types. In ventral mesodiencephalic (mdDA) neurons the orphan nuclear receptor *Nurr1* is a critical direct activator of most of this dopamine gene battery, often acting in concert with *Pitx3* in the capacity of terminal selector.[165,166] Notably, *Nurr1* and *Pitx3* together also activate many genes in these neurons outside of the dopaminergic subroutine, thus they are not restricted

in function to activating only a dopaminergic gene battery.[167] In olfactory bulb dopaminergic neurons, the AST-1 ortholog, *Etv1*, is required for expression of the key TH biosynthetic gene, echoing a degree of conservation from *C. elegans*, but the TF's that may act as terminal selectors of the dopaminergic gene battery in these neurons are not known.[164]

To summarize, we propose a broadening of the terminal selector concept to include any postmitotically and relatively selectively expressed TF(s) that acts in a terminally differentiating cell to direct the expression of effector genes that underlie unique features of a certain neuron/glia subtype, including morphology, neurotransmitter expression, and electrophysiological properties.

Combinatorial Codes That Diversify Final and Unique Cell Subtypes

Unlike results from *C. elegans*, there is no identified case of a final unique cell subtype fate being mostly dictated by a single terminal selector or terminal selector code in *Drosophila* and vertebrates. Instead, certain terminal selectors may execute specific subroutines, but mostly it is combinatorial codes of terminal selector TF's that appear to be the logical principle governing final and unique subtype fates. Here, we discuss two well-defined examples of combinatorial codes in vertebrates and *Drosophila* that highlight the complexities and dynamism of their activities to diversify unique, robust subtypes among closely related postmitotic neurons. In these examples, genetic loss of a TF acting within a combinatorial code may be observed as a change in subtype fate to one that resembles another closely related subtype in which that TF is not normally expressed. Similarly, genetic gain of a TF appears to reconstitute another subtype's combinatorial code with a resulting predictable conversion of subtype identity.

Motor neuron subtype differentiation provides an illustrative example for combinatorial codes in determining unique neuron subtype fates, primarily because axon pathfinding phenotypes are relatively easy to score, and seminal studies have revealed that motor neuron subtype identity is specified by TF codes.[135,138] Motor neuron determination and differentiation are remarkably well conserved. *Drosophila* and vertebrates share a core motor neuron differentiation pathway that determines generic motor neuron properties, but also motor neuron subtype properties (best defined for axon pathfinding).[168] The key players in the *Drosophila* (and vertebrate) motor neurons that contribute to terminal selector coding include *oli* (*Olig2*), *nkx6* (*Nkx6.1,6.2*), *exex* (*Mnx1/Hb9*, *MNR2*), *islet (also tailup)* (*Islet1,2*), *lim3* (*Lhx3,4*), and also includes *vvl* (*POU3f1/scip*) and *zfh1* (*ZEB1.2*).[38,33,169–173]

In vertebrates, a combination of *Lhx3/4*, *Isl1/2*, and *Mnx1* (also *Hb9*) determines a generic motor neuron identity in young postmitotic motor neurons.[136,174] However, this initial motor neuron code rapidly breaks down into a more complex motor neuron subtype terminal identity code defined by partially overlapping expression of LIM-HD factors *Isl1*, *Isl2*, *Lhx3*, *Lhx4*, and *Lhx1* (among others) that instructively differentiates subtype identities for motor neurons.[175] For example, the LMC-lateral motor neuron division is *Lhx1+/Isl1−* and their axons project to the dorsal limb bud, whereas the LMC-lateral motor neuron division is *Lhx1−/Isl1+* and their axons project to the ventral limb bud. Altering *Lhx1* or *Isl1* expression predictably alters these axon pathway choices via changes in downstream axon guidance effector gene expression.[138,162,176,177] Underlying this discrimination of *Lhx1* and *Isl1* expression is the activity of retinoic acid signaling that promotes *Lhx1* at the expense of *Isl1*, as well as mutual antagonism between these two TF's.[176,178] Also, specific retinoic acid signaling and *Lhx1/Isl1* discrimination requires the activity of *Hoxc6* and *Hoxc8*.[40,179]

