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Transcriptional regulation of neuronal identity

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Abstract

Neuronal diversity is an intrinsic feature of the nervous system. Transcription factors (TFs) are key regulators in the establishment of different neuronal identities; how are the actions of different TFs coordinated to orchestrate this diversity? Are there common features shared among the different neuron types of an organism or even among different animal groups? In this review, we provide a brief overview on common traits emerging on the transcriptional regulation of neuron type diversification with a special focus on the comparison between mouse and *Caenorhabditis elegans* model systems. In the first part, we describe general concepts on neuronal identity and transcriptional regulation of gene expression. In the second part of the review, TFs are classified in different categories according to their key roles at specific steps along the protracted process of neuronal specification and differentiation. The same TF categories can be identified both in mammals and nematodes. Importantly, TFs are very pleiotropic: Depending on the neuron type or the time in development, the same TF can fulfil functions belonging to different categories. Finally, we describe the key role of transcriptional repression at all steps

We at EJN are pleased to introduce Nuria Flames in our "Trailblazers in Neuroscience" series, see Editorial https://onlinelibrary.wiley.com/doi/10.1111/ejn.15201. Nuria Flames' reflections on undertaking a career as a neuroscientist can be found at the end of the review. You can read other Trailblazers Reviews in the series here.

Abbreviation list: ADAMTS-like, A Disintegrin And Metalloproteinase with Thrombospondin motifS like; ASE, *C. elegans* Amphid neurons, single ciliated endings; *ast-1*, *C. elegans* Axon STeering defect gene; bHLH, basic Helix loop Helix; BMP, bone morphogenetic protein; Brn3a, POU domain, class 4, transcription factor 1 (Pou4f1); *che-1*, *C. elegans* abnormal CHEmotaxis gene 1; COE, Collier/Olf1/EBF transcription factor family; DNA, deoxyribonucleic acid; EBF, Early B-Cell Factor; *egl-18*, *C. elegans* EGg Laying defective gene 18; *egl-43*, *C. elegans* EGg Laying defective gene 43; *egl-46*, *C. elegans* EGg Laying defective gene 46; ETS, Erythroblast Transformation Specific transcription factor family; GATA, GATA transcription factor family (bind GATA sequence); Gli, glioma-associated oncogene 1; HD, homeodomain; *hlh-3*, *C. elegans* Helix Loop Helix gene 3; HSN, *C. elegans* Hermaphrodite Specific Neuron; Lhx2, LIM/homeobox protein 2; *lsy-6*, *C. elegans* Laterally SYmmetric (defective in lateral asymmetry) gene 6; *madd-4*, *C. elegans* Muscle Arm Development Defective gene 4; Ngn1, neurogenin 1; Nrl, neural retina-specific leucine zipper protein; Pbx1a, pre-B-cell leukaemia transcription factor 1 isoform a; Pbx1b, pre-B-cell leukaemia transcription factor 1 isoform b; Pet1, FEV transcription factor, ETS family member; POU, Pit-1, Oct, Unc-86; Rbpj, recombination signal binding protein for immunoglobulin kappa J region; SAB, *C. elegans* Interneuron, (sublateral) motor neuron; scRNAseq, single cell RNA sequencing; *sem-4*, *C. elegans* SEx Muscle abnormal gene 4; Shh, sonic hedgehog; SMAD, *C. elegans* SMA ('small' worm phenotype) and MAD family ('Mothers Against Decapentaplegic') in Drosophila; *tbx-37/38*, *C. elegans* T Box family transcription factor gene 37 and 38; TCF/LEF, T cell factor/lymphoid enhancer factor family; TF, transcription factor; *unc-3*, *C. elegans* uncoordinated gene 3; unc-86, *C. elegans* uncoordinated gene 86; VIP, vasoactive intestinal peptide; wnt, wingless and Int-1; ZF, zinc finger; ZIC2, zinc finger protein 2.

controlling neuronal diversity and propose that acquisition of neuronal identities could be considered a metastable process.

KEYWORDS
enhancer, neuronal differentiation, neuronal identity, regulation of gene expression, regulatory genome, repression, transcription factor

1 | INTRODUCTION

Nervous systems, even in more rudimentary organisms, are characterized by an enormous cellular diversity. The nematode *Caenorhabditis elegans* contains only 302 neurons in the adult hermaphrodite (White et al., 1986), a minuscule number compared to the millions of neurons present in the mouse brain. Astonishingly, this small number of neurons can be classified already into 118 different neuron types (White et al., 1986). Transcription factors (TFs) are the main players in the establishment of neuronal diversity. Many fascinating questions regarding their mechanisms of action are still largely unsolved: How are the actions of different TFs integrated to drive neuronal diversity during development? Are there common features in the transcriptional regulation of neuronal identity among the different neuron types of an organism? Are any of these features shared between different animal groups?

In this review, we will provide a brief overview of recurring themes emerging on the transcriptional regulation of neuronal identity, mainly focusing in recent findings and a comparison between mouse and *C. elegans* nervous systems. Due to the broad scope of the review and to space constrains, we often refer the reader to recent reviews on specific subjects; we deeply apologize to all authors whose important contributions are not directly cited in the text. Additionally, we provide a list of 471 mouse TFs and 93 *C. elegans* TFs, whose mutations are associated to neuronal phenotypes (Table S1).

2 | NEURON IDENTITIES OR NEURONAL TYPES

Most neurons have complex morphologies with dendrites and axonal projections that are employed to target different regions of the organism; they establish specific synaptic contacts, display particular electrophysiological properties, signal through and receive signals from specific neurotransmitters and neuropeptides and respond to particular external stimuli. Genes encoding the components required for these functions, such as ion channels, neurotransmitter biosynthesis enzymes, neuropeptides, neurotransmitter receptors and adhesion molecules, are known as neuronal effector genes. Many of these genes and their corresponding functions are highly conserved in different animal groups. The expression of a particular subset of the whole catalogue of neuronal effector genes provides the neuron with specific functionalities and determines its neuronal identity (or neuron type). Genes exclusively expressed in a unique neuron type are very exceptional; the transcriptome as a whole is a better reflection of specific neuronal identities.

