Published in final edited form as:

Nat Neurosci. 2014 July ; 17(7): 899–907. doi:10.1038/nn.3731.

Maintenance of postmitotic neuronal cell identity

Evan S. Deneris¹ and Oliver Hobert²

Evan S. Deneris: esd@case.edu; Oliver Hobert: or38@columbia.edu

¹Case Western Reserve University School of Medicine, Department of Neurosciences, Cleveland OH, 44106

²Columbia University Medical Center, Department of Biochemistry and Molecular Biophysics, Howard Hughes Medical Institute, New York, NY 10032

Abstract

The identity of specific cell types in the nervous system is defined by the expression of neuron type-specific gene batteries. How the expression of such batteries is initiated during nervous system development has been under intensive study over the past few decades. However, comparatively little is known about how gene batteries that define the terminally differentiated state of a neuron type are maintained throughout the life of an animal. We provide here an overview of studies in invertebrate and vertebrate model systems that have carved out the general and not commonly appreciated principle that neuronal identity is maintained in postmitotic neurons by the sustained, and often autoregulated expression of the same transcription factors that have initiated terminal differentiation in a developing organism. Disruption of postmitotic maintenance mechanisms may result in neuropsychiatric and neurodegenerative conditions.

The classic definition of the identity of specific neuron types in the nervous system relies on anatomical and electrophysiological features. These features have provided a rich description of the enormous diversity of cell types in animal nervous systems¹. With the advent of molecular biology, the definition of cell type identity has started to include molecular features of individual neurons². While such molecular markers have remained relatively sparse for a great number of neuron types in vertebrate nervous systems, molecular profiling studies over the past decade have nevertheless significantly broadened the spectrum of genes known to be coexpressed in specific neuron types, thereby defining neuron-type specific gene batteries^{3,4}.

While specific features of individual neurons are remarkably plastic⁵, many neuron identity features remain stable throughout the life of a terminally differentiated, postmitotic neuron. For example, with some notable exceptions to be discussed below⁶, neurotransmitter identities, as assessed by expression of enzymes and transporters that synthesize, synaptically package and reuptake a specific neurotransmitter, remain stable in many adult neuron types. One question that immediately arises from such enduring stability is how the tremendous diversity of neuron-types and their elaborate connectivity patterns are maintained for up to many decades of life in mitotically quiescent cells. A number of studies over the past few years have shown that there is a critical need for active maintenance of postmitotic neurons. What is the nature of the gene regulatory programs that are required across the lifespan of postmitotic neurons to preserve their subtype identity, morphology,

and connectivity and thereby sustain normal neural circuit function? In contrast to the intense focus over the past two decades on understanding the gene regulatory programs that specify different neuron types⁷ the regulatory programs that function later in life to maintain postmitotic neuron–type identities has received far less attention. The recent application of these tools has revealed that at least one answer to how maintenance of neuronal identity is achieved lies in considering how terminally differentiated features of a postmitotic neuron are induced in the context of development⁸. Indeed, a common lesson to emerge is that gene regulatory programs launched early in fetal life to specify neuronal–types identities continue to function later in life to maintain postmitotic identities. Here we review key findings from these studies that illustrate the impact of disrupted maintenance mechanisms on neuronal identity, function and circuitry with potential relevance to neurodegenerative and neuropsychiatric disorders.

Maintenance of neuronal identity in invertebrates

The identity of neurons in the nervous system of the nematode *C.elegans*, composed of 118 anatomically distinct neuron types, have been well–defined not only on the level of anatomy but also lineage and function⁹. A key advantage of studying the neuronal identity problem in *C. elegans* is the availability of many hundreds of reporter genes that monitor the expression of terminal identity markers of individual neuron types, such as neurotransmitter receptors, neurotransmitter synthesizing enzymes and transporters, sensory receptors, ion channels and neuronal signaling factors. Classic screens for mutants that affect specific neuronal lineages identified transcription factors (TFs) such as the Atonal homolog *lin-32* or the POU homeobox gene *unc-86* (which gave the POU domain its “U”) which control neuroblast identity and fate⁹. Genetic screens for behavioral mutants (e.g. *Unc* – uncoordinated, *Ttx* – thermotaxis–defective, *Odr* – odortaxis defective, *Che* – chemotaxis defective), as well as screens for mutants in which the expression of *gfp*–tagged terminal identity features of specific neuron types is affected, have identified a large number of TFs that act in postmitotic neurons, either alone or in specific TF combinations, to initiate neuron–type specific differentiation programs⁹. The deep knowledge of functional, morphological and molecular features of individual worm neuron types has enabled a fine–grained probing of these TF mutant phenotypes. A shared theme of many of these TFs, dubbed “terminal selectors”¹⁰, is that their genetic removal results in the loss of many functionally unrelated terminal identity features, such as for example, neurotransmitter identity, expression of neurotransmitter receptors, ion channels etc.; however, generic features shared by all neurons, e.g. the expression of synaptic vesicle proteins, are generally unaffected. Currently, distinct TFs or TF combinations are known to initiate the differentiation program of almost one half of all the 118 neuron classes of the worm⁹. In many of these cases, it was demonstrated that terminal selectors directly act on *cis*–regulatory elements in proximity of terminal identity genes to initiate their expression^{11–16}.

