New insights into the roles of retinoic acid signaling in nervous system development and the establishment of neurotransmitter systems

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Abstract

Secreted chiefly from the underlying mesoderm, the morphogen retinoic acid (RA) is well known to contribute to the specification, patterning and differentiation of neural progenitors in the developing vertebrate nervous system. Furthermore, RA has been shown to influence the subtype identity and neurotransmitter phenotype of subsets of maturing neurons, although relatively little is known about how these functions are mediated. This review provides a comprehensive overview of the roles played by RA signaling during the formation of the central and peripheral nervous systems of vertebrates and highlights its effects on the differentiation of several neurotransmitter systems. In addition, the evolutionary history of the RA signaling system is discussed, revealing both conserved properties and alternate modes of RA action. In conclusion, we propose that comparative approaches should be employed systematically to expand our knowledge of the context-dependent cellular mechanisms controlled by the multifunctional signaling molecule RA.

Key words: Comparative neurobiology, development, evolution, neurotransmitter, nervous system, neural crest, placodes, retinoic acid

1. Introduction

Pondering the general purpose of the nervous system, Sherrington wrote: *“The nervous system is in a sense the highest expression of that which French psychologists term the milieu interne. With the transition from the unicellular organism to the multicellular a new element enters general physiology. The phenomena of general physiology in the unicellular organism can be divided into two great groups; namely, those occurring within the cell, intracellular, and those occurring at the surface of the cell, in which forces that are associated with surfaces of separation have opportunity for play at the boundary between the organism and its environment. But in the multicellular organism a third great group of phenomena exists in addition; namely, those which are intercellular, occurring in that complex material which the organism deposits in quantity in the intercellular interstices of its mass as a connecting medium between its individual living units”* (Sherrington, 1906).

Thus, the nervous system can be understood as a means of internal interconnection that enables multicellular animals to coordinate their different physiological activities and interact with their environment. Depending on the morphology of an organism and the tasks it needs to perform throughout its life history, more or less specialized connections are required to receive, process and distribute information in a targeted manner.

Accordingly, animals have evolved a wide range of different neural cell types and networks (Arendt et al., 2016; Hartenstein and Stollewerk, 2015).

Wolpert aptly described metazoan evolution as “the brilliant result of altering developmental programs” (Wolpert, 1992). In order to form different characters, embryos largely rely on instructive signals that differentially modulate target gene transcription in specific cellular subsets, thus inducing them to commit to different developmental fates

and to ultimately assume different properties and functions. While the number of cell types varies considerably between different animals, ranging from a maximum of about 16 in sponges to approximately 244 in vertebrates (Niklas et al., 2014), only a surprisingly limited number of well-conserved signaling pathways appears to be required for generating and organizing this cellular diversity. The focus of this review is on one of these pathways, retinoic acid (RA) signaling, and its roles during the formation of the vertebrate nervous system. First, an overview is provided of the general characteristics of developmental signaling cascades, the importance of retinoids, the metabolism of RA as well as its different signaling mechanisms and major developmental functions. Next, the actions of RA signaling during the regionalization and patterning of the neural plate and neural tube, during neural progenitor proliferation, differentiation and survival as well as during neural crest and cranial placode development are explored in detail. Special attention is given to the still poorly understood influence of RA signaling on the neurotransmitter identity of specific neuronal subsets, including catecholaminergic, serotonergic, glutamatergic and GABAergic neurons. In addition, the evolutionary background of RA signaling is discussed, which can provide interesting new insights into the conserved properties of this multifunctional morphogen, but still remains elusive outside of the chordate phylum.