3 | NEURON IDENTITIES CORRESPOND TO DISCRETE TRANSCRIPTIONAL STATES

The advent of next-generation and single-cell RNA sequencing (scRNA-seq) technologies has transformed the study of neuronal diversity, enabling for the first time the description of transcriptional profiles for many cell types in many different species. In general, there is a good correlation between classical classifications of neuron types based on morphology or electrophysiological properties and their transcriptional profiles, both in mice and in *C. elegans* (Gouwens et al., 2020; Hobert et al., 2016; Scala et al., 2021).

One important conclusion arising from single cell transcriptional studies, both in rodents and in *C. elegans*, is that in most cases well-defined transcriptomes correlate with specific neuronal identities. In other words, there is not a continuum of smoothly varying gene expression profiles among neuron types but rather discrete transcriptional states (Cao et al., 2017; Taylor et al., 2021; Zeisel et al., 2018). As will be explained in the last section of the review, this might be a consequence of the transcriptional regulatory networks implemented to establish specific neuronal identities that combine gene activation and repression to exacerbate transcriptional differences among cell types.

Nevertheless, in some cases, discrete transcriptional states (presence or absence of specific gene sets) in two closely related neuron types are not observed (at least with current technologies). For example, continuous variations in gene expression and gradual transitions have been recently described for subtype classification of

intratelencephalic neurons of mouse primary motor throughout different cortical layers (Scala et al., 2021; Yao et al., 2021) or transcriptionally different Vasoactive intestinal peptide (Vip) subclasses of cortical interneurons display continuous variation in electrophysiological properties (Scala et al., 2021).

These findings suggest that establishing discrete and/or continuous variation in gene expression might constitute two different strategies implemented to increase the diversity of neuronal cell types. Further investigation is needed to better understand the mechanisms and evolutionary origins of these two strategies.

Finally, it is important to bear in mind that neuronal identities are not only distinguished by transcriptional differences. Two transcriptionally equivalent neuron types can still correspond to two different neuronal identities whose differences are determined by additional mechanisms such as differential splicing, translational or posttranslational modifications or transient transcriptional differences during development.

4 | REGULATION OF GENE EXPRESSION IS MODULAR

An important, yet unresolved question in neurobiology, is how these terminal transcriptomes and cellular properties are acquired during development. How is the expression of a single gene regulated in different neuron types? This question can be answered in light of general concepts of transcriptional regulation of gene expression that apply to all animal groups and to different cell types and tissues (Nord & West, 2020).

TFs act in combinations binding to non-coding regions in the genome known as enhancers that integrate TF inputs to eventually promote or inhibit the expression of the target gene (Reiter et al., 2017). Expression of a single effector gene in different neuronal types is achieved through enhancer modularity: Several enhancers control the expression of a single gene (Figure 1a). Thus, each enhancer is active in specific neuron types, and the complete expression pattern of the gene is the result of all parallel activities in the different enhancers (Figure 1a). For example, in *C. elegans*, three different serotonergic neuron types (NSM, ADF and HSN neurons) use cell type specific enhancers to regulate the expression of tryptophan hydroxylase gene, the rate limiting enzyme for serotonin synthesis (Lloret-Fernández et al., 2018). Similar cell-type modularity is observed comparing four different neuron types of the mouse nervous system for broadly expressed genes (Closser et al., 2021).

Enhancer modularity is also important when considering the temporal dimension. In a given cell, transcriptional activation of a gene can be regulated by different enhancers at different developmental times (Figure 1b) (Rhee et al., 2016; Velasco et al., 2017).

Therefore, the number of enhancers broadly outcompetes the number of genes in the genome. Consequently, the collection of enhancers that are active in a cell type at a specific moment represents a fingerprint more specific than the transcriptome itself as it reflects not only gene expression but also the precise transcriptional regulatory state of the cell. Moreover, this modularity increases the genome potential to generate different neuronal transcriptomes and hence to generate cell type diversity. It has been recently described that the non-coding regulatory genome of vertebrate neuronal genes has greatly expanded compared to non-neuronal genes to accommodate this complex regulatory logic and cell type diversity (Closser et al., 2021).

Finally, enhancer modularity also provides a mean of phenotypic robustness of cell identities (Figure 1a). The expression of a gene in a cell type, at a particular developmental time, is often controlled by more than one enhancer module simultaneously: These redundant enhancers have been termed shadow enhancers (Hong et al., 2008). Shadow enhancers have been described in several species including, flies, mice and *C. elegans* (Kvon et al., 2021). For example, in mice, expression of Neurogenin 1 (Ngn1), a bHLH proneuronal transcription factor or Sonic hedgehog (Shh) morphogen in the hindbrain and other regions of the nervous system are redundantly regulated by shadow enhancers (Jeong et al., 2006; Nakada et al., 2004). In *C. elegans*, enhancer redundancy characterizes the expression of panneuronally expressed genes (Stefanakis et al., 2015). Shadow enhancers are not mere duplications of ancestral enhancers as in some cases they are known to be regulated by the input of different combinations of TFs, increasing not only robustness to regulatory mutations but also to TF expression noise (Waymack et al., 2020). Indeed, enhancer redundancy might provide an important mechanism for buffering gene expression against mutations in non-coding regulatory regions. Such mechanisms are particulary important in the regulatory regions of genes implicated in human disease (Kvon et al., 2021).