The maintained expression of *C. elegans* terminal selector transcription factors throughout the postmitotic life of the respective neurons suggests that these factors not only initiate but also actively maintain the expression of terminal identity features of individual neuron types. To address this possibility directly, a number of studies have tested whether postembryonic and hence postdevelopmental removal of a number of terminal selectors from fully

differentiated neurons affects the maintenance of the differentiated state of specific neuron types, as assessed by examining the expression of continuously expressed terminal identity markers. Temporally controlled removal of terminal selectors was achieved in a number of different manners and in all cases examined, it was shown that the factors that initiate terminal differentiation programs are also continuously required to maintain the differentiated state (Table 1). For example, the Zn finger TF *che-1* is continuously required to maintain the differentiated state of the glutamatergic ASE sensory neurons¹⁷, the Prd-type homeodomain TF *unc-42* is continuously required to maintain ASH sensory neuron identity¹⁸, the EBF-type TF *unc-3* is continuously required in cholinergic motor neurons¹², and the combinatorial TF signature composed of the ETS-domain TF *ast-1* and Dlx-type homeodomain TF *ceh-43* is continuously required to maintain the differentiated state of all dopaminergic neurons in *C. elegans* (Table 1)^{16, 19}.

A continuous requirement has not only been observed for *C. elegans* terminal selector-type TFs that very broadly define the identity of a neuron type, but also for TFs that only affect select subsets of identity features. Specifically, the *die-1* Zn finger TF initiates the expression of a gene expression program that distinguishes the left and right ASE sensory neuron²⁰. These two neurons are very similar to one another, but differ in the complement of chemoreceptors that they express. *die-1* is expressed in the left neuron to initiate left-specific chemoreceptor and repress right-specific chemoreceptors. Postembryonic removal of *die-1* demonstrated that *die-1* is continuously required to define chemoreceptor gene expression²¹.

The *die-1* example also provides an entry point to tie the role of chromatin modifications into maintenance of gene expression. Genetic analysis suggests that *die-1* operates with the MYST-type histone acetyltransferase complex to initiate and maintain left vs. right ASE subtype features²¹. The use of a temperature-sensitive allele of a MYST complex component has underscored the continuous need of MYST in maintaining the identity of the left ASE neuron²¹. This specific case demonstrates chromatin modification states are not self-sufficient in maintaining a specific gene expression state, but also depend on the continuous presence of a transcription factor, *die-1*, that likely recruits histone modifying complexes.

Studies in *Drosophila* have provided additional support for the notion that TFs that initiate differentiation programs also continuously support the differentiated state (Table 1). A combination of seven transcription factors is required to initiate and maintain olfactory receptor expression patterns in fly olfactory neurons²². In photoreceptor neurons, active maintenance mechanisms ensure that the expression of distinct types of rhodopsin (rh) genes remains restricted to specific photoreceptor neurons. For example, the Erect wing (Ewg) TF is continuously required to prevent expression of Rh6 in R8-type photoreceptors²³. Moreover, the peptidergic Tv1 and Tv4 neurons require a combination of TFs (including the bHLH TF Dimm and the LIM homeodomain TF Apterous) to acquire a specific identity and, as revealed through the use inducible dsRNA transgenes, also require these TFs for maintenance of their differentiated state²⁴.

Maintenance of neuronal identity in vertebrates

In contrast to more short-lived invertebrate neurons, vertebrate neurons can be preserved through many decades of life. Like in invertebrates, many vertebrate transcription factors involved in the coordinate induction of batteries of genes encoding key features of neuron-type identity, such as the neurotransmitter phenotype, continue to be expressed across the life span in the mature neurons they initially helped to generate. Over the past several years, conditional Cre/Lox gene-targeting approaches have been implemented to investigate these terminal-selector-like TFs in the stage-specific maintenance of neuronal identity, morphology, and connectivity (Table 1).

Our current understanding of vertebrate neuronal maintenance mechanisms has been gained, in part, through studies of mouse serotonergic, dopaminergic, and noradrenergic neurotransmitter identity^{25–30}. The majority of the TFs examined were previously shown to perform crucial functions in the acquisition of specific monoaminergic features through activation of neurotransmitter-type gene batteries that encode the capacity for synthesis, reuptake, and plasma or vesicular membrane transport of a particular monoamine^{31–39}. The expression of all of these factors extends into adulthood and therefore conditional targeting strategies were used to determine their ongoing functions in the maintenance of identity features.

A consistent finding from the various studies of monoaminergic identity is that genetic removal of individual TFs within a few days after the acquisition of transmitter identity causes a profound loss of expression of various markers of transmitter phenotype within a particular postmitotic neuron type^{25–28, 30, 40, 41}. In fetal postmitotic 5-HT neurons, for example, expression of all markers of the 5-HT neuron-type gene battery and 5-HT itself was severely reduced within a day of targeting either *Pet-1* or *Lmx1b*^{30, 40}. Similarly, conditional targeting of *Gata-3* in fetal 5-HT neurons resulted in early, albeit less severe, maintenance defects in *Tph2*, *AADC*, *Sert*, and *VMAT2* expression⁴⁰.

Severe losses of noradrenergic identity followed targeting of *Hand2* and *Phox2* in differentiated fetal sympathetic neurons^{25, 28}. Targeting of the nuclear hormone receptor gene, *Nurr1*, in fetal or neonatal mesencephalic dopamine (DA) neurons led to a severe and progressive loss of DA identity and tissue DA transmitter levels particularly in the SN (Substantia nigra) but also in the VTA^{26, 27}. SN DA neuron cell bodies disappeared after early fetal targeting. Likewise, ongoing expression of the LIM-homeodomain factors, *Lhx7* and *Islet1*, are required at fetal stages to maintain cholinergic identity of postmitotic striatal interneurons and expression of several sensory neuron-type identity features in maturing dorsal root ganglia neurons, respectively^{42, 43}.

