A critical role for retinoid receptors in axial patterning and neuronal differentiation

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Retinoid signaling

Vitamin A is required for growth, vision, reproduction, morphogenesis, hematopoiesis, immune function, and differentiation of normal and malignant tissues [115]. Vitamin A deficiency during development leads to a spectrum of well-characterized defects collectively called the fetal vitamin A deficiency (VAD) syndrome. RA excess produces a spectrum of developmental defects affecting many of the same tissues as VAD, e.g., the heart, CNS, eyes, ears and reproductive tissues. Sensitivity of the same embryonic tissues to RA deficiency or excess suggested that endogenous RA levels required precise regulation for development to proceed correctly.

The biologic effects of RA are primarily mediated through two different families of ligand-activated transcription factors. The retinoic acid receptors (RARs) are activated by all-trans retinoic acid (atRA) at nanomolar concentrations [44, 99]. The retinoid 'X' receptors (RXRs) are high affinity receptors for 9-cis-RA (9cRA) [54, 79] and lower affinity receptors for two other natural ligands, phytanic acid [66, 78] and docosahexaenoic acid [25]. Both families contain three genes, each of which encodes a distinct receptor subtype (α, β, or γ). Each subtype has two or more isoforms that are the products of differential promoter usage and/or alternative mRNA splicing. RARs heterodimerize with RXR to bind specific DNA targets, termed retinoic acid response elements (RAREs) whereas RXRs bind to RXREs as homodimers. Both types of receptors regulate the transcription of response element-containing genes and provide a direct mechanism to link retinoid concentration and gene expression.

All of the RAR and RXR genes have now been disrupted in transgenic mice ([70], reviewed in [60]). The most dramatic phenotypes are seen in mice where multiple receptor subtypes have been eliminated. The phenotypes observed vary depending on the exact combination of receptors missing. The knockouts recapitulate many aspects of fetal VAD but also introduce novel phenotypes such as homeotic vertebral transformations and atavistic skeletal structures [60]. Interestingly, while multiple receptor knockouts are required to obtain observable phenotypes in the mouse model [60], removing a single receptor isoform, RARα2.2 causes dramatic effects in *Xenopus* embryos [67]. Thus, there appears to be a compensation mechanism in mammals to moderate the effects of losing a particular receptor whereas this does not seem to be the case for *Xenopus*. This makes *Xenopu*s an ideal system to study the role of individual receptor subtypes and isoforms in development.

A large body of evidence exists linking retinoids to the regulation of developmentally significant genes. Among these are genes involved in determining positional identity along the anteroposterior axis. Of particular interest is the requirement for retinoids in specifying the vertebrate anteroposterior (A/P) axis, especially their potential role in patterning the central nervous system (CNS). There is an interesting general correlation between the posteriorizing effects of RA excess and anterior Hox gene overexpression and between the anteriorizing effects of RAR compound knockouts and Hox gene knockouts (reviewed in [22]. Many excellent reviews are available which treat various aspects of axial patterning (especially in the mouse) and what role retinoids might have in this process [4, 9, 22, 31, 59, 82, 86, 127]. In this review, we will describe the known functions of signaling through the retinoic acid receptors in two distinct but related processes: patterning of the A/P neural axis and the regulation of neuronal differentiation.

Anteroposterior neural patterning

The isolated animal pole (animal cap) of a blastula stage Xenopus embryo will form epidermal tissue when cultured in a simple saline solution. However, when molecules such as n*oggin*, *follistatin*, *chordin or xnr3* are present in the solution, this naive ectoderm becomes neuralized in the absence of mesoderm induction [49, 52, 53, 56, 73]. *Noggin*, *follistatin* and *chordin* are expressed in all the Spemann organizer layers, while *xnr3* is solely expressed in the organizer's epithelial layer and is rapidly turned off when the cells begin to invaginate. These neural inducers are required in the ectoderm to block activity of *BMP-4*, a secreted molecule belonging to the TGFβ growth factors family [49, 56, 126]. BMP-4 is expressed in the ectoderm and acts to prevent the expression of neural genes. When animal caps are dissociated into individual cells and reaggregated, neural markers are expressed since BMP-4 is now absent. Similarly, overexpression of a dominant negative bone morphogenetic protein (BMP)-4 receptor or ligand causes Xenopus animal caps to express neural markers [49, 110, 125]. In accord with its biological activity, *BMP-4* transcripts are distributed evenly in the animal cap and ventrolateral marginal zone of the early gastrula embryo, but excluded from the Spemann organizer [39]. Organizer expressed genes such as noggin, follistatin and chordin neutralize BMP activity by directly binding to BMPs, thus blocking BMP inhibition of neural fate [38, 100, 129].

All of the direct neural inducers described above induce neural tissue of anterior character [49, 52, 53, 56, 73]. These findings all support a model wherein the basal state of the neural ectoderm is anterior and additional factors are required to generate the posterior parts of the nervous system [37, 94]. It is currently believed that the major components of the activation signal are FGF and Wnt signals that act before gastrulation to induce the organizer to secrete inhibitors of BMP and Wnt signaling such as noggin, chordin, cerberus, follistatin, and dickkopf during gastrulation (reviewed in [47]). In turn, these induce the neuroectoderm to adopt an anterior fate.

The transformation signal has been more elusive and is only recently becoming better understood. It had been previously shown that basic fibroblast growth factor (bFGF aka FGF2) could posteriorize anterior neuroectoderm in vitro. Many investigators inferred an endogenous role for FGFs in neural induction and patterning [23, 64, 72] (reviewed in [28]). Isaacs and colleagues showed that eFGF (FGF4) could posteriorize the axis via induction of downstream genes Xcad3 and Hox-A7 in vivo [103]. eFGF (aka FGF4) was proposed to be a good candidate for at least part of the posterior transforming signal. It is appropriately expressed in the notochord and posterior mesoderm. Inhibition of FGF signaling via overexpression of the dominant negative FGF receptor, XFD, reduced the expression of the posterior markers Hox-A7 and Xcad-3, but not the anterior markers Hox-B1 and Otx-2 [103]. However, others demonstrated that transgenic embryos expressing XFD (and non-mosaically deficient in FGF signaling) showed strong inhibition of posterior mesoderm but had only limited effects on A/P patterning of the nervous system. This suggested that signaling through FGFR1 is not essential for neural posteriorization [71] although there is not complete agreement on this point [102]. Papalopulu and colleagues proposed that FGF8 acting through FGFR4 (rather than FGF4 acting through FGFR1) is likely to be the major FGF pathway in neural posteriorization [46].

Retinoid signaling in A/P patterning

Retinoic acid (RA) was first proposed to be involved in neural development when it was demonstrated that exogenously applied RA produces a concentration-dependent truncation of anterior and enhancement of posterior structures in Xenopus embryos [30, 114] through its influence on the embryonic mesoderm and ectoderm [108, 113]. Carrasco and colleagues showed that RA treatment led to an anterior expansion of Hox-B7 and C6 expression in chicken and

Xenopus (posteriorization), whereas treatment with an RAR antagonist, R0-41, 5253 [63] reduced the levels and extent of Hox-B7 and C6 expression (anteriorization) [80, 81]. The expression of the hindbrain marker Krox-20 was shifted anteriorly by RA and posteriorly by the antagonist, which is also consistent with RA functioning to posteriorize the axis. Maden and colleagues showed that vitamin A deficient (VAD) quail had numerous axial defects and altered expression of A/P markers such as Hox-A2, Hox-B1, Hox-B4, Krox-20 and FGF-3 but no changes in the D/V markers sonic hedgehog, islet-1 and Pax-3 [84]. They concluded these animals were missing the hindbrain posterior to rhombomere 4 and that retinoid signaling is only required for A/P and not for D/V patterning. Experiments using VAD rats confirmed these results. Temporally regulated depletion of retinoic acid produced specific defects in the neural crest, eyes, and nervous systems [26]. Pharmacological doses of RA were able to rescue embryonic hindbrain defects and fetal resorption caused by VAD [124]. Taken together, these experiments suggest that retinoid signaling is indeed critical for A/P patterning.

