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REVIEW

Retinoic acid signaling and neuronal differentiation

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Abstract The identification of neurological symptoms caused by vitamin A deficiency pointed to a critical, early developmental role of vitamin A and its metabolite, retinoic acid (RA). The ability of RA to induce post-mitotic, neural phenotypes in various stem cells, in vitro, served as early evidence that RA is involved in the switch between proliferation and differentiation. In vivo studies have expanded this "opposing signal" model, and the number of primary neurons an embryo develops is now known to depend critically on the levels and spatial distribution of RA. The proneural and neurogenic transcription factors that control the exit of neural progenitors from the cell cycle and allow primary neurons to develop are partly elucidated, but the downstream effectors of RA receptor (RAR) signaling (many of which are putative cell cycle regulators) remain largely unidentified. The molecular mechanisms underlying RA-induced primary neurogenesis in anamniote embryos are starting to be revealed; however, these data have been not been extended to amniote embryos. There is growing evidence that bona fide RARs are found in some mollusks and other invertebrates, but little is known about their necessity or functions in neurogenesis. One normal function of RA is to regulate the cell cycle to halt proliferation, and loss of RA signaling is associated with dedifferentiation and the development of cancer. Identifying the genes and pathways that mediate cell cycle exit downstream of RA will be critical for our understanding of how to target tumor differentiation. Overall, elucidating the molecular details of RAR-regulated neurogenesis will be decisive for developing and understanding neural proliferation–differentiation switches throughout development.

Keywords Neurogenesis · Retinoic acid receptor · Proliferation-differentiation switch

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Introduction

The role of retinoic acid (RA) in neurogenesis has been known indirectly for as long as haliver (halibut) and cod liver oils were used to remedy neurological and ophthalmic disorders. Vitamin A, from which RA is derived, was discovered to be fat-soluble in 1891, labeled as vitamin A in 1920, chemically described in 1931, and synthesized in 1946 (reviewed in [1]). Prior to receiving its name, vitamin A had been known for years to be essential to life—the ancient Egyptians used extracts of (vitamin A rich) beef liver to treat night blindness more than 3,500 years ago and the Greeks prescribed the eating of liver to cure night blindness since at least 300 BC [2]. The link between vitamin A and vision was firmly established in the 1930s when George Wald identified vitamin A in the retinas and melanin-containing choroid layers of eye [3]. "Pigs born without eyeballs" was the title of one researcher's report about a pregnant gilt (young female pig) that received a vitamin A-deficient (VAD) diet and gave birth to 11 piglets without eyes [4]. The etymology of retin-ol, -al, -oic, -oid was derived from the retina, where these molecules were first discovered [5].

The first VAD animals possessed many other neuropathies in addition to blindness. Early studies found spinal

cord abnormalities in swine fed a diet consisting nearly entirely of wheat [6]. Although the authors noted that the abnormalities disappeared when the diet was supplemented with "Fat soluble A" (later identified as vitamin A), they attributed neural degeneration to wheat toxicity rather than a dietary deficiency [6]. A more definitive study was later conducted in pigs fed an otherwise nutritious diet that was solely deficient in vitamin A [7]. Nerve degeneration was found in the spinal cord, optic, femoral, and sciatic nerves, as well as the lateral geniculate body [7]. A severe neuromuscular phenotype, complete with hind limb paralysis, was observed in rats deprived of vitamin A prenatally, whereas only partial paralysis was seen in rats deprived of vitamin A postnatally [8]. Indeed, replicating paralysis in other animal systems proved difficult since vitamin A is readily stored in the fetus [8]. Therefore, historical examples of neurological symptoms from VAD pointed to a critical, early developmental role of vitamin A, and by association, RA.

In the early 1980s, it was discovered that RA induced multipotent P19 embryonal carcinoma cells to differentiate into neuronal and glial tissue, in vitro [9]. F9 embryonal carcinoma cells differentiated into neurons with the addition of RA and dibutyryl cAMP [10]. The ability of RA to induce neural phenotypes on various stem cells in vitro is summarized in [11]. Shortly after the cloning of the first retinoic acid receptors (RARs) [12, 13], it was observed that the P19-derived cell line, RAC65, was incapable of neuronal differentiation due to a 70-amino acid truncation in the RARα ligand-binding domain [14, 15]. Since DNA-binding was still intact, but activation by ligand was inhibited, the receptor acted as a dominant transcriptional repressor, aka, a dominant negative (DN) receptor. Further investigation showed that the RAC65 line was also unable to up-regulate $p27^{Kip1}$, a negative cell cycle regulator, and key effector of RA-mediated inhibition of cell cancer growth [16, 17]. These studies demonstrate that RARα is an essential factor in neuronal differentiation, in vitro, and links RA signaling to the cell cycle and a proliferation–differentiation switch.

Studies from several groups showed that both the number of primary neurons an embryo develops and the time that those neurons appear depends critically on the level of RA signaling [18–21]. Whole mount in situ hybridization studies revealed that both *RARα* and *RARγ* are localized in the neural plate and neural tube in neurula-stage embryos; hence, the receptors are expressed at the correct time and place to regulate primary neurogenesis [22]. DN-RARα injected embryos lacked primary neurons and were paralyzed and unresponsive to touch; microinjection of the constitutively active VP16-RARα, or xRXRβ together with xRARα2 created ectopic neurons [19, 21]. Reduced RA signaling in VAD quail led to a paucity of neurons in the spinal cord, concurrent with loss of proneural genes, such as *Neurogenin1/2* [23, 24]. Increasing RA levels in chick spinal cord explants resulted in increased expression of *NeuroD*, a basic helix–loop–helix protein that promotes neural differentiation [23]. Treatment of *Xenopus* embryos with RA or the RAR-selective agonist TTNPB led to ectopic primary neuron formation in the neural plate; antagonist treatment, or loss of either RARα or RARγ led to the loss of primary neurons and subsequent paralysis of embryos [22].

In this review, we discuss neurogenesis primarily in terms of how RA facilitates differentiation of neurons at the expense of proliferation. We explore the evolution and mechanism of RARs in neurogenesis and identify key molecular cell cycle regulators of neuronal development as well as potential downstream effectors of RAR signaling. Finally, we propose how cancer differentiation therapy can benefit from knowledge of RA and proliferation–differentiation switches.