Importantly, transcriptional maintenance programs continue to be crucial during early postnatal and even adult stages for preservation of monoaminergic-type and cholinergic-type neuron identity and transmitter levels^{44, 26, 29, 40}. For example, tamoxifen-inducible dopaminergic targeting of *Nurr1* in early adulthood led to progressive and severe decreases in the expression of the DA neuron gene battery encoding tyrosine hydroxylase, dopamine transporter and vesicular monoamine transporter 2 (VMAT2) and reduced striatal DA levels

but normal numbers of mutant cell bodies remained²⁶. Whole genome RNA sequencing of adult DA neurons obtained by laser capture revealed that Nurr1 determines DA identity primarily through activation of gene expression. In addition to maintaining expression of DA transmitter identity genes, Nurr1 is also required for maintenance of expression of genes involved in oxidative phosphorylation and mitochondrial function, which as discussed below, is potentially of significant clinical relevance. In 5-HT neurons, sustained expression of tryptophan hydroxylase, serotonin transporter, VMAT2, and vesicular glutamate transporter III was significantly weakened after adult targeting of *Pet-1* or *Lmx1b*^{29, 40}. A paired homeodomain orthologue, *Pitx*, appears to function similarly to maintain 5-HT neuron identity in planaria⁴⁵.

Although sustained expression of key identity genes, such as those encoding rate-limiting enzymes for monoaminergic neurotransmitter synthesis, are highly sensitive to loss of adult TFs other transmitter identity genes become independent of the TFs that were necessary for their initial expression in fetal postmitotic precursors. For example, *Pet-1* is required for the initial fetal expression of AADC and the 5-HT autoreceptor, Htr1a; however, targeted loss of *Pet-1* in adult 5-HT neurons had no effect on the maintenance of their expression. Similarly AADC expression becomes insensitive to knock out of *Lmx1b* in adult 5HT neurons²⁹. Interestingly, while VMAT2 becomes resistant to loss of *Pet-1* in adult 5-HT neurons it remains dependent on *Lmx1b* for its maintenance in adult 5-HT neurons^{29, 40}. These findings indicate evolving terminal selector requirements as the animal matures for maintenance of individual identity features. Individual identity features that lose dependence on a particular terminal selector may remain dependent on another for stable life long expression. Alternatively, terminal selectors can compensate for one another and even cooperate synergistically in the joint regulation of individual identity features^{16, 46}. These types of neuronal terminal selector interactions have not yet been explored in vertebrates and therefore it is not clear whether maintained expression of an identity feature that is resistant to knock out of one or another terminal selector is lost or significantly reduced when both factors are targeted.

Autoregulation

The continuous expression of terminal selectors raises the question of how their expression is sustained given that the upstream regulatory factors that induced their expression in postmitotic precursors are generally downregulated at this stage. In several cases, such maintained expression has been explicitly shown to be mediated by direct autoregulation, the simplest form of a classic feedback loop^{11, 14, 4748}. For example, the LIM homeobox TF *mec-3* (the “M” in LIM domain) autoregulates in *C. elegans* touch sensory neurons, which require *mec-3* for their terminal differentiation⁴⁹, the Zn finger TFs *che-1* and *odr-7* autoregulate in the *C.elegans* ASE and AWA sensory neurons, respectively^{11, 50}, in which they initiate the respective differentiation programs and the LIM homeobox TF *ttx-3* and the Prd-type homeobox TF *ceh-10* jointly autoregulate their expression in the cholinergic AIY interneurons, aside from initiating the terminal differentiation program of AIY¹⁴. In all cases examined, autoregulation is direct. For example, TTX-3 and CEH-10 jointly control downstream, terminal effector genes via a cognate binding site, the “AIY motif” and this AIY is found in the *ttx-3* and *ceh-10* loci themselves, where it is functionally required for

their maintained expression^{14, 51}. Not only do terminal selectors autoregulate, but factors that only regulate specific subaspects of a neuron type, such as the above-mentioned *die-1* transcription factor that control chemoreceptor expression, also autoregulate⁵².

Experiments aimed at testing the autoregulatory concept for mouse terminal selector-type factors are limited but do provide clear support for it at least in adult 5-HT neurons. The *ePet::CreER^{T2}* transgenic construct developed for targeting *Pet-1* in adult 5-HT neurons was used as a reporter to determine whether *Pet-1* expression in adulthood was needed to maintain transgenic CreER expression. In control animals injected with vehicle strong expression of CreER mRNA was detected in the cell bodies of DRN 5-HT neurons. However, in tamoxifen treated mice, which resulted in a severe knock down of *Pet-1* expression, the expression of CreER was not sustained. Chromatin immunoprecipitation assays with chromatin isolated from embryonic hindbrain demonstrated that *Pet-1* occupies sites in the *Pet-1* upstream regulatory region where conserved *Pet-1* ETS binding sites are located. Together these studies suggest that sustained expression of *Pet-1* requires positive autoregulation through direct binding of *Pet-1* to its own cis regulatory region⁴⁰.

Autoregulation of terminal selector transcription factors can be viewed as a “lock in” mechanism. Regulatory factors that act in immature neurons or neuroblasts to induce terminal selectors are usually only transiently expressed. Through the activation of autoregulating terminal selectors, a regulatory state that is driven by these earlier acting determinants is stably maintained (Fig. 1). This is well illustrated in the case of the AIY interneurons, in which transiently expressed *Zic* and *bHLH* transcription factors as well as a temporally controlled Wnt signal activate expression of *TTX-3* and *CEH-10*, which then autoregulate their own expression⁵¹. A further illustration of this concept is provided by activation of autoregulating *Pet-1* by transient *Gata-2* expression in serotonergic precursors⁵³. *Gata-2* appears to directly control *Pet-1* through binding to conserved *Gata* sites in the *Pet-1* enhancer region⁵⁴. However, *Gata-2* expression begins to decline shortly after initiation of *Pet-1* expression and is not detectable a few days later in 5-HT neurons⁴⁰.

Neuronal survival, morphology, and connectivity

A remarkable outcome of studies aimed at determining “late” functions of continuously expressed TFs in the vertebrate brain is that their roles are not limited to maintenance of neurotransmitter identity features. Indeed, over the past few years the critical need for neuronal maintenance throughout life has been powerfully reinforced with findings demonstrating that targeted disruption of postnatal TF expression trajectories can lead to dendritic and axonal atrophy, neuron death, transdifferentiation and dramatic behavioral changes that model human neurodegenerative and neuropsychiatric diseases.