A key determinant underlying motor neuron pool subtype differentiation that appears to shape subtype expression of LIM-HD TF's, among other TF's, is an instructional Hox gene coding system that is established and operates in young postmitotic motor neurons. However, a role for these Hox genes as terminal selectors for motor neurons awaits evidence that they directly contribute to effector gene expression, in addition to their role in determining the motor neuron subtype-specific terminal selector codes. Intriguingly, numerous of these Hox genes are activated in postmitotic motor neurons in ignorance of typical A–P axial expression domains; perhaps being co-opted evolutionarily to assist in generating the increasing motor neuron diversification required for fin/limb and sympathetic nervous system evolution of vertebrates.[39] Initially, young motor neurons express multiple Hox genes and the *Hox* co-factor *Meis1*,[180] but these become rapidly refined through their cross-repressive interactions to become differentially restrictively expressed within specific LMC motor neuron pools at limb levels. For example, *Hox4*, *Hoxa7*, and *Meis1* cross-repress one another to generate three motor neuron pools co-expressing either *Hoxa7* or *Hox4* or *Hox4 + Hoxc6* pools within a *Hoxc8* context, that each are instructive for the innervation of different forelimb muscles.[180] Such interactive Hox codes underlie the diversification of subtype-specific TF

expression that is required for the subtype-specific differentiation of limb-level LMC and also preganglionic motor neuron subtype.[39] Remarkably, these Hox gene activities are gated through the activity of *FoxP1*, although the mechanism is unknown. In *FoxP1* mutants, all Hox-dependent phenotypes are erased resulting in a loss of pool-specific TF expression and known guidance molecules. Although motor axons still innervate limbs, they appear to generically follow permissive pathways rather that target and innervate specific muscles.[181,182] The mechanisms of *FoxP1* gating are unknown, such as whether *FoxP1* interacts directly with Hox TF's or acts to prime *cis*-regulatory elements at which Hox TF's mediate their effects. This represents an interesting avenue for research in the future.

Similar to the specification of vertebrate motor neurons, *Drosophila* motor neurons are also specified by combinatorial action of terminal selector TF's[135] (reviewed in Refs 130, 171, 183). To a great extent, these TF's are evolutionary orthologs of the ones specifying vertebrate motor neurons. However, while these TF combinatorial codes act to specify motor neurons down to the subtype, with respect to the muscle field they innervate, *Drosophila* motor neurons are uniquely identifiable with respect to their stereotyped position and axon innervation of a single muscle fiber, and our current understanding of the combinatorial codes within these neurons does not explain this final diversification.

The *Drosophila* Ap1-4 neurons provide another excellent example of combinatorial coding, taking advantage of the highly specific ability to discriminate unique identities based on specific neuropeptide expression.[131,155,184,185] This example also provides a clear case of feedforward combinatorial coding for cell subtype differentiation. These neurons are generated from the final four divisions of the NB5-6T neuroblast, and acquire their final identity in a step-wise manner.[184] This regulatory cascade is initiated by the combinatorial action of broader spatial (Antp, Hth, and Exd), and late temporal (Cas) selectors, acting within the context of NB5-6T progenitor fate,[37,62] which is itself specified by the unique combination of other spatial selectors (segment polarity and columnar genes).[186] This combinatorial action triggers expression of several downstream TF's: the COE TF *knot* (also *collier*), the final temporal TF *grainy head*, and the subtemporal TF's *squeeze* and *nab*. These TF's in turn participate in an intricate feedforward and cross-repressive network that establishes terminal selector TF combinatorial codes wherein the four Ap1-4 neurons each differentially express a set of TF's including Apterous, Eyes absent, Dachshund, Dimmed, Seven Up, and phosphorylated Mad, in combination with subtype-specific maintenance of Knot and Grh expression.[62,63,184] Throughout the establishment of these terminal selector codes, the Knot TF shows extensive feedforward functions; acting first to activate Apterous and Eyes absent, then together with these TF's to activate Dimmed in two of the four cells, and finally with all three downstream TF's to activate specific neuropeptide gene (Nplp1) expression in one of the four cells.[184] Thus, throughout this period of Knot expression, its regulatory function is changed by the context provided by the serial activation of other TF's. These extensive combinatorial feedforward and cross-repressive TF codes result in Ap1 neurons differentiating to express the Nplp1 neuropeptide under the combinatorial control of Collier, Apterous, Eyes Absent, and Dimmed, and Ap4 neurons differentiating to express the FMRFa neuropeptide under the control of Apterous, Eyes Absent, Dachshund, Grainy head, Dimmed and phosphorylated Mad (Figure 5(b)). Finally, the specific axon pathfinding of the Ap neurons is under control of subsets of these regulators, exemplified by pathfinding errors in *ap* and *eya* mutants.[128,187]