We and others used dominant negative RARs to provide strong evidence for the necessity of retinoid signaling in A/P patterning. We showed that locally decreasing RAR signaling led to a loss of posterior markers such as Hox-B9, N-tubulin, and Xlim-1 [10]. Positional changes were observed in the hindbrain along with posterior coordinate shifts in the expression of anterior markers. In contrast, locally increasing RAR signaling yielded the opposite result [10]. Using different mutant RARs and molecular markers, other groups showed that retinoid signaling was required to specify positional identity in the hindbrain [68, 122]. Overexpression of the Xenopus retinoic acid hydroxylase (CYP26) that is believed to target RA for degradation, leads to expansion of anterior structures [24, 55]. Conversely, overexpression of the RA biosynthetic enzyme RALDH2 leads to reduction of anterior structures [16].

RALDH2 loss-of-function in mice led to a variety of axial defects including loss of posterior rhombomere identity, posterior mesoderm, limbs, and retinoic acid inducible molecular markers [93]. These defects were partially rescued by maternal administration of RA during gestation, confirming that loss of RA, rather than another RALDH2 product was the likely explanation. Lumsden and colleagues recently showed that RA is the endogenous transforming factor active during hindbrain patterning and that it acts in a graded fashion to specify the identity of rhombomeres 5-8 [29]. They further showed that generation of the RA gradient was a local process, likely involving an active mechanism to distribute RA along the hindbrain at the

appropriate concentrations while simultaneously restricting its diffusion to adjacent tissues [29]. The conclusion from these results is that retinoid signaling through xRARs is essential to correctly restrict the expression of anterior genes and to enable the expression of posterior marker genes. Considering the FGF experiments described above, it should also be noted that RA could posteriorize anterior neuroectoderm injected with XFD whereas FGF could not [6]. Therefore, both retinoid and FGF signaling can posteriorize anterior neural tissue in vitro, perhaps acting synergistically as was suggested previously based on transplantation experiments [20].

Wnt, FGF and retinoid signaling converge on Xcad3

Several studies demonstrated a role for Wnt signaling in posteriorizing the embryonic axis by showing that overexpression of Xwnt3A could posteriorize anterior neuroectoderm [89, 90]. This view was reinforced by experiments that showed blockade of Xwnt8 signaling could cause loss of posterior fates [5, 40, 89]. Furthermore, inappropriate activation of Wnt target genes caused by loss of the headless/TCF-3 gene resulted in severe anterior defects [65]. Two groups independently used MO loss-of-function and genetic analysis to show that Wnt8 is an important transforming factor in zebrafish and Xenopus, concluding that either Wnt8, or a factor critically dependent on Wnt8 for its expression is the endogenous neural transforming factor [36, 77]. Krumlauf and colleagues recently showed that the Wnt/β-catenin pathway posteriorizes Xenopus neural tissue via an indirect mechanism requiring FGF signaling, suggesting that the posteriorization pathway might be Wnt->FGF->Xcad3-> posterior Hox genes [27]. This model does not account for the observation that inhibiting RAR signaling with a dominant negative receptor blocks the expression of posterior neural markers [9, 10].

Retinoid and FGF signaling participate in many of the same processes during vertebrate development. These include gastrulation movements, axial posteriorization and the differentiation of primary neurons. The observations that both FGF [58, 103] and retinoid signaling [10] are required for the expression of posterior Hox genes led us to hypothesize that these pathways converge on one or more common target genes. Since Xcad3 is a key downstream gene in the FGF-mediated posteriorization pathway and retinoids have been shown to influence the expression of caudal family genes in other systems [1, 57, 104], we hypothesized that Xcad genes were likely targets for both retinoid and FGF signaling. Indeed, we found that

modulating retinoid signaling with RAR agonists or antagonists predictably altered the expression of Xcad3 and that RARα2.2 is required for the expression of Xcad3 and Hox-B9 [61]. Therefore, we concluded that RAR signaling is required for the expression of Xcad3 and that FGF and RAR signaling converge on Xcad3 in Xenopus development.

Interaction between RAR and FGF signaling during Xenopus A/P patterning

Overexpression of the dominant negative FGF receptor XFD suppresses mesoderm formation [2] and the expression of posterior neural genes [62, 102, 103]. To test whether RAR signaling was downstream of FGF signaling, epistasis experiments were performed. Microinjection of the constitutively active VP16-xRARα2.2 completely rescued Xcad3 and Hox-B9 expression in XFD-injected embryos whereas xRARα2.2 led to partial rescue and RA treatment did not rescue at all [61]. The inference is that XFD is downregulating a critical component of retinoid signaling. The failure of RA to rescue suggests that RA synthesis is not limiting in XFD-injected embryos. Partial rescue by restoring receptor expression suggests that the receptor itself is the key component missing. The constitutively active receptor does not require endogenous RARs or RA and therefore would be expected to rescue if retinoid is downstream of FGF signaling.

xRARα2.2 loss-of-function was achieved by microinjecting an antisense morpholino oligonucleotide (RAR-MO) that inhibits translation of the endogenous mRNA [61, 67]. RAR-MO microinjection led to downregulation of Xcad3 and Hox-B9 expression [61]. This downregulation could be rescued by coinjecting xRARα2.2 mRNA but not by overexpressing FGF8 or Xcad3 mRNAs [61]. Downregulation of FGF signaling by microinjecting XFD led to suppression of RAR pathway components xRARα2, RALDH2 and CYP26 [62]. Thus, FGF signaling can modulate RAR signaling by regulating the availability of components of the RAR signaling pathway and xRARα2.2 expression is required for the expression of Xcad3 and Hox-B9. We also showed that xRARα2.2 was required for the expression of FGFR1, FGFR4 and FGF8 [61]. Therefore, RAR signaling is both upstream and downstream of FGF signaling.

Zygotic expression of xRARα, RALDH2 and CYP26 colocalize with FGF pathway components at the onset of gastrulation in the circumblastoporal region [61]. It is possible that FGF signaling could be required for the initiation of the zygotic expression of RAR pathway components. xRARα, xRARγ, and bioactive retinoids are all present in the unfertilized egg [11].

Since RAR signaling is required for the expression of FGF pathway components [61], it is possible that the maternally expressed RARs are permissive for FGF signaling which is, in turn, instructive for the zygotic expression of RAR pathway components. Taken together, these observations suggests that RAR and FGF signaling cross-regulate each other, perhaps in a feedback loop that allows these posteriorizing factors to maintain each other's expression [61].

RA signaling is involved in multiple steps in posteriorization of the neural tube.