An early indication of more diverse functions of continuously expressed TFs was the finding in conditionally targeted *Lmx1b* mice that following the loss of serotonergic neuron identity markers at fetal stages nearly all 5-HT neuron cell bodies were missing from the neonatal brain suggesting *Lmx1b* is also required for 5-HT neuron survival³⁰. *Pet-1* does not appear to be necessary for survival of 5-HT neurons as *Pet-1* deficient cell bodies remain in normal numbers within the DRN after conditional targeting of *Pet-1*. Thus, although these TFs

share many common targets in 5-HT postmitotic precursors, *Lmx1b* likely regulates a distinct gene battery important for early postnatal neuron survival. *Lmx1b* becomes dispensable for maintenance of 5-HT neuron survival at some undefined postnatal stage as targeting of *Lmx1b* in adult 5-HT neurons does not lead to disappearance of *Pet-1*⁺ 5-HT cell bodies²⁹.

Interestingly, ongoing expression of the paired-type homeobox TF, Pax6, is intrinsically required, in a highly specific manner, for survival of dopaminergic neurons in the olfactory bulb through maintenance of crystalline α A expression, which prevents caspase-3-mediated programmed cell death⁵⁵. Similarly, the zinc finger factor, Gata-3, is continuously required in adult sympathetic neurons for survival (but not identity) of virtually all sympathetic neurons through transcriptional maintenance of proper levels of expression of several anti-apoptotic and pro-apoptotic genes⁵⁶. Lastly, a targeted investigation of the role of homeobox genes in the maintaining adult glutamatergic neurons in the mouse CNS has identified the LIM homeobox gene *Lhx1* and the POU homeobox gene *Brn3* as being continuously expressed in glutamatergic neurons in inferior olivary neurons in the brain stem. Removal of *Lhx1* in adult mice resulted in the loss of this neuron type¹⁸. Whether *Lhx1* is exclusively required for neuronal survival, or whether loss of these neurons is a secondary consequence of loss of the neurotransmitter phenotype of these neurons is currently not known.

The retinoic acid-related orphan nuclear receptor α (*Rora*) is expressed at high levels in fetal Purkinje cells (PCs) and is essential for their survival and maturation⁵⁷. As *Rora* expression continues across the life span conditional targeting approaches were used to determine whether it is also required for maintenance of PCs⁵⁸. Conditional disruption of ongoing *Rora* expression between postnatal days 10–21, when PC maturation is nearly complete, led to dendritic atrophy and distal spine loss⁵⁸. Furthermore, substantially decreased numbers of PCs were detected several weeks later. In addition, loss of *Rora* led to reversion of the mature mono-innervation pattern of climbing fiber input onto PCs to a multi-innervation pattern indicative of an earlier stage of development. Significantly, dendritic atrophy and morphological alterations of climbing fiber input to PCs also occurred after targeting of *Rora* in adult PCs. A progressive dendritic and axonal dystrophy also resulted from loss of *Nurr1* in adult DA neurons²⁶. These findings provide intriguing examples of the requirement for transcriptional maintenance of adult neural connectivity by the very TFs that initially setup the neural circuitry⁵⁸.

Transcriptional maintenance in the adult retina has recently been demonstrated to be crucial for preservation of visual activity and the prevention of late onset photoreceptor degeneration. *Otx2*, a paired-type homeodomain protein, is an essential regulator of multiple early steps in eye development including determination of the retinal pigment epithelium, photoreceptor cell fate and bipolar cell differentiation^{59, 60}. The persistent expression of *Otx2* in the retinal pigment epithelium (RPE), photoreceptors and bipolar cells led to studies of its potential postnatal functions in preserving retinal integrity and function. Conditional targeting of *Otx2* at P30 resulted in a progressive and ultimately an apoptotic-driven total loss of photoreceptors⁶¹. A more rapid degeneration of the RPE occurred resulting in disruption of RPE cell adhesive contacts with photoreceptors. RPE-specific

targeting of *Otx2* disrupted maintenance of RPE specific gene expression and led to a near total degeneration of photoreceptors similar in magnitude to that resulting from deficiency of *Otx2* from all *Otx2*+ retinal cell types. These findings demonstrated that ongoing *Otx2* function, intrinsic to RPE cells, regulates photoreceptor survival through cell non-autonomous interactions with the RPE⁶².

Another noteworthy study illustrated the requirement for *Nrl*, a basic leucine zipper TF, in the postnatal maintenance of retinal photoreceptors. *Nrl* initially functions in retinal precursors as a switch for specification of rod photoreceptors over cones⁶³. Montana et al. have shown that conditional targeting of *Nrl* in adult retina led to loss of rod-enriched gene expression and derepression of some cones genes, hence a partial rods-to-cones transdifferentiation. Further evidence in support of transdifferentiation in adult targeted cells was the appearance of photoreceptors with the morphological features of cone-like cells and retinal physiological changes indicative of enhanced cone activity. The potential for rod to cones switching with targeted *Nrl* loss of function was highest at birth and progressively declined as the animal matured. The reprogramming of rods to cones in *Nrl* postnatal conditional mutant mice indicates that ongoing *Nrl* expression is necessary for maintenance of rod photoreceptor identity and suppression of cone traits⁶⁴.