Neurogenesis as a model to study proliferation–differentiation switches

Developing systems exhibit a dynamic balance between cell proliferation and differentiation. The molecular mechanisms regulating this equilibrium remain an important, yet poorly understood question in development. The opposing signal model is a significant conceptual advance in developmental patterning [23]. In its most general terms, the model holds that mutually inhibitory interactions between factors promoting proliferation, and those promoting differentiation, regulate developmental patterning processes. For example, fibroblast growth factors (FGFs) promote proliferation while largely inhibiting differentiation; RA is a differentiation-inducing molecule that inhibits cell proliferation. Examples of RA mediating proliferation–differentiation switches occur frequently in developmental biology. RA-regulated processes include somitogenesis and axial elongation, cardiogenesis, neurogenesis (e.g., primary neurogenesis, hindbrain patterning, eye morphogenesis), limb development, and visceral organ formation [23, 25–28]. Regulating the switch between proliferation and differentiation is fundamental to vertebrate neurogenesis.

Vertebrate neural induction requires inhibition of bone morphogenetic protein (BMP) signaling [29]. FGFs and other growth factors play important, but incompletely understood roles in facilitating neural induction or A-P patterning (reviewed in [30]). Neural induction is associated with the expression of a suite of pro-proliferation transcription factors, downstream of FGF signaling (*Foxd4l1* and *Zic3*) or BMP inhibition (*Zic1*) [29, 31–36] (Fig. 1). *Zic1* and *Zic3* stabilize the neural fate immediately after neural induction, promoting proliferation of neural progenitors, up-regulating Notch signaling, and inhibiting

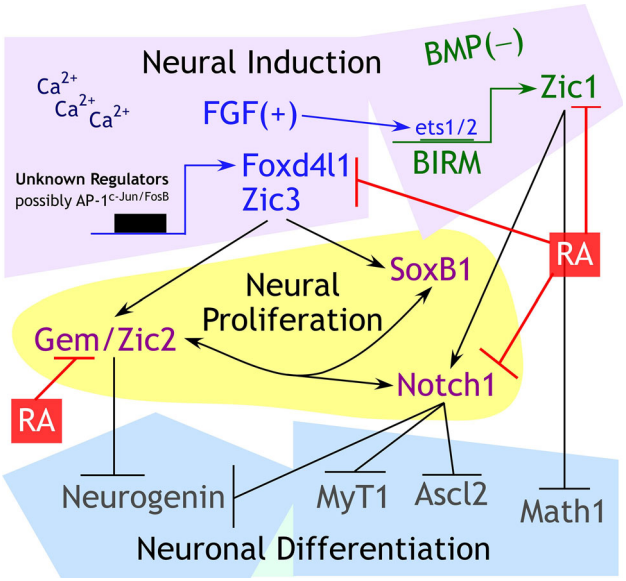

Fig. 1 Important proliferation factors that mediate the early transcriptional response of BMP inhibition and FGF signaling downstream of neural induction. After neural tissue is induced through active FGF and Ca^{2+} signaling and BMP inhibition, *Zic1*, *Zic3* and *Foxd4l1* are up-regulated [29, 34–36, 48, 49]. *Foxd4l1* and *Zic3* are downstream targets of FGF signaling, possibly mediated by AP-1 [32–34, 263, 264], whereas *Zic1* is an immediate early gene of BMP inhibition and is driven by a BMP inhibitor-responsive promoter module (BIRM) [34, 35]. *Geminin* (Gem) and *Zic2* are regulated by *Foxd4l1* and promote *Notch* signaling and inhibition of proneural gene *Neurogenin* [31, 52–55]. *Zic1* also promotes *Notch* signaling and directly represses proneural gene *Math1* [40, 41]. Cross-regulation between *Geminin*, *Zic*, *SoxB1* (*Sox2* and *Sox3*), *Sox11*, and *Notch* maintains proliferation in the neuroectoderm [39, 50, 54–56]. Potential inhibitory interactions between *Sox11* and *Zic* genes are explored in Moody 2013, but not displayed here. Collectively, proneural genes *Neurogenin* and *Math1* are repressed by these proliferative signals, and primary neurogenesis is inhibited as a result. RA inhibits neural proliferation quite early in this process by downregulating the expression of *Geminin*, *Zic1/2/3*, and *Notch* [22]

differentiation [37–41]. *Zic3* is a direct target of the pluripotency factors *Oct4*, *Nanog*, and *Sox2* [42–44], and its expression is diminished after differentiation with RA [43]. Calcium signaling through L-type calcium channels is also a major player in neural induction and is required for the expression of *Zic3* and *Geminin* [45–49]. *Geminin*, a gene that postpones lineage commitment of cells [50], is associated with proliferating neural progenitors [51] and interacts with *Brahma-related gene 1* (*Brg1*) to inhibit neuronal differentiation [52]. *Zic2* is downstream of *Foxd4l1* and is expressed in an alternating pattern with *Neurogenin; Zic2* inhibits *Neurogenin*, and therefore, neurons do not differentiate where *Zic2* is expressed [53]. *FoxD4L1, Geminin*, *Zic1* and *Zic3* promote *Notch*, *Sox2* and *Sox3* expression [39, 50, 54–56]. The concerted action of this group of genes promotes proliferation and maintenance of immature neural precursors (Fig. 1).

Through an as yet unclear mechanism, neural progenitors exit from the cell cycle and differentiate into primary neurons under the control of the proneural and neurogenic transcription factors such as *Neurogenin*, *Math1*, *Ascl2*, *MyT1* (Fig. 1) (reviewed in [57, 55]). Primary neurons are defined as four sets of neurons (sensory, interneurons, motor, and trigeminal) visible in the open neural plate stage. Primary neurons differentiate from the deep neuroectoderm layer of the embryo, whereas the superficial neuroectodermal layer maintains an immature, proliferative state [58]. We and others showed that RA is required for primary neurogenesis [19, 21, 59]. RA inhibits the expression of *Zic*, *Geminin*, *Notch*, and *Foxd4l1* while promoting expression of proneural and neurogenic genes [18, 22] (Fig. 1).

Primary neurogenesis in anamniotes versus amniotes

Primary neurogenesis is an important model for understanding proliferation–differentiation switches in all species throughout development; it also describes how the adult brain can regenerate new neurons, and how cancer cells arise. However, primary neurogenesis, per se, only occurs in anamniote embryos, which develop neurons as early as the neural plate stage, later enabling the larvae to swim and feed precociously [60, 61]. Since amniote (reptilian, avian and mammalian) embryos are protected from the external environment and develop completely before hatching, primary neurons are not required for survival and were probably lost during evolution [62]. The earliest born neurons of the cortex are often referred to as secondary neurons in anamniotes, although this process corresponds to primary neurogenesis in amniotes. Terminology aside, it is quite likely that the first cortical neurons in amniotes will be initiated using a mechanism similar to primary neurogenesis in anamniotes.