The alteration in the expression of FGF signaling components by the RAR-MO together with the requirement for FGF signaling to express RAR pathway components supports the existence of a mutual feedback loop. Isaacs and colleagues [58] showed using gain- and loss-of-function experiments that Xcad3 upregulates Hox-B9 expression in Xenopus embryos [58, 102, 103]. This contention is supported by the identification of caudal/Cdx homeodomain binding sites in the putative promoter region of several mouse and chick Hox genes [15, 117]. However, we note that ectopic expression of HoxB9 induced by Xcad3 overexpression is restricted to the neural tube even when the coinjected lineage tracer and presumably Xcad3 mRNA was distributed in other sites [61]. Therefore, we tested the effect of coinjecting the RAR-MO together with Xcad3 mRNA. Xcad3 injection alone could induce ectopic expression of HoxB9, even anterior to where it is normally expressed [61]. Co-injection of the RAR-MO led to severely reduced expression of HoxB9, suggesting that RAR function is required for Xcad3 to induce expression of HoxB9. This places RAR both upstream and downstream of Xcad3 and FGFs, indicating that RAR signaling is involved in multiple steps of the genetic cascade regulating neural posteriorization [61]. Figure 1 depicts our current thinking regarding how these pathways interact.

Neurogenesis - a brief story

The molecular mechanisms that control neuronal differentiation during neural patterning have been extensively studied in *Drosophila melanogaster* and are conserved in vertebrates (reviewed in [109]. Amphibian neurogenesis has two differentiation stages. First, a simple neuronal network that allows tadpoles to swim and feed is built during neurulation by primary neurogenesis. Primary neurons may be visualized in the developing neural plate as three longitudinal bands of cells on both sides of the midline that express the neuronal marker *N*-

tubulin. The medial band will differentiate into ventral motor neurons, the intermediate band into interneurons, and the most lateral band into sensory neurons [19]. Subsequently, a secondary wave of neurogenesis completes the refinement and shaping of the neural tube.

Neuronal differentiation is facilitated and regulated by a prepatterning process initiated during gastrulation. This cascade of molecular interactions leads to a balanced expression of the prepattern genes *Gli* and *Zic* whose zinc finger transcription factor products [13, 74, 88, 92] delineate the basic neural domains. For instance, *Gli1* is restricted to the neural plate midline (future floor plate), Gli2 is expressed in the rest of the neural plate and *Gli3* in a lateral to medial gradient. *Zic2* is expressed in domains of the neural plate intercalated with the longitudinal bands of the future primary neurons. *Zic2* locally inhibits neuronal differentiation by competing with the *Gli* proteins for DNA target sequences; therefore, *Gli* and *Zic2* have opposite functions. The prepattern genes broadly outline sites where primary neuron formation will be permitted by regulating the expression of proneural genes, (transcription factors with basic helix-loop-helix (bHLH)) domains such as *neurogenin* and *Xash-3* [83, 128].

Neurogenin (*ngn*) is expressed in three longitudinal bands on both sides of the neural plate midline. These expression domains mirror those of the future primary neurons, but precede the expression of the *N-tubulin* differentiation marker. *Ngn* expression defines restricted domains where cells may become neurons, however, not all the cells of such proneural clusters will form mature neurons. Within these proneural clusters, one cell develops into a neuronal precursor. Its neighbors are prevented from doing so by a process called lateral inhibition, mediated by the neurogenic genes *Notch* and its membrane-bound ligand *Delta* [19]. In neuronal precursors, *ngn* promotes the expression of *Delta* [83], which interacts with its receptor *Notch* on the surface of the neighboring cell. *Notch* is cleaved to release the intracellular domain ($Notch^{ICD}$) which associates with the intracellular transducers, suppressor of hairless (*X-Su(H)*), migrates to the nucleus and activates transcriptional repressors such as *HES* genes that block neuronal fate [123] (reviewed in [3, 12, 91]. The *Delta*-expressing cell goes on to become a primary neuron.

Most components of this complex genetic program have been identified in vertebrates, for the most part by homology to their fly counterparts (reviewed in [75, 109]. In addition to those molecules already mentioned above as components of the primary neurogenesis cascade, other proteins have been described that transiently promote neurogenesis, e.g. *Xash-1* [42]**,** *Xash-3* [41], and neuronal differentiation, e.g., *NeuroD*. Little is known about the events that follow

neural induction and precede the selection of neuronal precursors in vertebrates. In *Drosophila*, *iroquois* regulates the activity of proneural genes [8, 45]**.** *Iroquois* homologs have been identified in *Xenopus* (*Xiro1, 2, 3*). However, *Xiro1* activates *Xash3*, but not *neurogenin* or *atonal* in animal caps, suggesting that other factor(s) are involved.

Drosophila cubitus interruptus (*Ci*), a member of the *hedgehog* (*hh*) signaling pathway, is able to activate *Xiro1* and *Xiro2* in *Xenopus* embryos [45]. When the *hh* cascade is inactive, *Ci* is cleaved generating a transcriptional repressor fragment. When *hh* is present the cleavage is blocked, and *Ci* behaves as activator [107]. The *hedgehog* pathway was further modified in vertebrate evolution by separating the repression and activation activities of *Ci* by gene duplication. The *Gli* family comprises the vertebrate homologs of *Drosophila Ci***.** *Gli1* is a transcriptional activator while *Gli3* and *Gli4* are transcriptional repressors [74, 88]**.** The sonic hedgehog **(***Shh)* signaling pathway (homologous to *Drosophila hh)* pathway is completed with a complex of two membrane proteins *Patched* (*Ptc*) and *Smoothened* (*Smo*) that are proposed to transduce the *Shh* signal. In the absence of ligand, *Ptc* blocks the pathway, whereas *Shh* binding to *Ptc* relieves this inhibition and activates downstream intracellular events mediated by the *Gli* family.

Several observations suggested that Shh could diffuse from the notochord and floor plate and act directly on cells some distance way. First, a signal from the notochord or floor plate could induce motor neurons in transfilter assays [120]. This activity can be blocked by antibodies against Shh [35]. Second, the expression of a number of Shh-responsive genes are regulated at a significant distance from the source of Shh [87]. The differentiation of motor neurons requires the presence of Shh during S phase of the final cell division, suggesting a prolonged requirement for Shh in responding cells [35]. Although the diffusion of Shh extracellularly thought to be limited by cholesterol and palmitoyl modifications [98, 101] these data suggest that low levels of Shh are able to diffuse and act over a range of at least several cell diameters. Although the mechanism remains obscure in vertebrates, a *Drosophila* gene, *tout velu*, has recently been identified that appears to facilitate the diffusion of Hh possibly by regulating the interaction of Hh with extracellular matrix components [7].

RA induces the expression of N-tubulin in the neural plate

During Xenopus early neural development, neuronal differentiation is detected in the posterior neuroectoderm by stage 14, but it is retarded in the anterior region until after the completion of neural tube closure at stage 27. A similar delay in neuronal differentiation can be replicated when isolated animal caps explants are neuralized by treatment with the neural inducer noggin [73]. Treatment of neuralized animal caps with RA prematurely induced N-tubulin expression at the equivalent of stage 14, suggesting that RA plays an important role in the timing of neuronal differentiation [97]. In addition, this early differentiation of neurons is markedly increased by coexpressing the proneural gene, *Xash3*, suggesting cooperation between RA and Xash3 in regulating early neurogenesis. Treatment of whole Xenopus blastulae with exogenous RA produces extensive, premature anterior expression of N-tubulin in neurula stage embryos [97]. Despite this demonstrated effect of RA on neuronal differentiation in the anterior, there were until recently no studies on the potential role of RA in the posterior neural plate where primary neurogenesis begins.