Dedicated maintenance factors

The maintenance TFs discussed so far are also necessary for initial differentiation of neuron-type identities. Are there additional kinds of maintenance factors whose expression does not commence until after acquisition of neuron-type identity and therefore are not involved in initial specification? In other words is there a separate class of TF that is dedicated to maintenance of neuronal identity or survival? Dedicated maintenance factors have been described in *C.elegans*. The ASE chemosensory neurons require the *che-1* Zn finger transcription factor for the initiation and maintenance of many distinct terminal identity features of the ASE neurons, including the expression of putative chemoreceptors from the receptor-type guanylyl cyclase (rGC) family¹¹. The *Otx*-type transcription factor *ceh-36* has a minor role in initiating the expression of the rGC proteins, but becomes indispensable at later stages to maintain rGC expression⁵². Importantly, the *ceh-36* maintenance factor is a direct target of *che-1*¹¹ and the both operate via distinct binding sites in a feed forward configuration to control rGC expression¹¹. This demonstrates that initiators of differentiation programs also induce the expression of maintenance factors that will assist the initiating factors in maintaining gene expression programs (Fig. 1).

The engrailed (*En*) homeodomain paralogues, *En1* and *En2*⁶⁵, are also crucial for maintenance of mesDA neurons but not acquisition of DA identity. Simon et al., showed with *En1/tau-LacZ* null and *En2*^{-/-} double knock out mice that nearly normal numbers of TH⁺ mesDA neurons were generated and acquired their dopaminergic identity⁶⁶. However, soon thereafter DA cell bodies in the SNc and VTA completely disappeared from the fetal midbrain. These results are consistent with the finding that onset of *En* expression in postmitotic mesDA neurons occurs 2–3 days after acquisition of TH identity⁶⁷. Further studies suggested that *En* maintains postmitotic mesDA neurons cell autonomously by prevention of apoptosis⁶⁷. Therefore, at least for the majority of mesDA neurons, *En1/2* are

not required for acquisition of DA neuron identity but instead are dedicated maintenance factors essential for their postmitotic survival⁶⁸. In addition, their maintenance function may extend to neuroprotection as evidenced in MPTP, 6-OHDA, and α -synuclein models of Parkinson's disease⁶⁹.

En1/2 may play a similar dedicated maintenance role in serotonergic neurons. Conditional targeting of *En1/2* specifically in postmitotic fetal 5-HT neurons did not interfere with specification or early maintenance of 5-HT neuron identity. However, beginning at E16.5 a progressive perinatal decline in the numbers of 5-HT⁺ neurons in the dorsal raphe nucleus (DRN) occurred as a result of failed maintenance of Tph2 expression. The loss of serotonergic identity was followed by apoptotic loss of 5-HT cell bodies⁷⁰, which resulted in a significant deficit in adult forebrain 5HT. Since *En1/2* is also expressed earlier in 5-HT neuron lineage⁷¹, it cannot be excluded that apart from its maintenance role, it is also required at the progenitors stage. An intriguing preliminary finding is that *En1* expression appears to be reduced in *Pet-1* deficient 5-HT neurons and conserved *Pet-1* ETS binding sites are present in the *En1* upstream region. This suggests that similar to direct control of *ceh-36* by *che-1*, continuous autoregulated *Pet-1* expression is required not only for direct maintenance of 5-HT identity features but also to directly recruit other 5-HT maintenance factors (Fig. 1).

A different DA neuron class, juxtglomerular dopaminergic olfactory bulb (OB) interneurons, may also depend on a dedicated transcriptional maintenance factor, COUP-TF1 encoded by *Nr2f1*, for preservation of neuron-type identity. Although OB interneurons of various identities are continually generated throughout life from progenitors, COUP-TF1 expression is largely restricted to mature DA and GABAergic interneurons within the glomerular (GL) and granule cell (GcL) layers and is rarely detectable in subventricular zone-derived neuroblasts that will enter the bulb⁷². Early conditional targeting in the *EMX1*-derived OB interneuron lineage indicated that COUP-TF1 was not necessary for the generation or survival of DA interneurons. However, *Nr2f1*-deficiency led to a loss of TH expression in adult GL interneurons but not in DA interneurons generated in the early postnatal period. Moreover, targeting of *Nr2f1* with lenti-viral Cre injection into the adult rostral migratory stream did not affect TH expression in the GL after thirty days but led to a significant decrease in GL TH expression at 60 days post-injection. Together these findings suggest COUP-TF1 is not involved in acquisition of DA interneuron identity but instead is required for maintenance of adult DA identity⁷².

Plasticity of maintenance programs

Interestingly, olfactory sensory deprivation through naris closure caused a dramatic decrease in the number of COUP-TF1⁺ neurons in the GL. Decreased expression of COUP-TF1 following sensory deprivation was accompanied by a severe reduction in the numbers of TH⁺ neurons in the GL, which is in keeping with the well-documented control of TH expression in OB DA neurons by afferent synaptic activity^{73–75}. These findings support a model in which activity-dependent control of COUP-TF1 is essential for maintenance of OB DA neuron identity by COUP-TF1⁷² and reveal the potential for environmentally driven plasticity of gene regulatory maintenance programs in postmitotic neurons. Are other

transcriptional maintenance programs influenced by sensory–stimulation or other types of environmental signals such as secreted signaling molecules? Although gene regulatory programs that specify neuron–type identity are induced by the interaction of secreted morphogens, once launched, these programs appear to function cell autonomously in the specification of neuron–type precursors. However, recent studies in *Xenopus laevis* embryos and larvae have shown that early in development but after progenitor specification changes in spontaneous calcium spike activity can change the number of differentiating neurons that express serotonergic identity. Increased spike activity suppressed the number of *Lmx1b*⁺, *Tph*⁺ and 5-HT⁺ neurons, while decreases in spike activity led to increased numbers of these neurons, thus revealing early homeostatic control of serotonergic signaling in a reserve pool of neurons in response to altered activity^{6, 76}. Further studies showed that *Lmx1b* is required for changes in 5-HT identity in response to altered calcium spike activity. Thus, *Xenopus Lmx1b* is sensitive to changes in spontaneous activity in a subset of differentiating neurons that are serotonergic or are poised to become serotonergic. These results reveal neuroplasticity of gene regulatory programs that govern the differentiation or early maintenance of 5-HT neuron identity and raise the intriguing question of whether later in life maintenance of 5-HT neuron identity can be influenced by synaptic activity–dependent changes in the expression of terminal selectors.