Before any developmental mechanism can be elucidated, these incipient neurons for amniotes, secondary neurons for anamniotes, must be defined, yet this has remained elusive. Early-born axon tracts, nerve fibers that establish a scaffold for which other axons can follow, are predicted to serve as “pioneer” neurons in the developing brain (reviewed in [63]). Unlike primary neurons, these pioneer neurons exist not for basic survival, but rather, because the embryo is sufficiently small that guidance cues (e.g., chemoattractants) are close enough for axon trajectories to be established [64]. Recent labeling studies in aborted human embryos identified so-called “predecessor neurons” in the preplate, which are the earliest identified neurons to date [65]. Like primary neurons, these “predecessor neurons” are created prior to neural tube closure and are likely to be transient in nature [65]. Whether RA is involved in the differentiation of predecessor neurons from

proliferating neuroepithelial cells is unknown. However, considering that evolution often conserves developmental mechanisms, the possibility is very likely and remains an interesting open question.

Although there is no clear picture on how RA might foster differentiation of the earliest neurons in amniotes, and secondary neurons in anamniotes, RA does play other important, evolutionarily conserved roles in CNS development across most chordates, including three major patterning processes: (1) posteriorization of neuroectoderm; (2) D-V patterning of the spinal cord; (3) A-P patterning of the hindbrain. Excellent reviews have been written about these well-known patterning events [11, 25–27, 66, 67]. Briefly, RA together with Wnts and FGF signaling posteriorizes neuroectoderm, which would otherwise be anterior in character by default [19, 68]. Similarly, RA signaling is an important component of the neural posteriorizing pathway in the hindbrain—RA determines the identity and delineates borders of posterior rhombomeric segments (reviewed in [69]). During neurulation, RA secreted from paraxial mesoderm functions in the specification of nerve cell types in the spinal cord, in particular, motor neurons ([70], reviewed in [67]).

Most examples in amniotic systems concern the function of RA in patterning neural tissue—there is a paucity of information about how RA promotes neuronal differentiation. We consider three areas where RA plays a role in differentiation: in photoreceptors, hippocampus, and cortical neurons. RA can cause dissociated, neonatal rat retinas in culture to differentiate into photoreceptor cells expressing *rhodopsin* and/or *recoverin* [71]. Human, mouse, and monkey embryonic stem cells can also be differentiated into rod photoreceptors, albeit more laboriously, due to the requirement of an intermediate step of *Notch* inactivation, followed by a cocktail containing RA, Shh, FGFs, and taurine [72]. This suggests that RA is more important in the final steps of rod photoreceptor differentiation than in the early process of differentiating ES cells into retinal progenitors. In contrast, both mature ($NeuN^+$) and immature (dcx^+) neurons of the hippocampal dentate gyrus are reduced in retinoid-deficient mice, indicating that RA affects very early steps of the neuronal differentiation pathway in the hippocampus [73]. Neural stem cells in the proliferative ventricular zone of the cortex also require RA to differentiate into intermediate progenitor cells of the sub-ventricular zone and post-mitotic neurons of the cortical plate [74]. Meninges are thought to be the source of RA due to high levels of RA-synthesis enzymes, ALDH1A2 and RDH10 [74, 75]. *Foxc1* mutant mice that fail to form meninges normally exhibit increased proliferation, and are deficient in mature, $Ctip2^+$ neurons [74]. However, when forebrain explants from *Foxc1* hypomorphs were co-cultured with wild-type meninges (from which RA diffuses), cell cycle exit was restored [74]. How RA regulates differentiation in other aspects of cortical development, and how these processes can be related to the gene networks observed in vertebrate primary neurogenesis remain to be explored.

What was the first RAR-regulated nervous system?

A neural plate or neural tube need not be present for neurogenesis to occur. For example, the primitive acoelomorph flatworm, *Symsagittifera roscoffensis* (aka the mint sauce worm), which is considered to represent the earliest extant bilaterian organism, lacks a nerve cord, but possesses neurite bundles that span the A-P axis of the body [76]. Prior to the emergence of more complex bilaterians, nerve "cords", "nets", and "rings" already existed in cnidarians [77, 78] and ctenophores [79, 80]. Secretory apparatus resembling synaptic vesicles required for neurotransmitter communication can be found in single-celled choanoflagellates [81]. Therefore, it is clear that neural tissue can adopt a variety of forms, yet retain the function of communication between one part of the organism and others. Where then do retinoids fit into this process?

Data from vertebrate embryology support an essential role for retinoid signaling in the development of primary neurons [19, 21, 22, 24]. However, it is equally clear that nervous systems of considerable complexity can be found in organisms for which RARs have not been identified (e.g., *Drosophila melanogaster*). The larvacean urochordate, *Oikopleura dioica* can form a functional nervous system that expresses homologs of marker genes for vertebrate forebrain, hindbrain and spinal cord (but not midbrain) in their CNS [82], yet it lacks important components of RAR signaling (RARs, CYP26), while retaining RXR and Adh3 [83]. Intriguingly, other urochordates, hemichordates, and cephaolochordates express RARs and RXRs, as do echinoderms [84, 85]. It is clear that the RA signaling machinery has been lost in *Oikopleura* [85], although, it is not known to what extent their nervous system function is altered compared with other urochordates.

The recent explosion in genome sequences from taxonomically diverse organisms reveals the presence of RARs and RXRs in a variety of invertebrates beyond the deuterostome superphylum. Components of the RAR signaling machinery have been reported from cnidarians, mollusks and nematodes [86] and 9cRA and other RXR activators perturb development in mollusks [87–90]. Uncovering RAR homologs in lower organisms is relatively straightforward in silico, and numerous examples have been identified [86]. A quick BLAST search for this review using the RARα ligand-binding domain, identified putative

RARs in Pacific oyster, *Crassostrea gigas* and the California sea hare, *Aplysia californica*, among others (Fig. 2). Bioactive retinoids and RARs exist in some Lophotrochozoan species, e.g., the owl limpet, *Lottia gigantean*, the bristleworm, *Capitella telata,* and the giant pond snail, *Lymnaea stagnalis* [86, 91]. *Lymnaea* were shown to contain atRA and 9cRA in the hundreds of nanomolar range, and treatment with either chemical induced neurite outgrowth and growth cone turning in cell culture [92]. Putative RAR [93, 94] and RXR [87, 95] orthologs were recently cloned in the rock shell, *Reishia clavigera* and dog whelk, *Nucella lapillus*. Both species of gastropods are susceptible to imposex induced by RXR activators (rexinoids) [87, 89].