Treatment with RA or RA antagonists (e.g., Ro 41-5253) [63, 81] was employed to modulate the amount of RA signaling and to evaluate its effects on N-tubulin in the early neurula embryo (st 14). In control embryos, N-tubulin expression is organized as three longitudinal bands of cells (medial, intermediate and lateral) that correspond respectively to ventral (motor) neurons, interneurons and dorsal (sensory) neurons. A second expression domain is observed in the trigeminal ganglion neurons. N-tubulin expression in anterior neural plate is not detected prior to neural tube closing [19, 48]. RA-treated embryos showed a marked increase in N-tubulin expression. The number of N-tubulin expressing cells was increased, leading to the fusion of dorsal and intermediate expression bands. The ventral band of cells was less affected. In contrast to the results with RA treatment, antagonist treatment led to a reduction of N-tubulin expression and an increase in the separation between the bands of N-tubulin positive cells.

Similar results were observed by manipulating retinoic acid receptor function with mutant receptors such as dominant negative forms of RARα1 [9, 10] and RARα2 [112]. Overexpression of either dnRAR led to a reduction in the number of primary neurons as measured by N-tubulin expression. In contrast, increased RAR signaling by overexpressing RARα and RXRβ [111] or a constitutively active form of RAR (B. Blumberg unpublished results) leads to an increase in the number of primary neurons. Downregulating the synthesis of RA with the aldehyde dehydrogenase inhibitor, citral [111] or RAR antagonists (Blumberg and Carrasco, unpublished)

also suppresses the formation of primary neurons. While these results demonstrate that signaling through the RARs positively regulates N-tubulin, they do not speak to the mechanism of action since N-tubulin is a terminal marker for neuronal differentiation.

One important observation regarding the role of retinoid signaling in neuronal differentiation is that the induction of primary neurons is never ectopic, i.e., it does not occur in the non-neural ectoderm. This suggests that retinoid signaling interacts with other factors within the neural plate to influence the production of primary neurons. Significantly, N-tubulin expression within the neural plate remains within its normal boundaries, even when the number of primary neurons is upregulated by increasing retinoid signaling. Therefore, retinoids may require interactions with localized, specific cofactors to influence the formation of primary neurons.

The RA-mediated increase in primary neurons does not result from alterations in proliferation or apoptosis.

Differential proliferation and apoptosis of neuronal cells are fundamental features of vertebrate CNS development. In addition to its known roles in promoting differentiation, RA has also been shown to regulate cell proliferation and apoptosis [18, 85]. Therefore, in principle, retinoid signaling could be acting to regulate the differentiation, proliferation or survival of neuronal precursors. Sharpe and colleagues demonstrated that the increase in primary neuron formation promoted by treating embryos with RA or by overexpressing RAR and RXR was not dependent on increased proliferation of neuronal precursors. In both cases, the number of primary neurons increased despite the block of cell proliferation with hydroxyurea and aphidocolon [112]. TUNEL analysis at the neurula stage (st 14) showed that RA treatment did not inhibit apoptosis but rather led to an increase in the number primary neurons as measured by N-tubulin expression (Carrasco, et al unpublished). Taken together, these data suggest that the increase in primary neuron number elicited by increasing retinoid signaling does not result from effects on the proliferation or apoptosis of neuronal precursors. Therefore, retinoids must act at one or more places in the neuronal differentiation pathway.

Retinoids affect the expression of genes involved in primary neurogenesis

Expression of the neuronal differentiation markers *X-ngnr-1*, *X-MyT1* and *Gli3* was upregulated or expanded in RA-treated embryos [43]. We inferred that RA positively regulates the primary neurogenic cascade. RA-treated embryos also exhibit a reduction in the spacing between the three neuronal domains, suggesting that RA might be modifying the activity of the prepattern genes and changing the size of their expression domains [43]. RA treatment leads to an expansion of the Gli3 expression domain in the posterior neural plate accompanied by a reduction in the expression domain of Zic2, a gene that inhibits neuronal differentiation [43]. Conversely, inhibition of RAR signaling using the antagonist Ro-41, 5253 reduced the expression of *X-ngnr-1*, *X-MyT1* and *X-Gli3* while enlarging the expression domain of Zic2 [43]. Taken together, these results demonstrate that RA signaling through the RARs is required to establish and/or maintain the correct expression patterns of these genes. Therefore, we concluded that retinoid signaling favors neuronal differentiation by increasing recruitment of neuronal precursors within neurogenic domains.

Shh signaling delays neuronal differentiation

The floor plate is an epithelial structure that occupies the ventral midline of the vertebrate neural tube. Floor plate is involved in the ventral identity of the neural tube, induces ventral motor neurons and guides axonal growth [74] (reviewed in [21, 116, 119]. When the chicken notochord is removed the floor plate does not develop, but appears ectopically induced when notochord is transplanted into the lateral regions of the neural tube [119]. It is generally accepted that *Shh* is the notochord signal responsible for the floor plate induction. Targeted *Shh* loss-of-function blocks floor plate formation in mice [17]. It has also been shown that the fork-head family transcription factor *HNF3-β,* activates *Shh* transcription in the notochord. It is believed that *Shh* protein secreted by the notochord activates *Gli1* in the midline of the neural plate. In turn, *Gli1* activates *HNF3-β/Pintallavis*, which leads to *Shh* activation in the floor plate cells. In addition to its role in motor neuron induction, *Shh* is important for the patterning of a variety of structures during embryonic development [32, 33, 69, 76, 105]**.**

We have found that retinoid and *sonic hedgehog* signaling modulate primary neurogenesis by counterbalancing the expression of prepattern genes with opposing functions. *Shh* inhibits neurogenesis by downregulating *Gli3 (*a gene that favors neurogenesis) and activating *Zic2* (an inhibitor of neurogenesis). Ectopic *Shh* expression delays primary

neurogenesis, leading to proliferation of neuronal precursors. *Shh* overexpression also increased the number of secondary motor neurons. Therefore, we proposed that *Shh* signaling could lead to withdrawal of neural precursor cells from retinoid, induced differentiation, maintaining them in a proliferative state [43]. In this model, the balance between shh and retinoid signaling plays a critical role in determining the number of neuronal precursors available for primary and secondary neurogenesis.

Retinoids inhibit shh expression to promote neuronal differentiation

Given that RA and Shh signaling act in opposite ways on neuronal differentiation, one immediate question that arises is whether either affects the expression of the other. We showed that modulating retinoid signaling affects the expression of Shh. RA treatment led to the downregulation of Shh expression whereas the inhibition of retinoid signaling by treating embryos with Ro-41, 5253 had the opposite effect – Shh expression was increased [43]**.** It has previously been reported that the zebrafish and mouse *shh* promoters contain both HNF3β and RAR response elements (RAREs) and that these directly regulate *shh* expression [14, 34]. It is paradoxical that these RAREs have been characterized as mediating transcriptional activation rather than repression but that RA treatment inhibits *shh* expression. However, RA inhibition of *shh* expression has been described in chicken and zebrafish [14, 50, 95, 105]. During chicken facial morphogenesis, *shh* expression is inhibited by teratogenic doses of RA [51] and RA can both induce [50] and inhibit [121] *shh* expression in the developing limb. The most likely mechanism is that retinoid signaling upregulates a repressor of Shh expression.

Additional evidence for a connection between shh and RA signaling comes from the analysis of human mutations and RA teratogenesis. Mutations in the human *shh* gene cause a syndrome known as holoprosencephaly. The most severe cases exhibit failed separation of the cerebral hemispheres accompanied by cyclopia (a single eye in the mid line) and prosboscis (primitive nasal structure). Less severely affected individuals present with microcephaly or only one central maxillary incisor [106]. The administration of RA at early stages of mouse embryonic development also produces craniofacial malformations similar to holoprosencephaly [118], and treatment of early *Xenopus* embryos with RA leads to forebrain defects and cyclopia [30, 108, 114]. These observations support a model wherein RA inhibits *shh* signaling thereby promoting neuronal differentiation (Fig 2).