In addition to activity–dependent control of transmitter acquisition in early development and maintenance of OB DA interneuron identity, there are striking examples of postmitotic neurons that acquire new transmitter identities as a result of cell extrinsic signaling or sensory input. In the adult hypothalamus, interneurons in the periventricular and paraventricular nuclei switch off their dopaminergic identity and acquire a somatostatin (SST) peptide identity in response to short and long–day photoperiods⁷⁷. The changes in peptide expression were correlated with parallel changes in mRNAs encoding TH and SST indicating that transcriptional activation and repression mediated the switch in identity. The transcriptional mechanisms underlying the switch have not been explored but perhaps involve activity–dependent modulation of continuous *Nurr1* expression⁷⁸.

A well–studied example of transmitter switching is the adrenergic to cholinergic transmitter switch that occurs postnatally in specific small populations of postmitotic sympathetic neurons that innervate rodent eccrine sweat glands in footpads⁷⁹. The switch is physiologically important, as it is required for the acquisition and maintenance of sweat gland secretion⁸⁰. Target–derived neurotrophic secreted cytokines acting retrogradely on sympathetic neurons through gp130 receptor signaling are required for transmitter switching⁸¹. Although the transcriptional mechanisms involved in switching are poorly understood a recent study in primary sympathetic neuron cultures has shown that knockdown of *Satb2*, a chromatin organizer that binds to AT sequence–rich nuclear matrix attachment regions, prevents cytokine–stimulated upregulation of the cholinergic markers, *Chat* and *VAcH*, and suppression of noradrenergic markers, *TH* and *DBH*⁸². An intriguing possibility is that cytokine signaling via the gp130 receptor modifies chromatin architecture in a way that suppresses *Phox2b* and/or *Hand2*–mediated maintenance of noradrenergic identity and induces cholinergic identity through increased expression of *Chat* and *VAcH* transcriptional activators.

The role of chromatin modifications

The studies discussed so far demonstrate that both in vertebrate and invertebrates, sequence-specific DNA binding proteins that initiate terminal differentiation programs in postmitotic neurons are also continuously required to maintain the differentiated state. *Cis*-regulatory control regions and transcribed genes are often marked by specific patterns of chromatin modifications. For example, histone H3 is methylated at lysine 4 across actively transcribed genes⁸³. Such types of chromatin modifications are important in maintaining differentiated features of neurons, as for example illustrated through the use of a temperature-sensitive mutant allele in a histone modification enzyme²¹. The continuous requirement of this enzyme is mirrored by the continuous requirement of a Zn finger transcription factor that likely recruits this enzyme²¹. However, the continuous requirement of TFs illustrates that histone modifications are alone not sufficient to maintain regulatory states. In other words, a TF that initiates specific patterns of gene expression and thereby is responsible for the introduction of histone modification patterns that mark transcriptionally active regions does not become superfluous after introducing these modification patterns. The insufficiency of chromatin modifications in maintaining gene expression states is unfortunately not generally appreciated in the literature⁸⁴. The importance of specific kinds of chromatin modifications in maintenance of neuronal identity is an intriguing question that will require conditional removal of histone writers and erasers in specific postmitotic neuron-types.

Potential late onset paths to nervous system dysfunction

The foregoing examples illustrate the necessity of transcriptional maintenance programs for preservation of long-lived postmitotic mammalian neurons. What is the potential relevance of disrupted postnatal neuronal maintenance to nervous system pathogenesis? Not surprisingly, given the severity of the molecular maintenance defects reported so far, disruption of ongoing TF expression causes clinically relevant late onset physiological phenotypes. For example, continuous *Otx2* expression in the RPE is required for prevention of photoreceptor degeneration. Heterozygous mutations in human *Otx2* have been linked to many different kinds of ocular malformations. Interestingly, several of the genes regulated by *Otx2* in the RPE have been linked to retinal diseases including genes associated with increased susceptibility to age-related macular degeneration⁶². Thus, disruption of ongoing *Otx2* expression is a potential path to susceptibility for late onset retinal degenerative diseases.

Postnatal *Rora* deficiency in Purkinje cells caused a progressive impairment of various sensorimotor functions including deficits in motor coordination, muscle strength, and balance mimicking the severe ataxic phenotype of spontaneous (*staggerer*) or germ line targeted *Rora* mutant mice⁵⁸. *Rora* is required for expression of several Purkinje cell genes involved in establishing normal motor learning and coordination⁸⁵. Further, postnatal loss of *Rora* expression promotes polyglutamine-expanded mutant ATAXIN1-mediated neurodegeneration of Purkinje cells⁸⁶. Thus, continued *Rora* expression might be required for maintained expression of key downstream genes important for preservation of normal cerebellar function and prevention of susceptibility to neurodegeneration.

The critical need for continuous transcriptional maintenance of neuron-type identity was demonstrated by the dramatically increased anxiety-like behaviors that resulted from adult targeting of *Pet-1*⁴⁰. These anxiety behaviors are reminiscent of the abnormal emotional behaviors that result from germ line targeting of *Pet-1* and other serotonergic identity genes^{33, 87, 88}. A large literature has demonstrated that altered expression of either *Tph2* or *Sert* in animals can lead to a range of behavioral abnormalities. Moreover, suppression of *Htr1a* autoreceptor expression in 5HT neurons during the early postnatal period increased anxiety-like behaviors later in life⁸⁹. Human genetic studies suggest allelic variation that influences *Tph2* or *Sert* expression or function is associated with increased susceptibility for various neuropsychiatric disorders^{90–92}. *Tph2* and *Sert* are direct targets of *Pet-1*^{40, 93} and their reduced expression along with the accompanying significantly reduced levels of forebrain 5-HT that resulted from adult targeting of *Pet-1* might therefore be solely responsible for the abnormal anxiety phenotype. However, it is likely that *Pet-1* is required for maintained expression of other genes in adult 5-HT neurons and therefore a complex combination of mis-expressed genes in adult 5HT neurons may have contributed to defective 5-HT signaling that precipitated altered anxiety behaviors. Although human susceptibility variants impacting expression and function of the *Pet-1* human ortholog, *FEV*, have not been identified it remains an intriguing possibility that variation in *FEV* or its autoregulatory sites could disrupt maintenance of the serotonergic gene expression and consequently contribute risk for mental health disorders.