2. Developmental Signaling

A common feature of intercellular signaling pathways is that they can alter cell physiology and development in disparate ways, depending on a spatial and temporal context. This flexibility is generated by several mechanisms that often act in combination, in order to determine the specificity of cellular responses. One important element is cell competence, which is acquired during the specific developmental history of a cell and is

based on differences in its molecular make-up at the epigenetic, transcriptional and/or translational level, e.g. chromatin compaction, expression of transcriptional regulators, presence of signaling pathway components and other interacting factors (Wang et al., 2015). These differences affect the capability of cells to interpret developmental signals and can be established as early as at the first cleavage, when maternal factors become unevenly distributed between the initial two blastomeres (Weitzel, 2004; Heasman, 2006; Kumano and Nishida, 2009; Peng and Wikramanayake, 2013; Wang et al., 2015). Another way to achieve cell type-specific responses is through the variation of signal intensity, for instance by producing a concentration gradient of signaling molecules (morphogens) (Kicheva et al., 2012). This process often involves the secretion of a morphogen from a localized source, followed by its non-directional spread throughout a tissue and its degradation (Kicheva et al., 2012). However, morphogen gradients can also be formed through lineage or directional transport of morphogens (Kicheva et al., 2012). Lastly, different signaling pathways do not function as isolated entities, but are integrated into highly complex molecular frameworks that enable contextual responses (Guruharsha et al., 2012; Attisano and Wrana, 2013). During the course of development, the spatial pattern of cell identities and behaviors is thus dynamically determined through the crosstalk between variable levels of morphogen signals and depends recursively on cellular competence.

Classical nuclear receptor (NR) pathways, function through ligand-induced transcription factors that regulate the expression of specific target genes in response to endocrine signals, such as vitamin D3, sex steroids (e.g. progestins, estrogens, androgens), adrenal steroids (glucocorticoids, mineralocorticoids), thyroid and retinoid hormones (McKenna et al., 1999). NRs contain a highly conserved zinc finger-based DNA binding

domain that allows them to associate with specific regulatory sites in the genome, i.e. enhancers or silencers (McKenna et al., 1999; Escriva et al., 2000; Kininis and Kraus, 2007). At their target promoters, NRs directly control transcriptional activity in a ligand-dependent manner, through both the regulation of RNA polymerase binding and chromatin remodeling, which in turn modulates gene expression via epigenetic changes (McKenna et al., 1999; Kininis and Kraus, 2007; Evans and Mangelsdorf, 2014). NR function generally involves the recruitment or release of co-regulators, with co-activators mediating the potentiating functions of activated NRs and co-repressors transducing the attenuating functions of non-activated NRs (McKenna and O'Malley, 2002). Due to post-translational modification and selective recruitment of co-regulators, NR dimerization as well as complex interactions between NRs, co-regulators and other transcription factors, NRs are able to govern a broad array of finely tuned transcriptional responses (McKenna and O'Malley, 2002; Kininis and Kraus, 2007; Evans and Mangelsdorf, 2014). Accordingly, these signaling pathways play very diverse roles in the regulation of physiological, developmental and metabolic processes (McKenna et al., 1999; McKenna and O'Malley, 2002; Kininis and Kraus, 2007).

3. Retinoids as Morphogens, Teratogens and Drugs

Retinoids are biologically active derivatives of vitamin A (retinol) that have great impact on vertebrate development and homeostasis (Dollé and Niederreither, 2015). A disruption of physiological retinoid levels during pregnancy, for instance, can cause abnormal morphological development: an excess of vitamin A can lead to exencephaly, cleft palate, spina bifida, eye defects, hydrocephaly as well as shortening of the mandible and maxilla, whereas vitamin A deficiency has been correlated with malformations in the

eye, urogenital tract, diaphragm, heart and lung (Collins and Mao, 1999). Certain retinoids, such as 13-*cis*-retinoic acid (RA) (isotretinoin, Accutane®), are well known to act as teratogens in both animals and humans (Collins and Mao, 1999). Therefore, a great environmental threat is posed by cyanobacteria blooms, which can produce significant levels of teratogenic retinoids, contaminating sources of food and drinking water (Jonas et al., 2014; Wu et al., 2012). Moreover, glyphosate-based herbicides have been shown to increase the physiological activity of retinoid signaling pathways and are thus considered responsible for the higher incidence of embryonic malformations and spontaneous abortions in populations exposed to pesticides (López et al., 2012). Nonetheless, retinoids, such as 13-*cis*-RA, are also being used for numerous pharmacological applications, including the treatment of skin diseases and various forms of cancer (Camacho, 2003; Collins and Mao, 1999; Dollé and Niederreither, 2015). In addition, their potential for attenuating neurological diseases is currently under investigation (Dollé and Niederreither, 2015; Endres et al., 2014; Lerner et al., 2012; Mizze et al., 2014). Taken together, retinoids are important as teratogens but also for medical applications and it is therefore crucial to improve our understanding of retinoid signaling functions.