5 | COMBINATORIAL, PLEIOTROPIC AND REDUNDANT ACTIONS OF TRANSCRIPTION FACTORS

Combinations of different TFs bind to and regulate individual enhancers (Zeitlinger, 2020) (Figure 1b). The combinatorial activity of TFs confers them with very pleiotropic actions: TFs are usually expressed in different

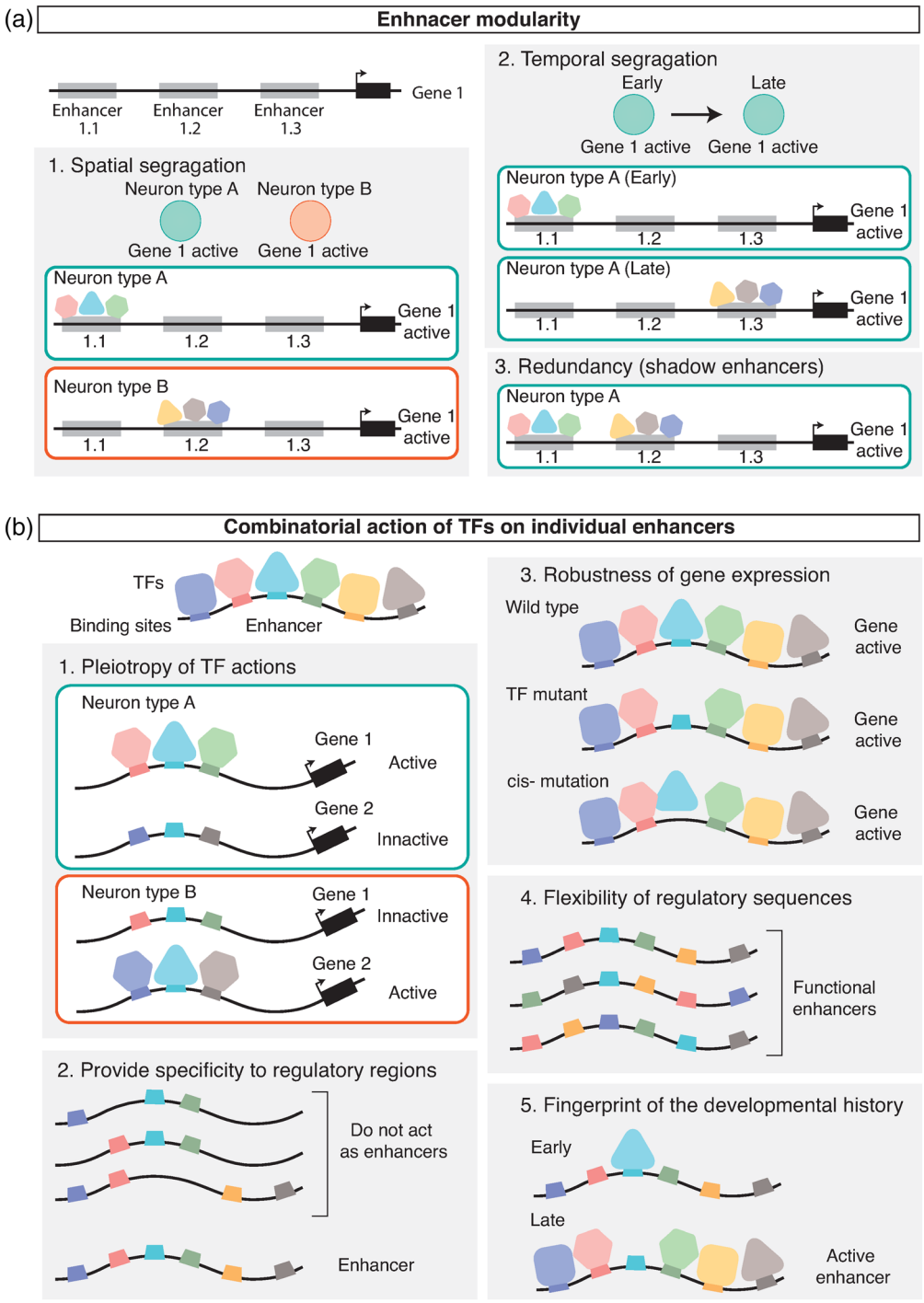

FIGURE 1 Basic concepts on transcriptional regulation of gene expression. (a) The complete expression pattern of each gene is the result of the combined action of several enhancer modules. This modularity allows for spatial and temporal segregation of enhancer function, as well as redundancy (shadow enhancers) and robustness of gene expression. (b) Transcription factors act in a combinatorial fashion on enhancers. Combinatorial actions of TFs allow for (1) pleiotropic functions. In the example, the TF represented as a blue triangle is expressed both in Neuron types A and B but activates different target genes because it works with cell type specific combination of TFs. (2) Increase enhancer specificity, as only regions with the complete collection of TF binding motifs will act as enhancers. (3) Robustness of gene expression, as the lack of a TF or a TF binding motif can be sometimes buffered by the rest of TFs. (4) Flexibility, because an enhancer can usually accommodate very flexible dispositions of TF binding motifs without losing activity. (5) Developmental fingerprint: An enhancer can be bound by different collections of TFs at different times in development; TF binding motifs present in the enhancer are a fingerprint of TF activities on enhancers at different developmental times

neuron types, or even in diverse tissues and at different developmental stages and their specific actions are highly context dependent. Generally, TFs are incapable of binding DNA wrapped into nucleosomes (also known as chromatinized DNA), with the exception of the so called pioneer TFs, which can displace nucleosomes to expose DNA (Zaret, 2020). Thus, in each cell type, TFs bind only a fraction of all their potential TF binding motifs present in the genome. Accordingly, TF binding and actions are modulated by the genomic architecture (chromatin accessibility) present in the cell at any given moment. Moreover, as TFs act in combinations, even if a TF is bound to an enhancer, it does not necessarily mean transcriptional activation of the target gene: The output will depend on the whole set of additional TFs bound at the enhancer (Figure 1b). In addition, splicing isoforms or posttranslational modifications can modify TF activity in a given enhancer. For example, in mouse olfactory bulb dopaminergic progenitors, Pbx1b isoform binds to regulatory regions in the Tyrosine hydroxylase gene but does not activate its expression (Grebbin et al., 2016), while Pbx1a isoform is expressed in postmitotic cells and activates tyrosine hydroxylase trasncription (Remesal et al., 2020).