Lastly, the time window wherein RA affects shh expression and primary neurogenesis was analyzed. Pulsed treatment of early Xenopus embryos with RA showed that a brief treatment of RA at the beginning of gastrulation was sufficient to inhibit shh expression at stage 10. The late gastrula loses the ability to downregulate shh expression in response to RA treatment (Carrasco, unpublished results). A similar window of sensitivity for RA-mediated loss of cephalic structures exists in the early embryo, declining at the end of the gastrulation (stage 12.5) [30, 108, 114]. RA treatment of late blastula embryos (st 9) induces premature cephalic N-tubulin expression [97]. These windows of sensitivity to RA correspond with the early expression of shh; hence, it is reasonable to infer that many of these early effects of RA are mediated through *shh*.

Conclusions: Retinoids regulate early A/P patterning and early steps in the neurogenic cascade

The experiments described above reveal complex interactions among FGF, Wnt and retinoid signaling. Retinoid signaling has been largely ignored in favor of growth factor signaling pathways in recent years; however, the data demonstrate convincingly that RARs act both upstream and downstream of growth factor signaling to pattern the A/P axis. Xenopus RARα2.2 is required for the expression of FGFs and FGF receptors and FGF signaling is required for the zygotic expression of RAR signaling pathway components. Embryos deficient in RARs do not form heads and primary neurons do not differentiate. This suggests that retinoid signaling is an essential component of the positional patterning system operational at the very earliest stages of embryonic development. The observations relating retinoid signaling and the expression of neurogenic markers all clearly point to a subsequent role for retinoids in neuronal differentiation. Activity of the RARs is required for the correct expression of proneural and prepatterning genes operating at the earliest steps of neural development [43, 96]. Considering that the timing of neuronal differentiation is coupled with that of A/P patterning, it is plausible that retinoid signaling may play a key role in linking these two processes.

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References

1. **Allan, D., M. Houle, N. Bouchard, B. I. Meyer, P. Gruss, and D. Lohnes.** 2001. RARgamma and Cdx1 interactions in vertebral patterning. Dev Biol **240**(1)**:**46-60.
2. **Amaya, E., P. A. Stein, T. J. Musci, and M. W. Kirschner.** 1993. FGF signalling in the early specification of mesoderm in Xenopus. Development **118:**477-487.
3. **Baker, N. E.** 2000. Notch signaling in the nervous system. Pieces still missing from the puzzle. Bioessays **22**(3)**:**264-73.
4. **Bally-Cuif, L., and E. Boncinelli.** 1997. Transcription factors and head formation in vertebrates. BioEssays **19:**127-135.
5. **Bang, A. G., N. Papalopulu, M. D. Goulding, and C. Kintner.** 1999. Expression of Pax-3 in the lateral neural plate is dependent on a Wnt- mediated signal from posterior nonaxial mesoderm. Dev Biol **212**(2)**:**366-80.
6. **Bang, A. G., N. Papalopulu, C. Kintner, and M. D. Goulding.** 1997. Expression of Pax-3 is initiated in the early neural plate by posteriorizing signals produced by the organizer and by posterior non- axial mesoderm. Development **124**(10)**:**2075-85.
7. **Bellaiche, Y., I. The, and N. Perrimon.** 1998. Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. Nature **394**(6688)**:**85-8.
8. **Bellefroid, E. J., A. Kobbe, P. Gruss, T. Pieler, J. B. Gurdon, and N. Papalopulu.** 1998. Xiro3 encodes a Xenopus homolog of the Drosophila Iroquois genes and functions in neural specification. Embo J **17**(1)**:**191-203.
9. **Blumberg, B.** 1997. An essential role for retinoid signaling in anteroposterior neural specification and neuronal differentiation. Seminars in Cell & Developmental Biology **8**(4)**:**417-428.
10. **Blumberg, B., J. Bolado, T. A. Moreno, C. Kintner, R. M. Evans, and N. Papalopulu.** 1997. An essential role for retinoid signaling in anteroposterior neural patterning. Development **124:**373-379.
11. **Blumberg, B., D. J. Mangelsdorf, J. Dyck, D. A. Bittner, R. M. Evans, and E. M. De Robertis.** 1992. Multiple retinoid-responsive receptors in a single cell: families of RXRs and RARs in the Xenopus egg. Proc. Natl. Acad. Sci. (U.S.A.) **89:**2321-2325.

12. **Bray, S.** 1998. A Notch affair. Cell **93**(4)**:**499-503.

13. **Brewster, R., J. Lee, and A. Ruiz i Altaba.** 1998. Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. Nature **393**(6685)**:**579-83.

14. **Chang, B. E., P. Blader, N. Fischer, P. W. Ingham, and U. Strahle.** 1997. Axial (HNF3beta) and retinoic acid receptors are regulators of the zebrafish sonic hedgehog promoter. Embo J **16**(13)**:**3955-64.

15. **Charite, J., W. de Graaff, D. Consten, M. J. Reijnen, J. Korving, and J. Deschamps.** 1998. Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements. Development **125**(22)**:**4349-58.

16. **Chen, Y., N. Pollet, C. Niehrs, and T. Pieler.** 2001. Increased XRALDH2 activity has a posteriorizing effect on the central nervous system of Xenopus embryos. Mech Dev **101**(1-2)**:**91-103.

17. **Chiang, C., Y. Litingtung, E. Lee, K. E. Young, J. L. Corden, H. Westphal, and P. A. Beachy.** 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature **383**(6599)**:**407-13.

18. **Chiba, H., J. Clifford, D. Metzger, and P. Chambon.** 1997. Distinct retinoid X receptor-retinoic acid receptor heterodimers are differentially involved in the control of expression of retinoid target genes in F9 embryonal carcinoma cells. Mol Cell Biol **17**(6)**:**3013-20.

19. **Chitnis, A., D. Henrique, J. Lewis, D. Ish-Horowicz, and C. Kintner.** 1995. Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature **375:**761-766.

20. **Cho, K. W. Y., and E. M. De Robertis.** 1990. Differential activation of Xenopus homeobox genes by mesoderm inducing growth factors and retinoic acid. Genes Dev. **4:**1910-1916.

21. **Colamarino, S. A., and M. Tessier-Lavigne.** 1995. The role of the floor plate in axon guidance. Annu Rev Neurosci **18:**497-529.

22. **Conlon, R. A.** 1995. Retinoic acid and pattern formation in vertebrates. Trends Genet. **11:**314-319.

23. **Cox, W. G., and A. Hemmati-Brivanlou.** 1995. Caudalization of neural fate by tissue recombination and bFGF. Development **121:**4349-4358.

24. **de Roos, K., E. Sonneveld, B. Compaan, D. ten Berge, A. J. Durston, and P. T. van der Saag.** 1999. Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and Xenopus laevis embryogenesis. Mech Dev **82**(1-2)**:**205-11.

25. **de Urquiza, A. M., S. Liu, M. Sjoberg, R. H. Zetterstrom, W. Griffiths, J. Sjovall, and T. Perlmann.** 2000. Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. Science **290**(5499)**:**2140-4.

26. **Dickman, E. D., C. Thaller, and S. M. Smith.** 1997. Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. Development **124**(16)**:**3111-21.