4. The Retinoic Acid Signaling Pathway

The structurally simple and diffusible molecule RA (Figure 1) is well known to act as a crucial regulator of gene expression during vertebrate embryogenesis (Blomhoff, 1994; De Luca, 1991; Sporn et al., 1994). Different stereoisomeric forms of RA can be found *in vivo*, including all-*trans*, 9-*cis* and 13-*cis* RA. While it has been established that in vertebrates all-*trans* RA is the physiologically active form, possible biological functions of other RA isomers are still being debated (Campo-Paysaa et al., 2008; Mic et al., 2003).

Endogenous RA synthesis requires the cellular uptake of retinol, which is tightly regulated. Due to its lipophilic nature, virtually all retinol present in the blood stream is bound to serum retinol binding proteins (RBPs) (Goodman, 1984). The transmembrane transporter STRA6 (stimulated by retinoic acid 6) functions as RBP receptor and mediates the bidirectional flux of retinol between extracellular RBPs and intracellular retinol binding proteins (CRBPs) (D'Ambrosio et al., 2011; Kawaguchi et al., 2012, 2007). The activity of STRA6 depends on both extra- and intracellular retinoid levels (D'Ambrosio et al., 2011; Kawaguchi et al., 2012; Sun, 2012). In addition, retinol uptake via STRA6 activity can be enhanced through coupling of LRATs (lecithin retinol acyltransferases), which are important enzymes for the metabolism of retinol in the visual system and in storage tissues (Golczak et al., 2008; Isken et al., 2008; Kawaguchi et al., 2007). Moreover, RBP can form a complex with transthyretin (TTR), a thyroid hormone distributor protein (D'Ambrosio et al., 2011; Richardson, 2009; Yamauchi and Ishihara, 2009) that blocks the ability of RBP to associate with STRA6, thus suppressing STRA6-mediated retinol uptake (Berry et al., 2012). Inside the cell, retinol is bound to CRBPs, since it is otherwise chemically unstable and has a low solubility in aqueous media (Newcomer, 1995). Four types of CRBPs (CRBP I, II, III, and IV) with distinct tissue distributions have been structurally characterized that bind either retinol or retinal and play an important part in the absorption, transport, metabolism and excretion of these retinoids (Conforti et al., 2000; Mezaki et al., 2012; Napoli, 1993; Noy, 2000).

The conversion of cellular retinol into RA begins with the reversible oxidation of retinol to retinal (retinaldehyde). This reaction is catalyzed either by cytosolic alcohol dehydrogenases (ADHs) or by short-chain dehydrogenases/reductases (SDRs) (Parés et al.,

2008). Subsequently, retinaldehyde dehydrogenases (RALDHs) irreversibly transform retinal into RA (Parés et al., 2008). In vertebrates, four RALDHs with distinct tissue-specific expression profiles are known to contribute to endogenous RA production: RALDH 1, 2 and 3 are class 1 aldehyde dehydrogenases (ALDH1s), whereas RALDH4 is a class 8 ALDH (Theodosiou et al., 2010). Aside from RALDHs, cytochrome p450 mono-oxygenases such as CYP1B1 can also oxidize retinol or retinal to RA (Chambers et al., 2007; Collins and Mao, 1999). As for retinol and retinal, RA is bound by specific cellular retinoic acid binding proteins, known as CRABP-I and CRABP-II, which are crucial for the intracellular balance between RA synthesis, degradation and transportation to RA receptors within the nucleus (Cai et al., 2012; Carvalho and Schubert, 2013; Dong et al., 1999; Napoli, 1999; Noy, 2000). CRABP-I is mainly involved in RA catabolism and, through interaction with RA degrading enzymes, even regulates cytoplasmatic levels of various types of RA-metabolites (Boylan and Gudas, 1992). In contrast, CRABP-II appears to be more directly involved in the process of RA signaling. Binding of RA induces CRABP-II to dissociate from the endoplasmic reticulum and undergo nuclear translocation, which ultimately leads to the formation of a complex between CRABP-II and the nuclear receptor of RA, through which RA is transferred from the binding protein to the receptor (Majumdar et al., 2011; Schug et al., 2007).