CONCLUSIONS

Advances in molecular genetic technologies are beginning to provide many of the required details that will allow us to define how TF's direct nervous system development, that requires a combination of (1) inducible loss- and gain-of-function genetics, (2) transgenic testing and genomic editing (such as by CRISPR) of gene *cis*-regulatory regions, (3) RNA-seq approaches to identify all cellular transcripts, and (4) ChIP-seq approaches to identify where specific TF's bind DNA, and also those chromatin modifications that correspond to gene activity, repression or alternate states such as being poised. These are all best performed *in vivo* where the native and complex context is provided as an experimental backdrop.

The concepts of spatial, temporal, tissue/cell type selector, terminal selectors, and combinatorial codes are useful to understanding the underlying principles of neural diversification. Satisfyingly, although these terms originate from the interpretation of genetic evidence, the increasing wealth of molecular/biochemical data lends support to the mechanistic differences between them. However, it should be underscored that the dividing lines between these functions can be blurred and that TF's should not always be viewed as having any one functional role, as most act in multiple

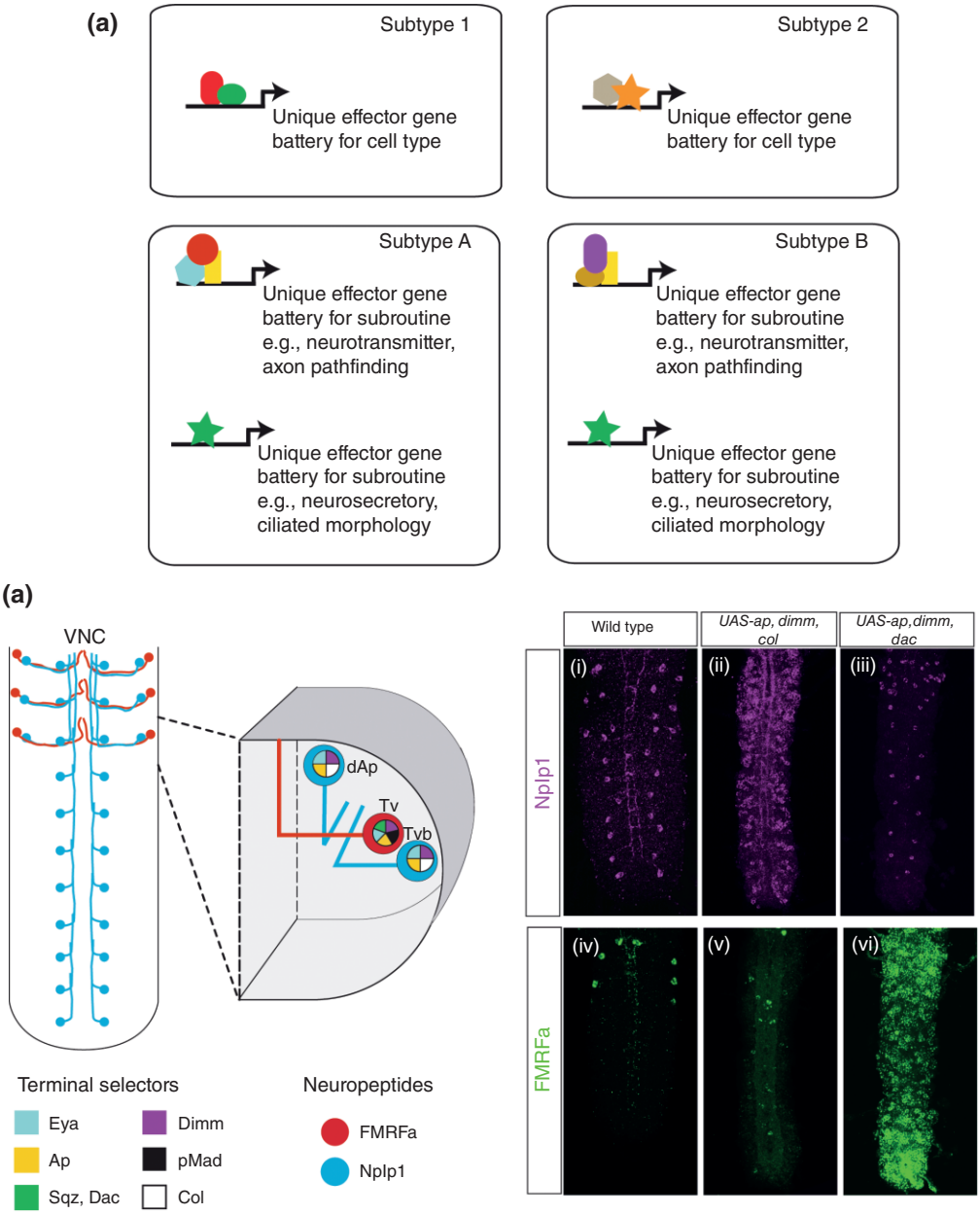

FIGURE 5 | Terminal selectors. (a) The original definition[139] of terminal selector genes focused on one or two TF's acting to direct a whole gene battery that uniquely identifies a specific neuronal subtype. For example, for cell subtypes 1 and 2, these neurons differ in the combination of TF's that synergistically activate most effector genes that are together unique to that neuronal subtype. In many other cases, terminal selectors can fall into two other categories. Comparing subtype A and B, a subroutine comprising most effector genes controlling neurotransmitter identity or axon pathfinding may be controlled by a combination of TF's that act as a terminal selector for that subroutine, but may not be used in other subtypes for their neurotransmitter or pathfinding. In parallel, single TF's (such as Dimmed or DAF-19) may singularly direct a battery of genes for a specific subroutine that is common to many neuronal subtypes, such as for neurosecretory phenotype (as for *Drosophila* Dimmed) or a ciliated