Combinatorial binding of different TFs is known to increase enhancer activation compared to homotypic collections of TF binding motifs (Grossman et al., 2017; Smith, Taher, et al., 2013) and also helps provide specificity for enhancer selection in a neuron-type specific manner (Figure 1b) (Lloret-Fernández et al., 2018). Finally, if TF combinations are complex enough, this complexity can also accommodate redundancy (and thus robustness of gene expression) (Figure 1b). For example, terminal differentiation of *C. elegans* HSN serotonergic neuron type is regulated by a combination of at least six different TFs. Predicted TF binding site clusters for all six TFs can be used to identify HSN active enhancers, yet TF single mutants often show weak gene expression defects while double mutants show strong synergistic effects (Lloret-Fernández et al., 2018).

Importantly, combinatorial actions of two different TFs in a given enhancer can be segregated in time. For example, in *C. elegans*, TBX-37/38 transiently binds an enhancer of *lsy-6* microRNA gene in the early progenitors of the ASE neuron. This transient binding is necessary to prime the enhancer and allow four cell divisions later, in the postmitotic ASE, the binding of an additional TF, the Zing Finger *che-1* (Charest et al., 2020). Remarkably, the two TFs are not co-expressed at any time in the cell (Figure 1b). Thus, each enhancer contains a collection of functional TF binding motifs, which might or might not be bound by TFs at each given moment, and that constitutes the fingerprint for the whole developmental history of the enhancer (Figure 1b).

To date, the mechanistic details of the combinatorial actions of TFs at enhancers have been poorly characterized (Zeitlinger, 2020). The lack of understanding of the rules underlying the non-coding regulatory genome constitutes a challenge for the study of the transcriptional regulation of neuronal identity as well as for the understanding of genetic variants associated to neurodevelopmental or neuropsychiatric disorders that most often lie in the non-coding genome.

6 | NEURONAL TERMINAL DIFFERENTIATION: SPECIFIC COMBINATIONS OF TERMINAL SELECTORS CO-REGULATE CELL-TYPE-SPECIFIC EFFECTOR GENES

In the following sections, we will provide a brief overview of the process of neuronal specification and differentiation, starting from the last step, terminal differentiation and progressively moving backwards towards earlier developmental time points.

Terminal differentiation refers to the last step of differentiation, when the postmitotic neuron starts the expression of cell-type effector genes required for its functions, such as neurotransmitter biosynthesis enzymes, ion channels or receptors, the expression of these genes is generally sustained throughout the life of the neuron. Transcriptional regulation of neuronal terminal differentiation has been best studied in *C. elegans*. In postmitotic immature neurons, specific TFs, termed terminal selectors, directly bind the cis-regulatory elements and activate the expression of a broad battery of neuron-type specific effector genes. The transcriptional activation of this set of effector genes provides each neuron type with its specific properties and functionalities (Figure 2) (Hobert, 2008). Currently, at least one terminal selector is known for most of the 118 neuron types in *C. elegans* hermaphrodite (Hobert, 2016). Similar to the action of any other TF, terminal selectors work in combinations to provide enhancer activation (Doitsidou et al., 2013; Gendrel et al., 2016; Lloret-Fernández et al., 2018; Zhang et al., 2014). A terminal selector collective of at least six different TFs from different TF families (*unc-86* POU HD, *egl-18* GATA, *sem-4* SPALT, *ast-1* ETS, *egl-46* ZF and *hlh-3* bHLH) control terminal differentiation of the serotonergic HSN neuron (Lloret-Fernández et al., 2018). However, the degree of complexity of other terminal differentiation regulatory networks in *C. elegans* is still largely unknown.

Among different TF families, HD TFs seem to display a prevalent role as neuronal terminal selectors. In *C. elegans*, each neuron type can be distinguished merely by the differential expression of HD TFs (HD code) (Reilly et al., 2020). It has been proposed that ancestral