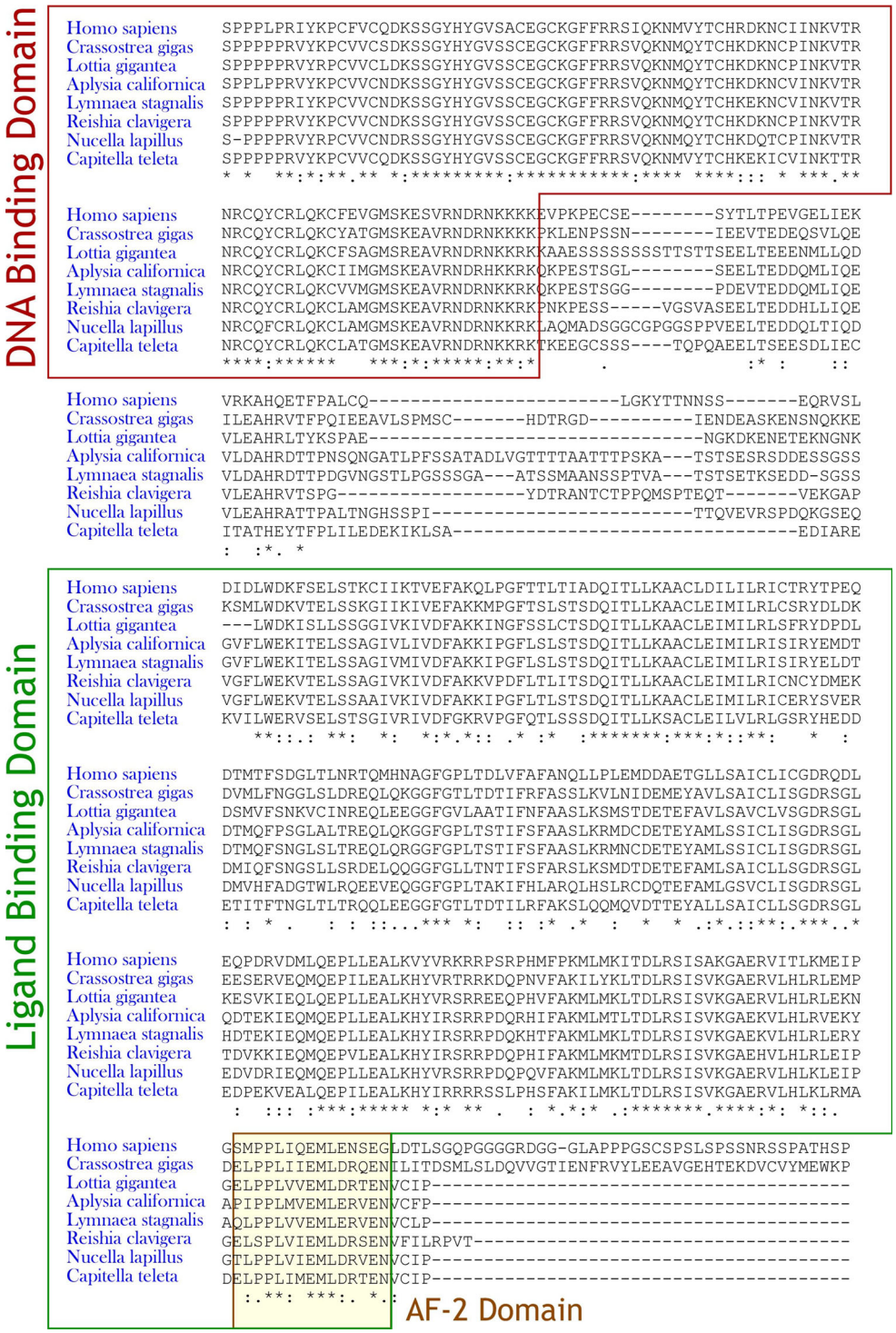

Fig. 2 MAFFT alignment of RARα2 in *Homo sapiens* versus Lophotrochozoan species. Alignment begins with the conserved DNA-binding domain of RARα2 (no conservation is observed in the N-terminal region)

It is important to note that the presence of an apparent RAR or RXR in a particular species does not conclusively show that the receptor will bind to RA and regulate gene expression in an RA-dependent manner. For example, *Reishia clavigera* and *Nucella lapillus* RARs and RXRs heterodimerize as do their vertebrate counterparts, but tcRAR and nlRAR appear not to be responsive to RA [87, 93]. Some investigators have speculated that many invertebrate nuclear receptors could have functions different from than regulating hormonal responses [96], although, others take the opposite position, that hormone-responsiveness was the ancestral state for nuclear receptors [97, 98]. Taken together, the evidence suggests that RA signaling is an ancient process that has been repeatedly lost in a variety of lineages [99]. While important for neural development and differentiation in vertebrates and most chordates, there is a considerable knowledge gap regarding requirements for RA signaling during invertebrate neurogenesis. It is tempting to speculate that advanced cephalopod mollusks with complex nervous systems, such as the octopus, have retained RA signaling in neurogenesis.

Neuronal differentiation and cell cycle genes

Proliferation within neural tissue is a direct consequence of self-renewal via symmetric (giving rise to two daughter stem/progenitor cells) or asymmetric (giving rise to one stem/progenitor cell and one lineage-restricted cell) cell division (reviewed in [100]). There are two schools of thought with respect to how cells make the proliferation–differentiation decision during the cell cycle. In the first, differentiation of neural stem cells is characterized by asymmetrical cell divisions leading initially to fate-restricted progenitors, and finally to terminally differentiated daughter cells that are incapable of dividing (reviewed in [101]). Critically, the proliferation/differentiation decision does not rely on cell cycle arrest, but rather a terminally differentiated cell is simply born in G0 and never makes a decision to proliferate or differentiate (reviewed in [101]). An alternative view is that cell cycle arrest turns a proliferative cell into a post-mitotic cell that differentiates into its final form. A checkpoint towards the end of G1 phase serves as a major restriction point where cells can continue to divide or enter the quiescent G0 phase [102, 103]. Signals favoring neuronal differentiation (e.g., RA) increase the expression of CDK inhibitors which promote cell cycle exit of G1 phase cells [104, 105]. While these two views appear to be mechanistically distinct, they are really quite similar because the genes promoting cell cycle exit and those responsible for stabilizing the G0 phase are identical. Thus, irrespective of whether the terminally differentiated cell was naturally born in G0 or came to G0 from G1, the molecular factors that got them to G0 are the same.