Due to the great importance of RA as a morphogen, physiological retinoid levels must be tightly controlled and buffered, for instance, against the variable bioavailability of retinol. This is achieved through a complex system of proteins and enzymes that bind, re-distribute, synthesize and degrade RA and its precursors (Dollé and Niederreither, 2015). The activity of most of these molecules directly depends on present retinoid concentrations and is regulated via multiple positive and negative feedback loops (Dollé

and Niederreither, 2015). For example, RA can repress its synthesizing enzymes and promote its degrading enzymes (Ross and Zolfaghari, 2011; Dobbsmcauliffe, 2004; Wang et al., 2002). For its removal from the cell, RA is oxidized by specific cytochrome P450 enzymes, the CYP26s (White et al., 1997). In mammals, the three CYP26s, namely CYP26A1, CYP26B1 and CYP26C1, can degrade RA into 4-oxo-RA, 4-hydroxy-RA, 5,6-epoxy-RA, 5,8-epoxy-RA and probably other polar metabolites (White and Schilling, 2008). In human fetal liver, RA can also be hydroxylated by CYP3A7, which might be crucial in the embryo for preventing RA-induced toxicity (Chen et al., 2000).

In general, RA signals are transduced by heterodimers of two nuclear hormone receptors, the RA receptor (RAR) and the retinoid X receptor (RXR). These RAR/RXR heterodimers bind directly to specific DNA motifs, called retinoic acid response elements (RARE) (Chambon, 1996). Typically, a RARE consists of two direct repeats that have the consensus nucleotide sequence (A/G)G(G/T)(G/T)(G/C)A, but the topology of and spacing between RARE halvesites varies significantly (Balmer and Blomhoff, 2005; Moutier et al., 2012). In the absence of a ligand, the association of RAR/RXR heterodimers with RAREs leads to the recruitment of co-repressor complexes that mediate chromatin compaction and target gene repression (Vilhais-Neto and Pourquié, 2008). However, the ability of the heterodimers to interact with the regulatory regions of certain target genes can also be dependent on ligand binding (Gutierrez-Mazariegos et al., 2014b; Moutier et al., 2012). Fixation of RA by RAR activates the ligand-dependent transcription factor function of the RAR/RXR heterodimer through a conformational change of RAR that causes the dissociation of the co-repressor complex and the recruitment of a co-activator complex, which ultimately induces target gene transcription (Gronemeyer et al., 2004; Rhinn and

Dollé, 2012). While the molecular mechanisms of RAR function have been studied extensively (Rochette-Egly, 2015; Samarut and Rochette-Egly, 2012; Urvalek and Gudas, 2014; Wei, 2015), the versatile modes of RXR action are less well understood (Ahuja et al., 2003; Evans and Mangelsdorf, 2014; Germain et al., 2002; Lefebvre et al., 2010). In mammals, three RARs (α , β and γ) and three RXRs (α , β and γ) have been identified, which show diverse expression patterns as well as divergent functions and are believed to associate into more than 30 different RAR/RXR heterodimers that transduce signals in the presence of all-*trans* RA (Gutierrez-Mazariegos et al., 2014b; Mark et al., 2009; Samarut and Rochette-Egly, 2012; Urvalek et al., 2014). The highly gene-specific transcriptional responses to RAR-mediated RA signals are further based upon a plethora of co-regulators as well as on the placement or removal of epigenetic marks on histones or on DNA (Gudas, 2013; Urvalek and Gudas, 2014; Wei, 2015).