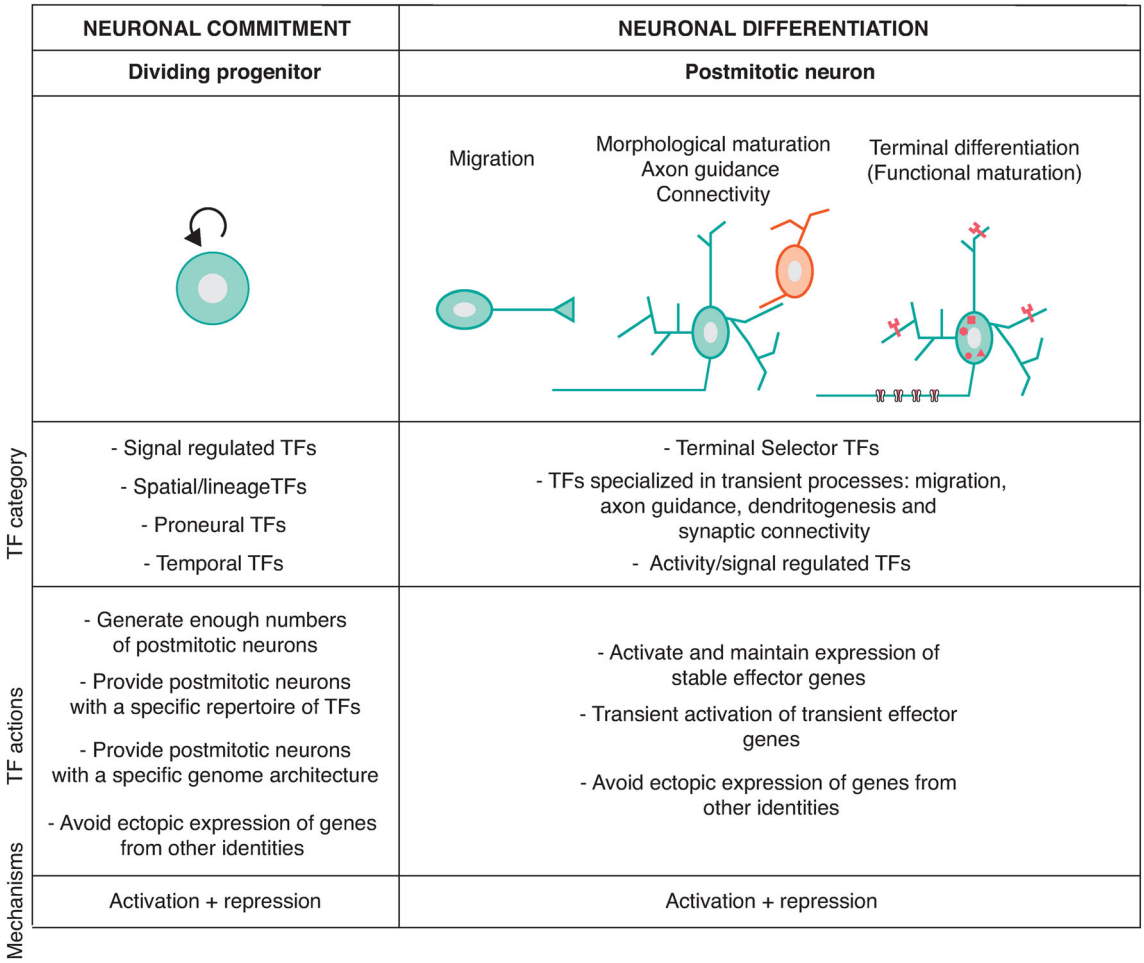

FIGURE 2 Main regulatory steps in the establishment of specific neuronal identities. Different concentrations of morphogens differentially modulate the activity of signal regulated TFs that in turn activate expression of specific spatial/lineage TFs and proneural factors. In *Drosophila*, the temporal progression of progenitors is delineated by the serial expression of temporal TFs that increases diversity of generated neuron types. These TF categories work together to control progenitor proliferation, to induce expression of downstream TFs, to impose specific genomic architectures and to avoid expression of alternative neuronal fates. Both activation and repression are combined to achieve the actions of this set of TFs. Postmitotic differentiating neurons combine transient processes of gene activation that control migration, morphological maturation, axon guidance and synaptic connectivity with the regulation of stable gene expression to induce terminal differentiation and functional maturation. Terminal selectors, transiently expressed TFs and activity/signal regulated TFs work together in these different steps. Again, both repression and activation of gene expression are required for correct neuronal differentiation

homeobox genes could be responsible for the regulation of the ancestral neuron types and that this functional linkage has been maintained and diversified throughout evolution (Hobert, 2021),

Not all genes present in the active transcriptome of a mature neuron type are equally dependent on terminal selectors: Ubiquitous, panneuronal or broadly expressed genes, such as cilia components, seem to be regulated by additional parallel regulatory routines (that is TFs other than terminal selectors) running in the neuron (Stefanakis et al., 2015; Swoboda et al., 2000).

Terminal selectors have also been described in the mouse nervous system (Hobert & Kratsios, 2019), suggesting they could constitute an evolutionary conserved strategy in the transcriptional regulation of neuronal identities. Examples of mouse terminal selectors are, among others, the Pet1 ETS TF that directly regulates serotonergic effector gene expression (Hendricks et al., 2003; Wyler et al., 2016) or the combined action of Lhx2 HD and TFs from the EBF family that directly control terminal differentiation of olfactory receptor neurons (Monahan et al., 2017). Further characterization of mouse TF targets in specific neuron types will help identify additional vertebrate terminal selectors and terminal selector combinations responsible for the activation of specific neuronal terminal transcriptomes.

7 | TRANSCRIPTIONAL REGULATION OF TRANSIENT DIFFERENTIATION PROCESSES: MIGRATION, AXON GUIDANCE, NEURONAL MORPHOLOGY AND SYNAPTOGENESIS

Prior to its complete terminal differentiation, postmitotic immature neurons often need to actively migrate to reach their final destinations. The capability to follow specific migratory routes is genetically encoded, and so different immature neuron types express specific sets of receptors, cytoskeleton components, adhesion and signalling proteins during this process. These sets of genes and their functions are often phylogenetically conserved among animal groups. Once in their final location, neurons go through a process of morphological maturation. Each neuron type extends dendrites and axonal projections to specific locations and establishes stereotyped synaptic connections characteristic of its neuron identity. Once more, tight gene expression control for specific sets of genes is required during this process.

In contrast to terminal differentiation that activates gene targets whose expression is sustained throughout the life of the neuron, transient events like migration, morphological maturation and synaptic connectivity often require dynamic and transient activation of effector gene expression. Indeed, scRNA-seq experiments in the fly brain show that highest transcriptional diversity among neuron types is achieved during the synaptogenesis process and declines in mature neurons (Özel et al., 2021).

The regulatory logic controlling the dynamic expression of these sets of effector genes is yet poorly characterized. One main limitation for these studies is that in most cases, for each neuron type, we still do not understand which is the specific set of neuronal effector genes required in these transient processes. Yet there is compelling evidence in both mice and worms that many TFs acting as terminal selectors are also required for correct migration, morphology maturation or synaptogenesis (Berghoff et al., 2021; de la Torre-Ubieta & Bonni, 2011; Desai et al., 1988; Donovan et al., 2019; Howell et al., 2015; Kessaris et al., 2014; Kwan et al., 2012; Lim, Mi, et al., 2018; Paolino et al., 2018; Remesal et al., 2020; Santiago & Bashaw, 2014; Smith, O'Brien, et al., 2013; Sze et al., 2002; Wyler et al., 2016; Zhang et al., 2014). Direct actions for these TFs on neuronal effector genes controlling these transitory events have been determined in some cases. In *C. elegans* SAB cholinergic motorneurons, UNC-3 COE-type TF acts as terminal selector directly activating expression of genes coding for enzymes and transporters that define the cholinergic neurotransmitter identity; in addition, UNC-3 controls synaptogenesis directly activating the transcription of ADAMTS-like gene *madd-4*/Punctin, a presynaptically secreted synapse-organizing molecule that clusters postsynaptic receptors (Kratsios et al., 2015). In mice, the establishment of serotonergic neuron forebrain projections requires the upregulation of protocadherin gene Pcdhac2 at late embryonic stages (E17) (Chen et al., 2017; Katori et al., 2009). Early in development (E12), Pet1 ETS TF acts as serotonergic terminal selector directly activating enzymes and transporters needed for serotonin synthesis; in addition Pet1, also directly activates Pcdhac2 transcription at later stages (Donovan et al., 2019). How Pet1 targets are temporally modulated is an important unresolved question.