Chromatin remodeling processes control chromosome assembly and segregation, regulating DNA accessibility throughout the cell cycle by condensing or decondensing DNA, thereby manipulating the inactivation or activation of the replication machinery. Chromatin remodelers are therefore, key determinants in proliferation–differentiation decisions. Brahma-related gene 1 (*Brg1*) is the catalytic subunit that provides ATPase activity to the chromatin remodeling complex SWI/SNF for nucleosome disruption [106]. *Brg1* is required for neuronal differentiation as demonstrated by loss-of-function studies facilitated by morpholino (MO) injection in *Xenopus* embryos [107]. Loss of *Brg1* function leads to an expansion of the neural progenitor population and decreased expression of the neurogenic basic helix–loop–helix (bHLH) genes *Neurogenin* and *NeuroD* as well as the neural differentiation marker *N-Tubulin* [107]. *Brg1* activity is antagonized by *Geminin* which suppresses neurogenesis and inhibits *Neurogenin* and *NeuroD* transcriptional activity [52]. Eyes absent homolog 1 (*Eya1*) and Six homeobox1 *(Six1*) also interact with *Brg1*, recruiting SWI/SNF to mediate the transcription of *Neurog1* and *Neurod1* in the otocyst and cochlea [108]. The role of *Brg1* in mammalian systems is more complex. Conditional deletion of murine *Brg1* led to reduced mitotic index in cultured neural progenitors [109]. However, another study found that deletion of *Brg1* did not alter cell cycle length or cause an increase in proliferation [110]. Rather, *Brg1* controls neuronal fate decisions: neural stem cells derived from *Brg1*-knockout mice differentiate towards the ependymal (glial) lineage, at the expense of the neuronal lineage [110].

Cyclin-dependent kinase inhibitors (CIKs), such as proteins belonging to the Ink4 and Cip/Kip families, inhibit different cyclin-CDK complexes at different time points, and govern cell cycle progression/withdrawal [111–115]. Cip/Kip proteins mediate the assembly of CyclinD-CDK4/6 in early G1 phase [111]. However, in the presence of Ink4, the CyclinD-CDK4/6 complex is disassembled and Cyclin D is targeted for degradation, thus freeing Cip/Kip proteins to bind and inhibit CyclinE-CDK2 [111]. The inhibition of CDK2 by Cip/Kip and CDK4/6 by Ink4 prevents the cell from progressing through the G1-S transition, causing G1 arrest [116, 117]. CKIs play important roles in neurogenesis, influencing cell fate decisions by controlling the timing of onset of neural determination gene expression [118]. In the developing mouse neocortex, ectopic expression of $p27^{Kip1}$ was shown to prolong G1 phase [119], a hallmark of cells proceeding to neuronal differentiation [101]. Loss of $p27^{Kip1}$ causes increased proliferation in the adult dentate gyrus due to a delay in cell cycle exit of immature neurons. $p27^{xic1}$, a *Xenopus* Cip/Kip

protein, is required for primary neurogenesis [120–122] and functions by CDK inhibition which prevents phosphorylation and promotes stabilization of the Neurogenin protein [120, 123]. Recently, *p27*^{xic1}, was shown to be phosphorylated by atypical protein kinase C (aPKC), which prevents CDK inhibition, shortens the G1 phase of neural progenitors, and encourages their proliferation [124]. The p21-activated serine/threonine protein kinase Pak3 also promotes cell cycle withdrawal and causes premature primary neurogenesis in *Xenopus* [125].

Another aspect of cell cycle control in neurogenesis concerns calcium (Ca^{2+}) influx. Barth and colleagues first identified a role for Ca^{2+} in neural induction by demonstrating that dorsal ectodermal explants could be differentiated into neural cells in the presence of Ca^{2+} and LiCl [126]. Later, it was demonstrated in *Xenopus laevis* and *Pleurodeles waltl* that explants exposed to dissociation medium (Mg^{2+} and Ca^{2+}-free) also differentiated into neural cells [127, 128]. The mere act of dissociation was enough to release Ca^{2+} into the cells [129]. Neuralization of explants could also be induced by Concanavalin A [46, 130, 131], which promotes Ca^{2+} influx/uptake [132, 133], and this was inhibited by Ca^{2+} channel antagonists [46]. However, during normal embryonic development cells are neither dissociated into individual cells nor is Concanavalin A (a legume glycoprotein) an endogenous factor. Hemmati-Brivanlou and Melton proposed the default neural model wherein inhibition of BMP signaling, per se, is necessary and sufficient for neural induction [134, 135]. Subsequent experiments showed that neural induction also requires FGF signaling [136] and that FGF4 activates Ca^{2+} channels required for neural gene expression [137]. BMP-antagonists such as Noggin trigger intracellular Ca^{2+} release [46], and high Ca^{2+} levels inhibit BMP signaling and simultaneously activate the FGF/Erk signaling pathway [138]. Critically, the presence of Ca^{2+} signaling is required for cells to become neural tissue; in the absence of Ca^{2+}, cells adopt an ectodermal fate [139].

Although Ca^{2+} plays a role in neuralizing tissue, it is unclear whether Ca^{2+} promotes proliferation of neuroectoderm, fosters differentiation, or both [140]. The role of Ca^{2+} in cell cycle is clear. Ca^{2+} is required in all growth and division stages of the cell cycle, encouraging cyclin D1/CDK4 accumulation in early G1, CDK2 activation in G1/S, and CDC2 activity in G2/M (reviewed in [141]). Proliferating fibroblast cells exposed to low Ca^{2+} media arrest in G1 and do not synthesize DNA [142]. Proliferation can be restored in these cells by the addition of Ca^{2+} [142]. The proliferative ventricular zone of the neocortex is reliant on ATP-dependent intracellular Ca^{2+} waves [143]. When Ca^{2+} waves were inhibited with an ATP receptor antagonist, cells failed to enter S phase, as indicated by reduced BrdU staining ([143], reviewed in [144]). Loss of L-type Ca^{2+} channels, which are differentially expressed in ectodermal tissues during early development [145], causes the down-regulation of *Zic3* and *Geminin* [129], two genes that are associated with the immature, proliferative phase of neurogenesis [57]. Much needs to be learned about other regulators of Ca^{2+} signaling, and which signaling pathway components that control proliferation/differentiation switches are sensitive to Ca^{2+} influx. For example, it has been two decades since Ca^{2+} release during neural induction was shown to increase protein kinase C and cyclic AMP activity, two pathways that are relatively disconnected from BMP and FGF signaling [146–149]. Curiously, this result has not been followed up despite its interest to the field [150].