Furthermore, RA can activate RAR/RXR heterodimers independently of DNA binding, as part of non-genomic signaling cascades, or bind and activate a number of other nuclear receptors, such as testicular receptor 4 (TR4 or NR2C2), chicken ovalbumin upstream promoter transcription factor 2 (COUP-TFII or NR2F2) or RAR-related orphan receptor β (ROR β or NR1F2) (Carvalho and Schubert, 2013). Apart from CRABP, intracellular RA can also be bound by fatty acid-binding protein type 5 (FABP5), which shuttles it to another nuclear receptor, peroxisome proliferator-activated receptor β/δ (PPAR β/δ), that transduces a different set of RA signaling functions (Berry and Noy, 2009; Schug et al., 2007). For instance, it was shown that in mice RA-induced neuronal differentiation is first mediated through RAR and subsequently through PPAR β/δ activity and that this switch in RA signaling is accomplished through changes in the CRABP-II/FABP5 protein ratio (Yu et

al., 2012). In addition, RA plays an important role in the transcription-independent activation of the PI3K/Akt (phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homolog) signaling pathway, which is involved in cell cycle regulation (García-Regalado et al., 2013; Lee et al., 2014; S. Zhang et al., 2015). RA can also convey developmental signals by modulating the activity of various types of other proteins, such as protein kinase C α , cAMP response element-binding protein, mitogen-activated protein kinase 1/2, extracellular-signal-regulated kinases 1/2 or ribosomal s6 kinase (Carvalho and Schubert, 2013). The interesting result can be a complex cross talk between the genomic and non-genomic effects of RA. For example, by RARs present in cell membrane lipid rafts RA induces kinase cascades, which phosphorylate several targets in the nucleus, including RARs and their co-regulators that thus induce the transcription of specific sets of target genes (Carvalho and Schubert, 2013; Rochette-Egly, 2015). Taken together, RA signals exert their biological functions through multiple interacting molecular cascades that can produce finely tuned transcriptional responses in a cell type-specific manner.

5. Major Functions of Retinoic Acid Signaling

In vertebrates, RA signaling is involved in a great variety of biological processes, during embryogenesis (Figure 2) as well as in the adult. For example, RA signaling functions are required for initiating and driving meiosis, which leads to the generation of haploid germ cells (Griswold et al., 2012; Koubova et al., 2014; Yu et al., 2013). In addition, RA signals have been implicated in germ cell maturation, survival and oocyte competence (Best et al., 2015; Endo et al., 2015; Ikami et al., 2015; Paik et al., 2014; Pauli et al., 2013). However, after fertilization, exposure of the developing embryo to exogenous RA is known to cause various teratogenic effects (Collins and Mao, 1999). Receptors for RA are

maternally expressed and it has been shown that the relative proportions of RAR and RXR subtype transcripts change dramatically during the first 24h of zebrafish development, coinciding with the transition from maternal to zygotic mRNAs (Blumberg et al., 1992; Oliveira et al., 2013). In response to treatments with exogenous RA, this early RAR/RXR signaling system was further found to up-regulate enzymes for the degradation and down-regulate enzymes for the synthesis of RA, suggesting a role in protecting the embryo against RA teratology (Oliveira et al., 2013). Nevertheless, exogenous RA still triggers an advancement of the zebrafish developmental program by precociously activating genes that normally remain silent until later developmental stages and by concomitantly inhibiting genes associated with earlier developmental stages (Oliveira et al., 2013).

Under normal conditions, RA synthesis commences at gastrula stages in trunk paraxial mesoderm and lateral plate mesoderm (Dobbs-McAuliffe et al., 2004; Duester, 2008; Niederreither et al., 1999; Swindell et al., 1999) and creates a linear two-tailed source–sink gradient of the morphogen, which is restricted anteriorly and posteriorly by the activity of RA degrading enzymes (Shimozono et al., 2013). These variable concentrations of RA are essential for axial patterning of embryonic tissues. The neural plate, for instance, is initially anterior-like and increasing levels of RA signaling are required in order to specify the more posterior domains of the hindbrain and spinal chord (Blumberg et al., 1997; Cai et al., 2012; Dupé and Lumsden, 2001; Grandel et al., 2002; Lara-Ramírez et al., 2013). Similarly, graded RA signals provide inductive positional cues in the foregut endoderm and trunk mesoderm that contribute to the correct specification and development of their derivatives, including the posterior pharyngeal arches (Kopinke et al., 2006), lung (Chen et al., 2010), pancreas (Huang et al., 2014; Zhang et al., 2013), somites