As exemplified by Pet1 actions, if terminal selectors are required for the regulation of these sets of transiently expressed genes, the emerging question is how is sustained expression of terminal selectors translated into dynamic regulation of target gene expression? One possibility is that, due to the combinatorial nature of TF actions on enhancers, terminal selectors could act with additional, transiently expressed TFs, in the regulation of these particular sets of genes. This intersectional regulatory logic has been described post-embryonically in *C. elegans* as a mechanism to control developmentally regulated synaptic remodelling and to generate plastic responses to environmental change or sexual maturation (Bhattacharya et al., 2019; Howell et al., 2015; Pereira et al., 2019).

In addition to TFs acting as terminal selector, other TFs have been described to fulfil specific roles on migration, axon guidance or morphological maturation of specific neuron types, without obviously affecting other aspects of neuronal identity, such as neurotransmitter synthesis, electrophysiological properties or other molecular markers (Figure 2). Examples for these TFs are found in *C. elegans* (Clark & Chiu, 2003; Desai et al., 1988; Wacker et al., 2003), *Drosophila* (Butler & Tear, 2007; Enriquez et al., 2015; Hoermann et al., 2020; Kurmangaliyev et al., 2019; Layden et al., 2006) and mouse (Butler & Tear, 2007; Escalante et al., 2013; Herrera et al., 2003; Lim, Pakan, et al., 2018; McKinsey et al., 2013; Morenilla-Palao et al., 2020; Murcia-Belmonte et al., 2019; Nóbrega-Pereira & Marín, 2009; Polleux et al., 2007; Srivatsa et al., 2015; van den Berghe et al., 2013). In *C. elegans*, EGL-43 Zinc Finger (ZF) TF regulates HSN serotonergic neuron migration but does not affect serotonergic identity (Baum et al., 1999; Garriga et al., 1993). In *Drosophila* visual system, T4 and T5 neuron subclasses differ in their axon and dendritic projections. Expression of five TFs in different combinations defines specific axon and dendritic morphologies

without affecting other cell identity features (Kurmangaliyev et al., 2019). In mouse retinal ganglion cells projecting ipsilaterally ZIC2, a ZF TF, activates expression of EphB1 tyrosine kinase receptor. Ephrin B2, the ligand for EphB1, is expressed by glial cells located at the midline inducing repulsion from the optic chiasm (García Frigola & Herrera, 2010). Similarly, in the spinal cord ZIC2 activates expression of EphA4 in ascending dorsospinal tracts to prevent midline crossing (Escalante et al., 2013). In both cases, other identity features seem unaffected by the loss of Zic2.

Finally, intrinsic genetic programs for migration and morphological maturation can be, in some cases, modulated by external signals or neuronal activity. Specific sets of TFs, whose activities are modulated by post-translational modifications, translate these external signals into specific transcriptional responses (Figure 2) (de la Torre-Ubieta & Bonni, 2011; Puram & Bonni, 2013; Simi & Studer, 2018; Wamsley & Fishell, 2017).

Additional studies will be required to better understand how the actions of terminal selectors, transiently active TFs and activity/signal regulated TFs are integrated in the regulation of migration, morphological maturation and synaptogenesis.

8 | NEURONAL PROGENITOR COMMITMENT: ESTABLISHMENT OF SPECIFIC GENOMIC ARCHITECTURES AND TRANSCRIPTION FACTOR EXPRESSION PROFILES

Developmental steps described so far take place in postmitotic neurons and, thus, are considered cellular differentiation steps. However, the establishment of specific neuronal identities starts earlier, already in the progenitor, a process known as neuronal commitment. Each neuron type arises from specific proliferating progenitors, and each committed progenitor has a limited capability to generate unique neuron types. How are progenitors committed to these unique neuron fates? Once more, TFs play a central role in this process.

In the mouse brain, neuronal progenitors are located near the walls of the ventricle. Early during development diffusible morphogens and cell-to-cell contacts (such as Wnts, BMPs, Shh, Notch and retinoic acid) act through specific signal-regulated TFs (TCF, SMADs, Gli and Rbpj) imposing spatial coordinates in the progenitors (Figure 2) (Azzarelli et al., 2015; Lai et al., 2016). Main targets of signal-regulated TFs are additional TFs. Thus, in each progenitor domain along the dorso-ventral and antero-posterior axis, morphogen gradients are eventually translated into the expression of specific combinations of TFs that are known as spatial TFs (Figure 2). This model is beautifully illustrated by the different progenitor pools present in the ventricle wall of the spinal cord (Lai et al., 2016). In *C. elegans* nervous system, most of the 118 neuron types are represented by only a pair of bilateral neurons, each neuron arising from a different progenitor (Sulston et al., 1983). Thus, there is no need for progenitor pool amplification or segregation of progenitors into spatial domains. Instead, different progenitor lineages are characterized by the expression of specific TFs, which could be considered the equivalent to mouse or *Drosophila* spatial TFs, but in *C. elegans* are named lineage TFs (Hobert, 2010; Ma et al., 2021). In summary, the expression of specific sets of spatial/lineage TFs commit progenitors to the generation of unique neuron types.