Members of the Id (Inhibitor of DNA-binding/differentiation) family are helix–loop–helix proteins that inhibit the ability of bHLH proteins to homo- or heterodimerize and bind to DNA [151, 152]; they foster proliferation, in most (but not all) biological systems. Id1$^{-/-}$Id3$^{-/-}$ mouse embryos exhibit premature neurogenesis characterized by ectopic and early onset of expression of neurogenic bHLH proteins, such as MATH1/2/3 and NeuroD1 [153]. Id3 promotes proliferation of neural crest precursors in *Xenopus* [154]. Loss of Id4 compromises the proliferative capacity of ventricular zone stem cells in the mammalian cortex [155]. Id proteins are intrinsically linked to the cell cycle in neurogenesis. Id2 inhibits bHLH factor E47 [156], which prevents heterodimerization of E47 with NeuroD1 [157]. An important downstream target of E47 is the CIK p57^{Kip2}; hence, Id2 effectively down-regulates *p57*^{Kip2} via E47 inhibition, thus promoting proliferation in neuroblastoma cells [156]. Activation of the anaphase promoting complex with *Cdh1* coactivator (*APC*^{Cdh1}) by differentiation signals (such as RA [158]) causes Id2 to be targeted for degradation, thus promoting neuronal differentiation [159]. When *Cdh1* is ablated in mice, increased proliferation in neurospheres and stabilization of substrates such as Geminin is observed [160]. Id1 also antagonizes a different CIK, p16^{Ink4a} [161], which is found specifically in adult nervous tissue [162]. Taken together, these data indicate that neurogenic transcription factors interact specifically with the cell cycle machinery to modulate proliferation during neurogenesis.

ETS proteins are nuclear targets for extracellular signaling pathways [163] and are modified by mitogen-activated protein kinases (MAPK), integrins, or Ca^{+2}/calmodulin-dependent protein kinases, often downstream of growth factor pathways [163, 164]. Members of the Ets family of transcriptional activators, such as Ets1, Ets2, Elk1 and repressors, such as Ets2 Repressor Factor (ERF) and Etv3, are important cell cycle regulators; their function is tightly regulated by MAPK-mediated phosphorylation. The phosphorylation state of ERF varies at different points

in the cell cycle and this affects ERF subcellular localization. ERF shuttles between the nucleus, where it functions as a repressor, and the cytoplasm where it is inactive [165]. Loss of ERF results in the complete depletion of primary neurons in *Xenopus* embryos [22]. Nuclear localization of ERF occurs in cells arrested in G0 or G1 [165]. Growth factor or serum stimulation of proliferation causes ERK to phosphorylate ERF, which leads to its export from the nucleus [165]. Phosphate mutant ERF proteins cannot be exported and cause cell cycle arrest in the G0/G1 phase [165] and increases the number of *Xenopus* primary neurons [22]. Thus, ERF is critical for negatively regulating transcription factors required for cell cycle re-entry from G0 or quite possibly, elongation of G1 (unpublished data from [166]), which is associated with neuronal differentiation [101]. The direct targets of ERF repression in neurogenesis are currently unknown. However, since Myc has been identified as an ERF target in fibroblasts [167], it is likely that ERF would also repress n-Myc, a gene that is functionally interchangeable with c-Myc [168]. n-Myc promotes expansion of neural progenitor populations, and would be a good candidate to be repressed by ERF to foster differentiation [169].

Many more cell cycle control genes that regulate the differentiation of neural progenitors have yet to be identified. Furthermore, which cell cycle genes are downstream mediators of known differentiation cues remain to be deciphered. Regulators of cell cycle progression during G0 and G1 phase and the G0/G1 transition are likely to be key determinants of the proliferation-differentiation decision. However, it is still controversial whether cells make that decision from G1, or if cells are simply born in G0 due to asymmetrical factors they inherited, or the niche in which they are residing. Various events during the cell cycle such as chromatin remodeling, the opposing effect of cyclin-CDK and CIKs, and the phosphorylation and nuclear shuttling of Ets and Ets repressors form a network that controls when and where neural progenitors commit to differentiate.

Downstream effectors of retinoic acid signaling related to neurogenesis

Throughout neurogenesis, RA is readily available due to the presence of retinaldehyde dehydrogenase 2 (ALDH1A2), which synthesizes RA in the paraxial mesoderm of the developing embryo. RA then diffuses to the neural plate and spinal cord to promote the differentiation of neural progenitors [23]. Due to the availability of RA, and known action of the RARs, most of the direct gene targets of RAR during neurogenesis are expected to be transcriptionally up-regulated; although, the possible recruitment of ligand-dependent transcriptional co-repressors cannot be completely excluded [170]. Very few direct targets of RA that are definitively involved in the neural proliferation–differentiation switch have been identified. The known direct RAR target, *HoxA1*, [171, 172], is required for the differentiation of embryonic stem (ES) cells into neurons [173]. HoxA1-null ES cells are refractory to treatment with RA as measured by reduced expression of post-mitotic neuron markers (e.g., *β-tubulin III, Nestin*); RA sensitivity can be restored by rescue with *HoxA1* cDNA [173]. *Btg2* is also a putative direct target of RAR [174] and is induced by RA in neurula-stage embryos [175, 176] and in various cell lines [174, 177]. *Btg2* decreases arginine methylation and lysine acetylation of histone H4 at RAR target genes, thus increasing the transcriptional activity of RAR [177]. *Btg2* is expressed in differentiating neuroblasts [178] and promotes neuronal differentiation in PC12 cells [179]. Loss of *Btg2* increases proliferation in the neural plate of *Xenopus* embryos [180], likely via repression of Cyclin D1 transcription [181].

Evidence from a variety of cell culture systems shows that RA directly and indirectly regulates the expression of many other genes, in addition to *Btg2*, that facilitate cell cycle exit and differentiation [182–185]. Considering that RAR activation promotes differentiation at the expense of proliferation, the most likely downstream effectors of RAR are inhibitors of the cell cycle. We previously showed that RA-induced ETS repressors are key components of the proliferation–differentiation switch during primary neurogenesis, in vivo [22]. ERF and ETV3 inhibit proliferative signals by displacing activating ETS proteins from promoters of cell cycle control genes while recruiting co-repressor complexes to facilitate cell cycle arrest [186, 187]. ETV3 was identified as an anti-proliferative factor induced during macrophage differentiation [186, 188] and neuronal differentiation [22]. ERF mediates the switch between proliferation and differentiation in macrophages, fibroblasts, extraembryonic ectoderm, and neuroectoderm [22, 167, 187, 189]. Importantly, both *Erf* and *Etv3* are upregulated by RAR agonists, down-regulated by RAR antagonists, and knockdown of ERF or ETV3 results in paralysis, loss of primary neurons and increased proliferation of undifferentiated neural progenitors. Thus, these Ets-repressors are key effectors that inhibit neural progenitor identity and promote differentiation.