27. **Domingos, P. M., N. Itasaki, S. A. Jones, S. Mercurio, M. G. Sargent, J. C. Smith, and R. Krumlauf.** 2001. The Wnt/b-catenin pathway posteriorizes neural tissue in Xenopus by an indirect mechanism requiring FGF signaling. Dev Biol **239**(1)**:**148-160.

28. **Doniach, T.** 1995. Basic FGF as an inducer of anteroposterior neural pattern. Cell **83:**1067-1070.

29. **Dupe, V., and A. Lumsden.** 2001. Hindbrain patterning involves graded responses to retinoic acid signalling. Development **128**(12)**:**2199-208.

30. **Durston, A. J., J. P. Timmermans, W. J. Hage, H. F. Hendriks, N. J. de Vries, M. Heideveld, and P. D. Nieuwkoop.** 1989. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature **340:**140-144.

31. **Durston, A. J., J. van der Wees, W. W. Pijnappel, and S. F. Godsave.** 1998. Retinoids and related signals in early development of the vertebrate central nervous system. Curr Top Dev Biol **40:**111-75.

32. **Echelard, Y., D. J. Epstein, B. St-Jacques, L. Shen, J. Mohler, J. A. McMahon, and A. P. McMahon.** 1993. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell **75**(7)**:**1417-30.

33. **Ekker, S. C., L. L. McGrew, C. J. Lai, J. J. Lee, D. P. von Kessler, R. T. Moon, and P. A. Beachy.** 1995. Distinct expression and shared activities of members of the hedgehog gene family of Xenopus laevis. Development **121**(8)**:**2337-47.

34. **Epstein, D. J., A. P. McMahon, and A. L. Joyner.** 1999. Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms. Development **126**(2)**:**281-92.

35. **Ericson, J., S. Morton, A. Kawakami, H. Roelink, and T. M. Jessell.** 1996. Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell **87**(4)**:**661-73.

36. **Erter, C. E., T. P. Wilm, N. Basler, C. V. Wright, and L. Solnica-Krezel.** 2001. Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo. Development **128**(18)**:**3571-83.

37. **Eyal-Giladi, H.** 1954. Dynamic aspects of neural induction. Arch. Biol. **65:**180-259.

38. **Fainsod, A., K. Deissler, R. Yelin, K. Marom, M. Epstein, G. Pillemer, H. Steinbeisser, and M. Blum.** 1997. The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. Mech Dev **63**(1)**:**39-50.

39. **Fainsod, A., H. Steinbeisser, and E. M. De Robertis.** 1994. On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. Embo J **13**(21)**:**5015-25.

40. **Fekany-Lee, K., E. Gonzalez, V. Miller-Bertoglio, and L. Solnica-Krezel.** 2000. The homeobox gene bozozok promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. Development **127**(11)**:**2333-45.

41. **Ferreiro, B., C. Kintner, K. Zimmerman, D. Anderson, and W. A. Harris.** 1994. XASH genes promote neurogenesis in Xenopus embryos. Development **120**(12)**:**3649-55.

42. **Ferreiro, B., P. Skoglund, A. Bailey, R. Dorsky, and W. A. Harris.** 1993. XASH1, a Xenopus homolog of achaete-scute: a proneural gene in anterior regions of the vertebrate CNS. Mech Dev **40**(1-2)**:**25-36.

43. **Franco, P. G., A. R. Paganelli, S. L. Lopez, and A. E. Carrasco.** 1999. Functional association of retinoic acid and hedgehog signaling in Xenopus primary neurogenesis. Development **126**(19)**:**4257-4265.

44. **Giguere, V., E. S. Ong, P. Segui, and R. M. Evans.** 1987. Identification of a receptor for the morphogen retinoic acid. Nature **330:**624-629.

45. **Gomez-Skarmeta, J. L., A. Glavic, E. de la Calle-Mustienes, J. Modolell, and R. Mayor.** 1998. Xiro, a Xenopus homolog of the Drosophila Iroquois complex genes, controls development at the neural plate. Embo J **17**(1)**:**181-90.

46. **Hardcastle, Z., A. D. Chalmers, and N. Papalopulu.** 2000. FGF-8 stimulates neuronal differentiation through FGFR-4a and interferes with mesoderm induction in Xenopus embryos. Curr Biol **10**(23)**:**1511-4.

47. **Harland, R.** 2000. Neural induction. Curr Opin Genet Dev **10**(4)**:**357-62.

48. **Hartenstein, V.** 1989. Early neurogenesis in Xenopus: the spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. Neuron **3**(4)**:**399-411.

49. **Hawley, S. H., K. Wunnenberg-Stapleton, C. Hashimoto, M. N. Laurent, T. Watabe, B. Blumberg, and K. W. Y. Cho.** 1995. Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction. Genes Dev. **9:**2923-2935.

50. **Helms, J., C. Thaller, and G. Eichele.** 1994. Relationship between retinoic acid and sonic hedgehog, two polarizing signals in the chick wing bud. Development **120:**3267-3274.

51. **Helms, J. A., C. H. Kim, D. Hu, R. Minkoff, C. Thaller, and G. Eichele.** 1997. Sonic hedgehog participates in craniofacial morphogenesis and is down- regulated by teratogenic doses of retinoic acid. Dev Biol **187**(1)**:**25-35.

52. **Hemmati-Brivanlou, A., O. G. Kelly, and D. A. Melton.** 1994. Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell **77:**283-295.

53. **Hemmati-Brivanlou, A., and D. A. Melton.** 1994. Inhibition of activin receptor signaling promotes neuralization in Xenopus. Cell **77:**273-281.

54. **Heyman, R. A., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller.** 1992. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell **68:**397-406.

55. **Hollemann, T., Y. Chen, H. Grunz, and T. Pieler.** 1998. Regionalized metabolic activity establishes boundaries of retinoic acid signalling. Embo J **17**(24)**:**7361-72.

56. **Holley, S. A., P. D. Jackson, Y. Sasai, B. Lu, E. M. De Robertis, F. M. Hoffmann, and E. L. Ferguson.** 1995. A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin [see comments]. Nature **376**(6537)**:**249-53.

57. **Houle, M., P. Prinos, A. Iulianella, N. Bouchard, and D. Lohnes.** 2000. Retinoic acid regulation of Cdx1: an indirect mechanism for retinoids and vertebral specification. Mol Cell Biol **20**(17)**:**6579-86.

58. **Isaacs, H. V., M. E. Pownall, and J. M. Slack.** 1998. Regulation of Hox gene expression and posterior development by the Xenopus caudal homologue Xcad3. Embo J **17**(12)**:**3413-27.

59. **Joyner, A. L.** 1996. Engrailed, Wnt and Pax genes regulate midbrain-hindbrain development. Trends Genet. **12:**15-20.

60. **Kastner, P., M. Mark, and P. Chambon.** 1995. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life. Cell **83:**859-869.

61. **Katsuyama, Y., T. Koide, J. Song, and B. Blumberg.** 2003. Multiple points of interaction between RAR and FGF signaling in vertebrate development. submitted for publication.

62. **Katsuyama, Y., and H. Saiga.** 1998. Retinoic acid affects patterning along the anterior-posterior axis of the ascidian embryo. Dev Growth Differ **40**(4)**:**413-22.

63. **Keidel, S., P. LeMotte, and C. Apfel.** 1994. Different agonist- and antagonist-induced conformational changes in retinoic acid receptors analyzed by protease mapping. Mol. Cell. Biol. **14:**287-298.

64. **Kengaku, M., and H. Okamoto.** 1995. bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in Xenopus. Development **121:**3121-3130.