In the progenitor, spatial/lineage TFs work together with proneural TFs, a specific set of bHLH TFs with conserved functions in mouse, *Drosophila* and *C. elegans*. Proneural TFs are necessary, and in some contexts sufficient, to induce neuronal fates (Figure 2) (Baker & Brown, 2018; Dennis et al., 2019; Guillemot & Hassan, 2017; Hobert, 2010; Huang et al., 2014; Johnson, 2020; Masoudi et al., 2021; Oproescu et al., 2021). Similar to signal-regulated TFs, important targets of proneural and spatial/lineage TFs are additional downstream TFs, including terminal selectors (Christensen et al., 2020; Masoudi et al., 2021). In addition, proneural TFs and some spatial/lineage TFs act as pioneer TFs modifying chromatin accessibility in a cell-type specific manner that will determine the binding profiles of downstream expressed TFs (Aydin et al., 2019; Sen et al., 2019). Thus, combinatorial actions of proneural and spatial/lineage TFs do not only control proliferation of neuronal progenitors but also provide the postmitotic neuron with a specific collection of activated downstream targets (including and most importantly other TFs, but also panneuronal features or other effector genes) and a particular chromatin accessibility landscape. Both features constrain (or commit) progenitor's potential to generate only specific neuronal types.

In mouse or *Drosophila*, progenitors go through several rounds of asymmetric divisions generating different types of postmitotic immature neurons serially along time. Thus, the temporal axis is an additional strategy to increase neuronal diversity from a single progenitor. In flies, neuronal progenitors show a temporal progression in the expression of specific TFs in each division; these TFs are termed temporal TFs (Figure 2). Temporal TFs series are specific for each region of the nervous system and determine the diversity of generated neuron types along development (Holguera & Desplan, 2018; Rossi et al., 2017). In mammals, progenitor outputs are also temporally controlled: In

some cases, the same progenitor generates different neuronal identities early or late in development, such as individual radial glia production of pyramidal neurons in different cortical layers (Gao et al., 2014) or the generation of both somatostatin and parvalbumin cortical interneurons from same progenitors at different developmental stages (Ciceri et al., 2013). However, the molecular mechanisms responsible for mammalian temporal specification remain poorly understood (Hu et al., 2017; Kawaguchi, 2019; Llorca & Marín, 2021), and the existence of temporal TFs is still under intense debate (Di Bella et al., 2021; Telley et al., 2019).

In *C. elegans* nervous system, most progenitors go through a unique terminal division to generate postmitotic neurons, excluding the possibility of diversification by temporal TFs (Sulston et al., 1983). There are however a few exceptions where a single progenitor goes through two rounds of asymmetric divisions that generate two different neuron types at different developmental times. In the V5 lineage, the POU homeodomain TF UNC-86, homologue of mouse BRN3a, is expressed in the progenitor in the second but not in the first asymmetric division. *unc-86* mutants fail to generate the late neuron (PVD neuron) and reiterate the generation of the early neuron (PDE neuron) (Finney & Ruvkun, 1990), a phenotype similar to *Drosophila* temporal TFs mutants. It will be interesting to explore if other TFs work analogously to temporal TFs in additional *C. elegans* lineages that generate several neurons along time.

9 | THE PREVALENCE OF REPRESSION IN THE ESTABLISHMENT OF NEURONAL IDENTITIES

In the study of neuron specification, most attention has been focused on transcriptional activation; however, repression, aimed to exacerbate differences among related neuron types, is an important driver of neuronal diversification and neuron type individualization. We propose that the establishment of neuronal diversity is a metastable process, that is, a process that is stable under normal conditions but upon system's disruption can change to an alternative state. Perturbations such as TF mutations lead not only to loss of the corresponding cell-type-specific effector gene expression but often also to the acquisition of gene expression from alternative fates. Homeotic transformations of neuronal identities (complete transformation of one neuron type into another) represent extreme examples of this metastable states and have been observed in mouse and in *C. elegans* (Arlotta & Hobert, 2015).

Repression is prevalent in vertebrate and invertebrate model systems and at all stages of neuron specification: (1) Signal regulated TFs act basally as repressors and upon signalling they switch to activators (Barolo, 2002); (2) progenitor spatial domains of the mouse ventricle are established by dorso-ventral cross repression of patterning TFs (Briscoe et al., 2000); (3) *Drosophila* temporal TFs transition to the next TF expression by repression decay (Averbukh et al., 2018); (4) there are multiple examples of repression of undesired effector genes coding for proteins involved in migration, axon guidance or morphological maturation (Cobos et al., 2007; Corty et al., 2016; Ding et al., 2012; Huynh et al., 2011; Marín et al., 2001; McKinsey et al., 2013; Pak et al., 2004; Ramos et al., 2009; Srinivasan et al., 2012; van den Berghe et al., 2013); (5) similarly, TF repression of alternative neuron-type effector genes during terminal differentiation has been widely reported (Baum et al., 1999; Borromeo et al., 2014; Gordon & Hobert, 2015; Kerk et al., 2017; Remesal et al., 2020; Smith, O'Brien, et al., 2013; Tsyporin et al., 2021; Zheng et al., 2018) and (6), finally, active repression of alternative fates in mature neurons has also been observed. Adult removal of Nrl bZIP TF, a TF required for rod photoreceptor terminal differentiation, produces loss of rod effector gene expression, concomitant de-repression of cone effector genes, including two cone opsins, and acquisition of some morphological and electrical properties of cone cells (Montana et al., 2013).

Mechanistically, repression can result from TFs controlling neuron identity acting as activators of a specialized TF repressor (Kovach et al., 2013). TFs can also display dual actions directly as activators or repressors depending on the enhancer they bind to, the collection of additional bound TFs and the recruitment of different cofactors (Sandberg et al., 2016; Wyler et al., 2016). Post-translational modifications or splicing isoforms can also modulate activating or repressing activities of TFs (Huynh et al., 2011; Shalizi et al., 2006). Finally, TFs can also exert repressive actions by competitive binding of shared TF partners to block TF function (Gordon & Hobert, 2015; Smith, O'Brien, et al., 2013).