Multiple genomics-based studies have elucidated some genes that respond to RA under a variety of differentiation conditions [175, 176, 190–196]. A subset of the genes identified in these analyses will be candidate neurogenic genes regulated by RAR. The pro-neural gene *Ascl1* (*Mash1*), a bHLH transcription factor and activator of Notch signaling, is an interesting downstream effector because *Ascl1* regulates both proliferation and differentiation in a temporally and spatially restricted manner [197].

Ascl1 promotes positive cell cycle regulators in the proliferating ventricular zone of the cortex, but later fosters differentiation in the post-mitotic cortical plate [197]. It is unknown whether there are inherent temporal and spatial differences in RAR signaling in the developing cortex, but RAR is known to create graded, spatially restricted expression of *Ascl1* in other systems. Low *Ascl1* expression is observed in the presence of RA in ventral spinal cord progenitors; high *Ascl1* is observed in the absence of RA in hindbrain serotonergic progenitors [198]. *Numb*, another gene in the Notch pathway, is also a potentially intriguing downstream effector of RA. *Numb* homologs were recently characterized in *Xenopus*—knockdown of *Numb-like* causes the complete loss of primary neurons, expansion of neural progenitor markers, and increased proliferation [199]. *Numb* can promote proliferation or differentiation, depending on which isoform is expressed [200]. A sharp change in *Numb* isoform expression was observed in P19 embryonal carcinoma cells when neuronal differentiation was stimulated by RA [201]. The molecular mechanism of *Numb* alternative splicing remains an open question, although RA-induced differentiation alters splicing machinery in P19 cells [202] and in SH-SY5Y neuroblastoma cells [203].

Other potential downstream effectors of RA include *ATP7A, Elongin A, Reelin*, and *Prdm12*. RARβ2 induces the expression of *ATP7A*, a Golgi-associated protein that removes copper from cells [204]. Knock down of RARβ2 inhibits expression of *ATP7A*, reducing copper efflux. Copper levels are apparently critical for the response of neuroblastoma cells to RA because copper supplementation induced proliferation, and copper chelation promoted differentiation [204]. *Elongin A* is an elongation factor that is essential for neuronal differentiation, in vivo [205]. *Elongin* $A^{-/-}$ embryos have widespread CNS defects, and ES cells derived from these embryos fail to differentiate into neurons in response to RA treatment [205]. The hypothesis is that *Elongin A* improves the processivity of RNA Polymerase II on genes that are upregulated by RA [205]. Increased RNAPII occupancy was observed on *Neurogenin1/2* and *HoxA7* genes in response to RA in *Elongin* $A^{+/+}$ embryonic stem cells; however, this was not observed in *Elongin* $A^{-/-}$ cells [205]. Reelin is an extracellular matrix glycoprotein that regulates the number of newborn neurons during development [206]. De novo neurogenesis in regions of the adult brain was decreased in *Reelin*-mutant mice [206]. RA increases occupancy of *Sp1* and *Pax3* promoters, and concomitant demethylation at the *Reelin* promoter in NT2 cells [207, 208]. In summary, some of the important players downstream of RA have been identified, but many of the detailed molecular interactions required for the proliferation of neural progenitors and their differentiation into neurons remain obscure.

Differentiation therapy for cancer

RA has been known to inhibit growth of many tumor-cell lines derived from cancers of different origin (e.g., neuroblastomas, adenocarcinomas, lymphomas, sarcomas, melanomas) for at least 40 years [209]. Neuroblastoma cell lines were commonly used to demonstrate the differentiation and anti-tumorigenic potential of RA. LA–N-1/2/5, CHP-134, KA [210, 211], SH-SY5Y [212], Neuro-2a [213], SK-N-BE2 [214], IMR-32 [215], SMS-KCNR [216], and D283 [217] can all be differentiated by retinoids into cells expressing neuronal markers and exhibiting neurite morphology. Although many cell lines can be differentiated in response to RA, the clinical response to RA treatment is variable. Neuroblastomas represent 11 % of all pediatric cancers [218]. The potential for neuroblastomas to be differentiated is so important that pathological classifications have been created to assess the degree of tumor differentiation—the higher the differentiation state, the better the prognosis [219].

The molecular mechanisms underlying RA-stimulated differentiation in neuroblastomas have not been resolved, nor is the ability of these tumors to become RA resistant completely understood. Some evidence indicates that RARs regulate the expression of microRNAs (miRNAs) that support neurite outgrowth and decrease cellular motility (e.g., invasion and metastasis) and proliferation in neuroblastoma cells (reviewed in [220]). miRNA profiles can predict survival of patients with neuroblastoma, therefore, making targeting miRNAs with antagomirs (oligonucleotides that block miRNA activity) a promising therapy [221]. In addition to altering miRNA expression, RA can induce genome-wide changes in DNA methylation by increasing expression of DNA methyltransferases and concomitant hypomethylation of promoters during neuroblastoma differentiation [222]. These changes in miRNA and DNA methylation alter the epigenetic landscape and ultimately affect the expression of oncogenes and tumor-suppressor genes. For example, microarray data demonstrate that expression of tumor-suppressors such as *Erf* and *Etv3* (see section above) are down-regulated in human medulloblastoma (a type of neuroblastoma) [223–225]. Whether this results from increased methylation of these genes, or if treatment with RA would up-regulate *Erf* and *Etv3* to accelerate differentiation in human neuroblastoma tissue is an intriguing, open question.

Another possible mechanism to explain the success (or lack of success) of tumor differentiation involves RAR coregulators. PRAME is a human tumor antigen that is overexpressed in a variety of cancers and is a prognostic indicator for poor survival in neuroblastomas [226]. PRAME functions as a dominant repressor of RAR signaling that renders cancer cells refractory to RA treatment

[227]. Furthermore, a synthetic lethal screen recently found that stimulating differentiation of neuroblastoma cells is dependent on the transcription factor, ZNF423, a ligand-independent, coactivator of RAR signaling [228, 229]. HDAC inhibitors increase RA sensitivity by promoting dissociation of repressive complexes from RAR, thus, accelerating the differentiation process [230, 231]. Taken together, these studies indicate that the presence or absence of RAR-modulators, such as ZNF423 and PRAME, in tumor cells is critically important for sensitivity of cells to RA differentiation therapy and disease outcome [227, 228].