The cellular and molecular pathology present in midbrain DA neurons following postnatal targeting of *Nurr1* suggests that deficient *NURR1* expression in postmitotic DA neurons might contribute to Parkinson's disease (PD) pathogenesis⁹⁴. Significant locomotor impairment accompanied the progressive cellular and morphological pathology of the DA system after disruption of adult *Nurr1* expression,²⁶. Further, loss of adult *Nurr1* led to dystrophic dopaminergic dendrites, which is a pathological feature reported in early stages of PD. *Nurr1* has also been implicated in regulation of genes involved in neuroprotection against oxidative and excitotoxic stress⁹⁵. *NURR1* expression is decreased in remaining SN DA neurons present in confirmed cases of PD, which parallels the loss of TH in the PD brain⁹⁶. Thus, although human *MURR1* has not been identified as a major susceptibility locus for PD it remains possible that rare genetic mutations⁹⁷ or environmental factors might lead to decreased *NURR1* expression and function in adulthood and thereby accelerate PD disease progression⁹⁴. For example, α -synuclein gene (*SNCA*) missense and gene duplication mutations are known to cause familial cases of PD and the level of α -synuclein overexpression correlates with disease severity⁹⁸. Significantly, overexpression of *SNCA* in the adult rat SN decreased the expression of *Nurr1* and the downstream DA neuron gene battery comprising *Th*, *Aadc*, *Vmat2*, *Dat*, demonstrating that α -synuclein interferes with *Nurr1*-dependent maintenance of DA neuron identity⁹⁹. In addition, *Nurr1* is required for maintenance of the GDNF neuroprotection signaling pathway in DA neurons, which is also disrupted with α -synuclein overexpression. Importantly, viral-mediated delivery of *Nurr1* in α -synuclein-overexpressing rats largely reversed α -synuclein-induced toxic effects on locomotor function, GDNF signaling and DA neuron identity⁹⁹. These findings demonstrate that disruption of transcriptional maintenance programs is likely to be a key element

underlying the progression of PD pathogenesis and that restoration of Nurr1 may offer a therapeutic avenue for treatment of the disorder⁹⁴.

Conclusions and Future Directions

The findings we have reviewed demonstrate that gene regulatory programs launched early in fetal life are required across the life span to maintain many vital features necessary for continued normal neuronal circuit function and behavior (Fig. 1). In addition, examples of TFs that are required exclusively for maintenance of neuron-type identity but do not appear to be involved in the initial specification process have also been described although maintenance of these factors depend on the initiation TFs. These studies constitute an important beginning in understanding the regulatory mechanisms that function to maintain postmitotic neuronal identity. Yet many fundamental questions remained unanswered. First, our knowledge of gene regulatory maintenance programs in specific neuron-types is sketchy. In addition, the breadth of neuronal features that are actively maintained is poorly understood although the findings we have reviewed have so far indicated that maintenance is not restricted to just neurotransmitter-type identity features. Whole genome expression profiling as was performed for Nurr1 in DA neurons will help to reveal the types of gene batteries and range of neuronal functions that are under active maintenance at different stages of life. These approaches may also reveal additional terminal selectors and other dedicated maintenance factors involved in postmitotic neuronal maintenance. Second, the requirements for terminal selector dependent control of individual identity features throughout life are far from clear, particularly in vertebrate neuron-types. Terminal identity genes that become independent of one terminal selector in postnatal life might remain dependent on another untested TF for stable life long expression or perhaps the dependency switches to another TF. Individual terminal selectors may also act cooperatively or compensate for one another in the joint control of identity genes. More complex targeting strategies will need to be implemented to investigate terminal selector compensation, cooperation, and potential switching. Third, sensory-stimuli and electrical activity can influence adult neuronal identity through changes in expression of neurotransmitters. It is presently unclear, however, how common this type of plasticity is in different neuron-types. An intriguing question is whether there are activity-dependent critical periods for transcriptional maintenance of certain features of terminal identity?

Although further investigation of gene regulatory maintenance programs will be more straightforward in *C. elegans* and will likely provide additional key conceptual advances into conserved maintenance mechanisms it will also be essential, depending on availability of suitable genetic tools, to continue the study of these programs in specific neuronal types within the vertebrate nervous system. Finally, an important untested hypothesis for future investigation is whether fetal or postnatal environmental influences (nutritional status, emotional stress, toxins, pathogens etc.) or the acquisition of *de novo* somatic mutations, perhaps in *cis*-regulatory regions of genes encoding neuronal identity features or terminal selector autoregulatory elements result in maintenance defects and consequent susceptibility for nervous system disease.

Acknowledgments

The authors are funded by the NIH (R01NS039996 and R01NS050266–03 to O.H. and R01MH062723 and P50MH096972 to ESD.) and the HHMI (OH). We thank Doug Allan, Paschalis Kratsios, Nuria Flames, Piali Sengupta, Steven Wyler, Clay Spencer for comments prior to submission. We also thank the three referees for their constructive comments. We are indebted to Mark Ptashne for his insightful comments and suggestions throughout the writing of this review.