sensory morphology (as for *C. elegans* DAF-19). (b) An example of a combinatorial code of TF's acting to dictate critical aspects of unique subtype identity. In the *Drosophila* ventral nerve cord (VNC) a set of TF's are expressed in subsets of Apterous neurons. While these TF's are expressed by many other neurons, their combinatorial co-expression is unique to two neuronal subtypes; the Tv neurons that express the FMRFa neuropeptide, and the Tvb and dAp neurons that express the Nplp1 neuropeptide. Mutants for any of these TF's disrupt establishment of cell fate. (i–vi) Combinatorial CNS-wide misexpression of these TF's can trigger widespread neuropeptide expression. The Nplp1 expression code consists of Ap (Apterous), Dimm (Dimmed), and Col (Collier). The FMRFa expression code consists of Ap, Dimm, and Dac (see Ref 184 for more details). In these same cells, Dimm further acts as a terminal selector for a gene battery controlling neurosecretory function. Importantly, each triple code selectively activates one neuropeptide and not the other, in spite of the mere substitution of one TF.

cellular contexts in different roles, even within a single lineage or single cell.

There are several important future avenues for studies regarding TF and nervous system development. First, it will be important to address the molecular nature of the regulatory flow from spatial, temporal, cell/tissue type, terminal selectors, onward to terminal effector genes and final cellular properties. Comprehensive molecular insights into these multistep genetic cascades are few, if any. Such studies should also be coupled to detailed epigenetic studies, to address the extent to which early selectors control final cell fate by epigenetic control, by direct and 'classical' gene regulation, or a combination of both. Second, it is still somewhat of an enigma how different members of the same TF family, which apparently bind similar DNA targets, can play such vastly different roles during development. Third, this molecular complexity further extends into how it is that many TF's can act both as activators on some genes, and repressors on others, often in the same cell. Finally, it will be intriguing to determine how the regulatory TF flow and function is utilized to maintain unique cell fates throughout the life of the cell.

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