65. **Kim, C. H., T. Oda, M. Itoh, D. Jiang, K. B. Artinger, S. C. Chandrasekharappa, W. Driever, and A. B. Chitnis.** 2000. Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. Nature **407**(6806)**:**913-6.

66. **Kitareewan, S., L. T. Burka, K. B. Tomer, C. E. Parker, L. J. Deterding, R. D. Stevens, B. M. Forman, D. E. Mais, R. A. Heyman, T. McMorris, and C. Weinberger.** 1996. Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR. Mol Biol Cell **7**(8)**:**1153-66.

67. **Koide, T., M. Downes, R. A. Chandraratna, B. Blumberg, and K. Umesono.** 2001. Active repression of RAR signaling is required for head formation. Genes Dev **15**(16)**:**2111-21.

68. **Kolm, P. J., V. Apekin, and H. Sive.** 1997. Xenopus hindbrain patterning requires retinoid signaling. Dev. Biol. **192:**1-16.

69. **Krauss, S., J. P. Concordet, and P. W. Ingham.** 1993. A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell **75**(7)**:**1431-44.

70. **Krezel, W., V. Dupe, M. Mark, A. Dierich, P. Kastner, and P. Chambon.** 1996. RXRg null mice are apparently normal and compound RXRa+/-/RXRb-/-/RXRg-/- mutant mice are viable. Proc. Natl. Acad. Sci. (USA) **93:**9010-9014.

71. **Kroll, K. L., and E. Amaya.** 1996. Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development **122:**3173-3183.

72. **Lamb, T. M., and R. M. Harland.** 1995. Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. Development **121**(11)**:**3627-36.

73. **Lamb, T. M., A. K. Knecht, W. C. Smith, S. E. Stachel, A. N. Economides, N. Stahl, G. D. Yancopolous, and R. M. Harland.** 1993. Neural induction by the secreted polypeptide noggin [see comments]. Science **262**(5134)**:**713-8.

74. **Lee, J., K. A. Platt, P. Censullo, and A. Ruiz i Altaba.** 1997. Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. Development **124**(13)**:**2537-52.

75. **Lee, J. E.** 1997. Basic helix-loop-helix genes in neural development. Curr Opin Neurobiol **7**(1)**:**13-20.

76. **Lee, J. J., D. P. von Kessler, S. Parks, and P. A. Beachy.** 1992. Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene hedgehog. Cell **71**(1)**:**33-50.

77. **Lekven, A. C., C. P. Thorpe, J. S. Waxman, and R. T. Moon.** 2001. Zebrafish Wnt8 encodes two Wnt8 proteins on a bicistronic transcript and is required for mesoderm and neuroectoderm patterning. Dev Cell **1:**103-114.

78. **Lemotte, P. K., S. Keidel, and C. M. Apfel.** 1996. Phytanic acid is a retinoid X receptor ligand. Eur J Biochem **236**(1)**:**328-33.

79. **Levin, A. A., L. J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzeisen, J. Rosenberger, A. Lovey, and J. F. Grippo.** 1992.

9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXRa. Nature **355:**359-361.

80. **Lopez, S. L., and A. E. Carrasco.** 1992. Retinoic acid induces changes in the localization of homeobox proteins in the antero-posterior axis of Xenopus laevis embryos. Mech Dev **36**(3)**:**153-64.
81. **Lopez, S. L., R. Dono, R. Zeller, and A. E. Carrasco.** 1995. Differential effects of retinoic acid and a retinoid antagonist on the spatial distribution of the homeoprotein Hoxb-7 in vertebrate embryos. Dev Dyn **204**(4)**:**457-71.
82. **Lumsden, A., and R. Krumlauf.** 1996. Patterning the vertebrate neuraxis. Science **274:**1109-1115.
83. **Ma, Q., C. Kintner, and D. J. Anderson.** 1996. Identification of neurogenin, a vertebrate neuronal determination gene. Cell **87**(1)**:**43-52.
84. **Maden, M., E. Gale, I. Kostetskii, and M. Zile.** 1996. Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. Curr Biol **6**(4)**:**417-26.
85. **Maden, M., A. Graham, E. Gale, C. Rollinson, and M. Zile.** 1997. Positional apoptosis during vertebrate CNS development in the absence of endogenous retinoids. Development **124**(14)**:**2799-805.
86. **Maden, M., and N. Holder.** 1992. Retinoic acid and the development of the central nervous system. BioEssays **14:**431-438.
87. **Marigo, V., and C. J. Tabin.** 1996. Regulation of patched by sonic hedgehog in the developing neural tube. Proc Natl Acad Sci U S A **93**(18)**:**9346-51.
88. **Marine, J. C., E. J. Bellefroid, H. Pendeville, J. A. Martial, and T. Pieler.** 1997. A role for Xenopus Gli-type zinc finger proteins in the early embryonic patterning of mesoderm and neuroectoderm. Mech Dev **63**(2)**:**211-25.
89. **McGrew, L. L., S. Hoppler, and R. T. Moon.** 1997. Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in Xenopus. Mech Dev **69**(1-2)**:**105-14.
90. **McGrew, L. L., C. J. Lai, and R. T. Moon.** 1995. Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. Dev. Biol. **172:**337-342.

91. **Mumm, J. S., and R. Kopan.** 2000. Notch signaling: from the outside in. Dev Biol **228**(2)**:**151-65.

92. **Nakata, K., T. Nagai, J. Aruga, and K. Mikoshiba.** 1998. Xenopus Zic family and its role in neural and neural crest development. Mech Dev **75**(1-2)**:**43-51.

93. **Niederreither, K., V. Subbarayan, P. Dolle, and P. Chambon.** 1999. Embryonic retinoic acid synthesis is essential for early mouse post- implantation development [see comments]. Nat Genet **21**(4)**:**444-8.

94. **Nieuwkoop, P. D.** 1952. Activation and organization of the central nervous system in amphibians. Part III. synthesis of a new working hypothesis. J. Exp. Zool. **120:**83-108.

95. **Niswander, L., S. Jeffrey, G. R. Martin, and C. Tickle.** 1994. A positive feedback loop coordinates growth and patterning in the vertebrate limb [see comments]. Nature **371**(6498)**:**609-12.

96. **Paganelli, A. R., O. H. Ocana, M. I. Prat, P. G. Franco, S. L. Lopez, L. Morelli, A. M. Adamo, M. M. Riccomagno, E. Matsubara, M. Shoji, J. L. Affranchino, E. M. Castano, and A. E. Carrasco.** 2001. The Alzheimer-related gene presenilin-1 facilitates sonic hedgehog expression in Xenopus primary neurogenesis. Mech Dev **107**(1-2)**:**119-31.

97. **Papalopulu, N., and C. Kintner.** 1996. A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in Xenopus neuroectoderm. Development **122**(11)**:**3409-18.

98. **Pepinsky, R. B., C. Zeng, D. Wen, P. Rayhorn, D. P. Baker, K. P. Williams, S. A. Bixler, C. M. Ambrose, E. A. Garber, K. Miatkowski, F. R. Taylor, E. A. Wang, and A. Galdes.** 1998. Identification of a palmitic acid-modified form of human Sonic hedgehog. J Biol Chem **273**(22)**:**14037-45.

99. **Petkovich, M., N. J. Brand, A. Krust, and P. Chambon.** 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature **330:**444-450.

100. **Piccolo, S., Y. Sasai, B. Lu, and E. M. De Robertis.** 1996. Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell **86**(4)**:**589-98.

101. **Porter, J. A., K. E. Young, and P. A. Beachy.** 1996. Cholesterol modification of hedgehog signaling proteins in animal development. Science **274**(5285)**:**255-9.