10 | CONCLUSIONS

This review briefly describes the transcriptional mechanisms employed in the establishment of neuronal identities shared by vertebrate and invertebrate nervous systems.

Key features to understand neuronal diversification are enhancer modularity and the combinatorial action of TFs on enhancers. Sometimes, actions of different TFs in an enhancer can be segregated in time. In addition, both activation and repression are combined to induce specific neuronal identities.

Moreover, TF activities can be further diversified through tight regulation of TF concentration, expression of different isoforms, regulation of mRNA translation and mRNA stability or through post-translational modifications, showing the ever-increasing complexity and pleiotropy of TF actions.

Most of our knowledge is still limited to the actions of individual TFs. Major challenges ahead are (1) to integrate these actions into more global gene regulatory networks, also known as Core Regulatory Complexes (CorCs), that act at different stages in development and direct the identity acquisition for each neuron type (Arendt et al., 2016), (2) to characterize the combinatorial activity of TFs on enhancers and how they translate into 'enhancer signatures', (3) to identify targets of transiently expressed TFs and how their actions are combined with stably expressed TFs and (4) to study repressive actions of TFs.

These studies are needed to better understand the generation of neuronal diversity in development and evolution, to improve the strategies employed in the generation of specific neuron types in vitro and to assign biological meaning to hundreds of non-coding variants linked to diseases of the nervous system.

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CONFLICT OF INTEREST

We declare no conflict of interest.

AUTHOR CONTRIBUTIONS

N.F. and E.S. wrote the manuscript. E.S. did the analysis to generate Table S1.

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SUPPORTING INFORMATION

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APPENDIX

NURIA FLAMES' ACHIEVEMENTS AND REFLECTIONS ON UNDERTAKING A CAREER AS A NEUROSCIENTIST

Nuria Flames: I obtained my PhD in Neurosciences at Instituto de Neurociencias de Alicante, under the supervision of Dr. Oscar Marín. During this period, I was trained in mouse cortical development, and I focused on the study of interneuron migration and cell fate specification. For my postdoctoral studies, I joined Dr. Oliver Hobert, at Columbia University/HHMI. There I learnt to use *Caenorhabditis elegans* as a model to study the transcriptional regulation of neuron type diversity. In 2012, I started my own independent group at Instituto de Biomedicina de Valencia. My lab combines the use of mouse and *C. elegans* to study gene regulatory networks that drive specific neuron fates, how these networks act on the regulatory genome and how they evolve to generate novel neuronal types.

What led you to become a neuroscientist?

Perhaps, it was my fascination with the complexity of the human brain. How such an amazing organ can be built from a single cell by decoding the genome in so many different ways? These were the questions that draw my attention to neurosciences.

What did you learn during your PhD/postdoc that helped you launch your independent career?

From both my PhD and my postdoc supervisor, I learned the importance of always giving your best, the importance of trying to build stories that you are proud of and that can provide significant contributions to the field. I also learnt the importance of being enthusiastic about your work and transmitting and sharing that enthusiasm with your group.

What external influences or mentors had a significant impact on your scientific career?

My PhD advisor Oscar Marín has been instrumental throughout all my career, and I am still constantly looking for his advice for most of my important decisions. For me, Oscar is a clear example of how critical and comforting it is to have a mentor you can rely upon.

Apart from that, many scientists are very inspirational for me. Among other aspects that I appreciate in my job, I love the many opportunities we have to interact with so many brilliant, motivated and hardworking people, who are also tremendously self-effacing, generous and willing to help. I get very inspired by their work but most importantly by their attitude.

Finally, as a woman, I am influenced by other woman colleagues. I really feel it is very important for all of us, man and woman, and for Science itself, to fight for gender equality.

How has the development of neuroimaging/optogenetics/chemogenetics and other modern techniques to interrogate brain networks influenced your research?

In our research field, the advent of deep sequencing together with single cell sequencing technologies has opened the door of a new era. We can now have information that was unimaginable 10–15 years ago, such as the whole transcriptomic profile for each of the 118 neuronal types in *C. elegans*. It also opens the possibility of interrogating many non-model species, which is becoming very important for evolutionary studies.

What are the main challenges young neuroscientists face to build an independent and successful career in research? How did you overcome those? Do you have any tips you'd like to share with early-career scientists based on your personal experience?

To me, the main challenge at the beginning was to build a motivated and cohesive group, in which everyone believes in the importance of their own project but also enjoys helping other team members. I was very lucky with the three founding members of my lab, two PhD students Carla Lloret and Laura Remesal and the lab manager Laura Chirivella, because they were able to create just the environment I wanted.

In addition, when you start your own lab, you have the pressure of publishing fast but also of publishing in reputed journals, which I found extremely difficult to achieve. I was fortunate enough to have stable funding for several years that allowed me to pursue my own scientific niche without the need of rushing for publications. It took me six years to publish my first paper as senior author; I know this is a long time, and in many cases, delaying your initial publication ends up causing funding difficulties and can leave you out of the science system, which is very competitive. I am not sure my decision was correct, but the influence of the PhD and postdoctoral training made me wait until I found that the "story" I could tell was worth to be shared and published.

How important do you consider the role of social medias in increasing the impact of your research? What platforms do you prefer to share your scientific work with the community? Why?

I know social media nowadays are very important to spread broadly and instantly new findings, but I am quite "lazy" about using them (no Facebook, no Twitter, no Instagram, barely use WhatsApp). I get lost in the middle of so much information, and I feel it will take me a lot of my time, which I rather spend in a different way. I still use the conventional way of sharing the unpublished work and talking to people in meetings, which hopefully will come back to "life" instead of "virtual" soon. In addition, as getting papers published is often such a long process, I like bioRxiv as a way of making results accessible to everyone as soon as possible.

What is your view of Impact Factor as a metric to choose the Journals to publish your scientific work? How do you personally assess the impact of your own work in your field?

I try to choose Journals that publish papers that I judge of high quality from other groups in my field. I also check the Impact Factor of the journal I pick, but I would say that this does now weight much in my choice.