The identification of neural stem cells (NSCs) [232–234] and CNS stem cells [235, 236] has expanded the possible applications of RA in differentiating tumors of neural origin, particularly in aggressive brain tumors such as glioma, meningioma, and neuroma. It is currently unknown whether most brain tumors originate from mutated NSCs within the perivascular niche [237, 238] or if normal cells acquire mutations that cause their dedifferentiation into immature, carcinogenic neural progenitors [239]. Tumorigenesis could take a hierarchical or linear pathway from cancer stem cells to malignant tumor cells or could result from normal tissue losing differentiation markers (e.g., β-Tubulin) and gaining proliferation markers (e.g., *Sox2, Nestin*) (reviewed in [240]). Within the last few years, dedifferentiation as a mode of action in tumorigenesis has been re-evaluated and re-popularized in a variety of cancers including intestinal [241, 242], respiratory [243], breast [244], and brain [239, 245]. Molecular evidence from *Drosophila* revealed that dedifferentiation is associated with the loss of a neural-specific zinc-finger protein Lola-N that normally functions to repress cell cycle genes like *cdc25* in post-mitotic neurons [245]. Loss of *lola* results in brain tumors, and *lola* mutant neurons express neuroblast genes and proliferate in regions of the brain where proliferation usually does not occur [245]. Similar mechanisms might be at work in human tumors.

Considering this renewed interest in dedifferentiation, one might expect RA to play an important role in the treatment of brain tumors. RA was once a prospective treatment for malignant glioma [246–249], but has not proved to be an effective treatment, mostly due to side effects and resistance. There is no doubt that RA is successful in cell culture models of gliomas. In glioblastoma progenitors [250], RA quickly induces cell cycle arrest, inhibiting growth and decreasing clonogenic capacity [251]. RA down-regulates *CD133, Msi-1, Nestin* and *Sox-2* while increasing differentiation markers in these cells [251]. However, retinoid signaling is more complex in human gliomas, in vivo, and resistance and side effects are common. One possible hypothesis for resistance is that RA can be channeled to a pro-proliferative, oncogenic pathway depending on the relative abundance of the RA-transporting proteins CRABP2 and FABP5 (reviewed in [252]). In high-grade, undifferentiated, metastasized glioma, CRABP2 is down-regulated and FABP5 is up-regulated [253, 254], which may divert RA towards the PPARβ/δ pathway (promoting cell survival) and away from RARs (which promote differentiation) [255, 256]. This hypothesis is intriguing, but much more research is needed to test it.

A new differentiation therapy for gliomas using IDH1 (isocitrate dehydrogenase 1) inhibitors [257, 258] has provided another link between retinoid signaling and differentiation. IDH1 mutant tumors produce 2-hydroxyglutarate which is associated with genome-wide hypermethylation of a select group of cancer genes which are reproducible and recognizable as a "glioma methylome" [259, 260]. Intriguingly, IDH1 inhibitor therapy absolutely requires retinoid signaling. All IDH1-mutant tumors have retinol binding protein 1 (RBP1) promoter hypermethylation, and decreased levels of RBP1 [261]. Decreased RBP1 ultimately implies that RA bioavailability is reduced, and thus tumors cannot differentiate. RBP1 hypermethylation serves as a unique biomarker of glioma, and might correlate with improved sensitivity to RA differentiation therapy [261]. This result has immediately produced a pre-clinical trial whereby RA has been repurposed in the treatment of IDH-mutant tumors [262].

Summary and future directions

As described above, it is now well-established that the timing of primary neuron appearance and the number of primary neurons produced is regulated by the levels of RA signaling during embryonic development [18–21]. RA acts in opposition to growth factor signaling to halt the proliferation of neural progenitors and stimulate neuronal differentiation. The effects of RA on primary neurogenesis are at least partly mediated by induction of Ets repressors that act at mid- to late gastrula stages to induce cell cycle exit of neuronal progenitors and their differentiation into primary neurons [22]. Although much is known about neuronal differentiation, we still know relatively little about the molecular mechanisms through which RA and its receptors regulate neuronal differentiation and many questions remain to be answered. Which receptors are the primary players in neurogenesis? Knockdown or antagonism of either RARα or RARγ blocks primary neurogenesis, suggesting that both may be required for primary neurogenesis [22]. Do these receptors act independently, or is one required for expression or maintenance of the other? While it appears that cell cycle genes are the most likely targets for RAR, it is unclear which of these are critical and whether RARs act indirectly as has been suggested [22], or if RARs recruit ligand-dependent

transcriptional repressors to directly repress expression of cell-cycle genes required for proliferation. Some candidates for downstream effectors of RA signaling can be identified from the literature (see above), but only whole genome approaches are likely to fully elucidate the RA-regulated gene network functional in neurogenesis. The rapid increase in the quality of the available databases from *Xenopus* (*laevis* and *tropicalis*) will facilitate the study of these important questions, in vivo.

While a role for RA signaling in primary neurogenesis in anamniote embryos has been demonstrated convincingly, much less is known about its potential roles in neuronal differentiation in amniote embryos. Is RA required for the development of "predecessor neurons" that may correspond to primary neurons, or is this process independent of RA? Apparently bona fide RARs have been identified from mollusks and other invertebrates—what is their function in neurogenesis? Can a role for RA in neuronal differentiation be demonstrated in the invertebrates that have apparent RARs, or is the situation more like that in *Drosophila* where neurogenesis does not require RA? Considering that *Oikopleura* has some components of RA signaling (*RXRs, Adh3* but not *RARs* or *CYP26*), is it possible that RXR signaling plays an important role in neurogenesis when RARs are absent? Or does RXR play a more fundamental role in neurogenesis of both vertebrates and invertebrates that has, so far, remained unknown?

Although RA was first identified as a differentiation agent that inhibited the growth of numerous tumor-derived cell lines in the 1970s and 1980s, the early promise of RA differentiation therapy was not realized. Recent studies have rekindled interest in RA differentiation therapy for cancers, particularly in aggressive brain tumors such as gliomas. A more complete understanding of how RA regulates cell-cycle exit may provide therapeutic targets for future generations of tumor-selective retinoids. The identification of novel components in RA-regulated signaling pathways in neuronal differentiation may also provide important cancer diagnostic and prognostic markers.

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