102. **Pownall, M. E., H. V. Isaacs, and J. M. Slack.** 1998. Two phases of Hox gene regulation during early Xenopus development. Curr Biol **8**(11)**:**673-6.

103. **Pownall, M. E., A. S. Tucker, J. M. W. Slack, and H. V. Isaacs.** 1996. eFGF, Xcad3, and Hox genes form a molecular pathway that establishes the anteroposterior axis in Xenopus. Development **122:**3881-3892.

104. **Prinos, P., S. Joseph, K. Oh, B. I. Meyer, P. Gruss, and D. Lohnes.** 2001. Multiple pathways governing Cdx1 expression during murine development. Dev Biol **239**(2)**:**257-69.

105. **Riddle, R. D., R. L. Johnson, E. Laufer, and C. Tabin.** 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell **75:**1401-1416.

106. **Roessler, E., E. Belloni, K. Gaudenz, P. Jay, P. Berta, S. W. Scherer, L. C. Tsui, and M. Muenke.** 1996. Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. Nat Genet **14**(3)**:**357-60.

107. **Ruiz i Altaba, A.** 1997. Catching a Gli-mpse of Hedgehog. Cell **90**(2)**:**193-6.

108. **Ruiz i Altaba, A., and T. Jessell.** 1991. Retinoic acid modifies mesodermal patterning in early Xenopus embryos. Genes Dev. **5:**175-187.

109. **Salzberg, A., and H. J. Bellen.** 1996. Invertebrate versus vertebrate neurogenesis: variations on the same theme? Dev Genet **18**(1)**:**1-10.

110. **Sasai, Y., B. Lu, H. Steinbeisser, and E. M. De Robertis.** 1995. Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. Nature **376**(6538)**:**333-6.

111. **Sharpe, C., and K. Goldstone.** 2000. The control of Xenopus embryonic primary neurogenesis is mediated by retinoid signalling in the neurectoderm. Mech Dev **91**(1-2)**:**69-80.

112. **Sharpe, C. R., and K. Goldstone.** 1997. Retinoid receptors promote primary neurogenesis in Xenopus. Development **124**(2)**:**515-23.

113. **Sive, H. L., and P. F. Cheng.** 1991. Retinoic acid perturbs the expression of Xhox.lab genes and alters mesodermal determination in Xenopus laevis. Genes Dev. **5:**1321-1332.

114. **Sive, H. L., B. W. Draper, R. Harland, and W. H.** 1990. Identification of retinoic acid-sensitive period during primary axis formation in Xenopus laevis. Genes Dev. **4:**932-942.

115. **Sporn.** 1994. The Retinoids: Biology, Chemistry, and Medicine. Raven, New York.

116. **Stoeckli, E. T., and L. T. Landmesser.** 1998. Axon guidance at choice points. Curr Opin Neurobiol **8**(1)**:**73-9.

117. **Subramanian, V., B. I. Meyer, and P. Gruss.** 1995. Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. Cell **83**(4)**:**641-53.

118. **Sulik, K. K., D. B. Dehart, J. M. Rogers, and N. Chernoff.** 1995. Teratogenicity of low doses of all-trans retinoic acid in presomite mouse embryos. Teratology **51**(6)**:**398-403.

119. **Tanabe, Y., and T. M. Jessell.** 1996. Diversity and pattern in the developing spinal cord. Science **274:**1115-1123.

120. **Tanabe, Y., H. Roelink, and T. M. Jessell.** 1995. Induction of motor neurons by Sonic hedgehog is independent of floor plate differentiation. Curr Biol **5**(6)**:**651-8.

121. **Torok, M. A., D. M. Gardiner, J. C. Izpisua-Belmonte, and S. V. Bryant.** 1999. Sonic hedgehog (shh) expression in developing and regenerating axolotl limbs. J Exp Zool **284**(2)**:**197-206.

122. **van der Wees, J., J. G. Schilthuis, C. H. Koster, H. Diesveld-Schipper, G. E. Folkers, P. T. van der Saag, M. I. Dawson, K. Shudo, B. van der Burg, and A. J. Durston.** 1998. Inhibition of retinoic acid receptor-mediated signalling alters positional identity in the developing hindbrain. Development **125**(3)**:**545-56.

123. **Wettstein, D. A., D. L. Turner, and C. Kintner.** 1997. The Xenopus homolog of Drosophila Suppressor of Hairless mediates Notch signaling during primary neurogenesis. Development **124**(3)**:**693-702.

124. **White, J. C., V. N. Shankar, M. Highland, M. L. Epstein, H. F. DeLuca, and M. Clagett-Dame.** 1998. Defects in embryonic hindbrain development and fetal resorption resulting from vitamin A deficiency in the rat are prevented by feeding pharmacological levels of all-trans-retinoic acid. Proc Natl Acad Sci U S A **95**(23)**:**13459-64.

125. **Wilson, P. A., and A. Hemmati-Brivanlou.** 1995. Induction of epidermis and inhibition of neural fate by Bmp-4. Nature **376:**331-333.

126. **Xu, R. H., J. Kim, M. Taira, S. Zhan, D. Sredni, and H. F. Kung.** 1995. A dominant negative bone morphogenetic protein 4 receptor causes neuralization in Xenopus ectoderm. Biochem Biophys Res Commun **212**(1)**:**212-9.

127. **Yamada, T.** 1994. Caudalization by the amphibian organizer: brachyury, convergent extension and retinoic acid. Development **120:**3051-3062.

128. **Zimmerman, K., J. Shih, J. Bars, A. Collazo, and D. J. Anderson.** 1993. XASH-3, a novel Xenopus achaete-scute homolog, provides an early marker of planar neural induction and position along the mediolateral axis of the neural plate. Development **119:**221-232.

129. **Zimmerman, L. B., J. M. De Jesus-Escobar, and R. M. Harland.** 1996. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell **86**(4)**:**599-606.

Figure Legends

Figure 1 RAR - signaling and A/P patterning

A schematic depiction showing the relationship between RAR and FGF signaling and the effects on downstream genes. RAR and FGF signaling are each required for expression of the other pathway's components. Retinoid signaling is required both upstream and downstream of Xcad3 signaling in posteriorization of the CNS.

Figure 2 - Retinoid signaling and neuronal differentiation

We propose that retinoid signaling through RARs is required early in development to inhibit shh expression, favoring neuronal differentiation over neuronal proliferation. Inhibitory actions of gene products are indicated by (-), whereas positive effects are indicated by (+).

Figure 1 – RAR signaling and A/P patterning.

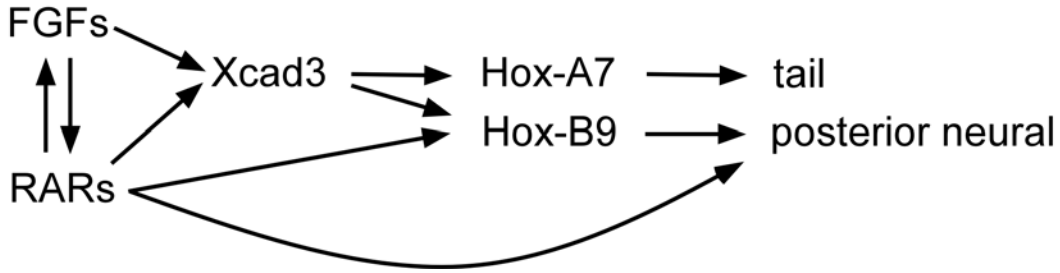

Figure 2 - Retinoid signaling and neuronal differentiation