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Box 1**KEY POINTS**

- Studies in *C. elegans*, *Drosophila*, and mice have demonstrated that neuronal identity must be actively maintained throughout life.
- Invertebrate and vertebrate terminal selector transcription factors that initially control acquisition of neuron–type identity continue to function throughout life to maintain identity.
- Additional transcription factors whose expression begins in postmitotic neurons after acquisition of neuron–type identity have been implicated in maintenance of neuron–type identity.
- Adult transcriptional maintenance programs play key roles in neuronal survival and preservation of neural circuit function.
- Expression of terminal selectors in postmitotic neurons is maintained through direct autoregulation.
- Chromatin modifiers are important for maintenance of differentiated states but depend on continuous recruitment by sequence–specific transcription factors.
- Disruption of terminal selector expression in adult mice leads to human disease–related pathophysiology.

Figure 1. Initiation and maintenance of neuronal identity

Regulatory factors expressed transiently in the lineage that gives rise to a specific neuron type induce the expression of terminal selector-type transcription factors. These factors then initiate terminal differentiation by directly activating the expression of terminal effector genes, such as genes coding for neurotransmitter synthesizing enzymes, transporter and receptors, ion channel, synaptic adhesion molecules and so on. The activated state of these effector genes is maintained via the terminal selectors that initiated their expression. Such maintenance is made possible through the often observed autoregulation of terminal selectors. Other factors that played no role in initiating effector genes may assist terminal selectors in maintaining the differentiated state. In at least one case examined, these maintenance factors are controlled by terminal selectors as well (see text).

Table 1

Transcription factors known to maintain neuronal identity

Species	Transcription factor name (type)	Neuron type that requires sustained TF expression to maintain its identity	Approach	Reference
<i>C. elegans</i>	<i>che-1</i> (Zn finger)	ASEL/R gustatory neuron class	postdevelopmental RNAi	17
	<i>die-1</i> (Zn finger)	ASEL subtype identity	postdevelopmental RNAi	21
	MYST histone acetyltransferase	ASEL subtype identity	temperature-sensitive allele	21
	<i>unc-3</i> (EBF)	Ventral nerve cord motor neurons	pulsing gene activity in a mutant background ¹	12
	<i>ast-1</i> (ETS)	Dopaminergic neurons	pulsing gene activity in a mutant background ¹	19
	<i>ceh-43</i> (Dlx-type homeobox)	Dopaminergic neurons	hypomorphic allele leads to initial generation, but then loss of identity	16
	<i>mec-3</i> (LIM homeobox)	Touch sensory neurons	pulsing gene activity in a mutant background	18
	<i>unc-42</i> (Prd-type homeobox)	ASH nociceptive neuron	postdevelopmental RNAi	18
<i>D. melanogaster</i>	Eya, Ap, Dimm, Dac	Tv1/Tv4 neurons	Temporally controlled-RNAi ²	24
	Acj6, E93, Fer1, Onecut, Aim, Xbp1, Zf30c	Olfactory neurons	Temporally controlled-RNAi ²	22
	<i>ewg</i>	Photoreceptors	Adult ewg rescue	23
<i>S. mediterranea</i>	<i>Pitx</i> (paired-type homeobox)	5HT neurons	RNAi	45
<i>M. musculus</i>	<i>Hand2</i> (bHLH)	Sympathetic neurons	<i>DBH-Cre</i>	28
	<i>Gata-3</i> (Zn-GATA)	Sympathetic neurons 5HT neurons	<i>DBH-CreER</i> <i>ePet-Cre</i>	56 40
	<i>Pet-1/FEV</i> (ETS)	5HT neurons	<i>ePet-Cre</i> <i>ePet-CreER</i>	61
	<i>Lmx1b</i> (LIM homeobox)	5HT neurons	<i>ePet-Cre</i> <i>Pet1-CreER</i>	29, 30
	<i>Gata-3</i> (Zn-GATA)	5HT neurons	<i>ePet-Cre</i>	40
	<i>Engrailed 1/2</i>	5HT neurons Dopaminergic neurons	<i>ePet-Cre</i> <i>null alleles</i>	70 66, 67
	<i>Nurr1</i> (NHR)	Dopaminergic neurons	<i>DAT-Cre</i> <i>AAV-Cre</i> <i>DAT-CreER</i>	26, 27
	<i>Foxa1/foxa2</i> (forkhead/winged helix)	Dopaminergic neurons	<i>DAT-Cre</i>	41
	<i>Pax-6</i> (paired-type homeobox)	Dopaminergic neurons (Olfactory bulb)	<i>DAT-Cre</i>	55
	<i>COUP-TF1</i> (NHR)	Olfactory bulb DA neurons	Lenti-Cre	72
	<i>Islet1</i> (LIM homeobox)	Dorsal root ganglia sensory neurons	<i>Isl1-CreER</i>	43
	<i>Nkx2.1</i> (homeobox)	Forebrain cholinergic neurons	<i>Chat-Cre</i>	44
	<i>Lhx7</i> (LIM homeobox)	Striatal interneurons	<i>Chat-Cre</i>	42
	<i>Phox2</i> (homeobox)	MNs. Sympathetic and Locus coeruleus Norepin. neurons	<i>Isl1-Cre</i> <i>βactinCreER</i>	25

Species	Transcription factor name (type)	Neuron type that requires sustained TF expression to maintain its identity	Approach	Reference
	<i>RORa</i> (NHR)	Purkinje cells	<i>Pcp2-Cre</i> <i>Lenti-Cre</i>	58
	<i>Otx2</i> (homeobox)	Photoreceptors	<i>CreER</i> <i>Lenti-Cre</i>	61, 62
	<i>Nrl</i> (bZIP)	Rod photoreceptors	<i>CAG-CreER</i>	64
	<i>Lhx1</i> (<i>LIM homeobox</i>)	Inferior olivary neuron	<i>Rosa25-CreER</i>	18

¹ initial pulse to successfully rescue mutant phenotype in early stage, then let gene activity peter out, then scored for loss of identity.

²TARGET system.

NHR, nuclear hormone receptor