(Cunningham et al., 2015; Moreno and Kintner, 2004; Retnoaji et al., 2014), kidney (Le Bouffant et al., 2012; Takayama et al., 2014), heart (Zaffran et al., 2014; Zaffran and Niederreither, 2015) and limbs (Cunningham and Duester, 2015; Monaghan and Maden, 2012). RA signaling has also been implicated in skeletogenesis and liver morphogenesis, but its precise roles are still poorly understood (Green et al., 2016; Huang et al., 2009; Ijpenberg et al., 2007; Spoorendonk et al., 2008). Furthermore, RA signaling controls dorso-ventral patterning of the neural tube (Pera et al., 2014; Wilson et al., 2004), heart and visceral organ laterality, left-right symmetry during somite formation (Samarut et al., 2015; Vermot and Pourquié, 2005) and the termination of body axis extension (Denans et al., 2015; Lara-Ramírez et al., 2013).

Apart from specifying patterns of cellular identities, RA signals are also important mediators of cell cycle behavior and can, for instance, determine the precise timing of stem cell differentiation by generating heritable epigenetic changes in gene expression, which modulate the ability of stem cells to respond to both retinoid and other signaling pathways (Gudas, 2013; Ikami et al., 2015; Paschaki et al., 2013a; Rochette-Egly, 2015; Urvalek and Gudas, 2014). Moreover, RA has been shown to have context dependent pleiotropic effects on cell survival (Schug et al., 2007). These functions are not only crucial for coordinating embryogenesis, but also for the regeneration and maintenance of adult tissues, during which RA signals further contribute to cell positional memory, via combinatorial Hox gene expression (Blum and Begemann, 2015; Gudas, 2012; Ikami et al., 2015; Lepp and Carlone, 2015; Monaghan and Maden, 2012; Paschaki et al., 2013a). Accordingly, RA signaling fulfills several key roles in the adult, such as epithelial and immune homeostasis (Chanchevalap et al., 2004; Choi and Fuchs, 1990; Raverdeau and Mills, 2014; Sun et al., 2007), adult

neurogenesis (Bonnet et al., 2008; Jacobs et al., 2006; Paschaki et al., 2013a) and spermatogenesis (Ikami et al., 2015).

6. Retinoic Acid Signaling during Nervous System Development

During vertebrate nervous system development, the RA signaling pathway is involved in the regulation of a multitude of different biological processes including rostro-caudal regionalization (Das et al., 2014; Paschaki et al., 2013b), neural crest induction (Knecht and Bronner-Fraser, 2002; Villanueva et al., 2002), the specification of neural progenitors and the coordination of neuronal maturation (Janesick et al., 2015). In addition, there is ample evidence that RA signals are important for the differentiation of certain neurotransmitter phenotypes, such as dopaminergic cells (Jeong et al., 2006; Samad et al., 1997; Valdenaire et al., 1998), norepinephrinergic cells (Dupin and Le Douarin, 1995a; Holzschuh et al., 2003; Matsuoka et al., 1997a), cholinergic cells (Berrard et al., 1993; Kim et al., 2001; Nilbratt et al., 2007), serotonergic cells (O'Reilly et al., 2007; Ruiz i Altaba and Jessell, 1991), neuropeptide Y expressing cells (Magni et al., 2000; Mannon and Kaiser, 1997) and GABAergic cells (Chatzi et al., 2011). Moreover, RA signaling has been implicated in synaptic transmission and plasticity, adult neurogenesis and several neuronal disorders (Bonnet et al., 2008; Bremner et al., 2012; Chen et al., 2014; Jacobs et al., 2006; Paschaki et al., 2013a).

6.1 Posteriorizing the Neural Plate

According to the activation-transformation model of Nieuwkoop, neural tissue is first specified as anterior neuroectoderm and requires posteriorizing signals that impose posterior identities in a dose-dependent manner (Nieuwkoop, 1952). First experiments

using exogenous RA treatments as well as injections of constitutively active and dominant negative RARs into African clawed frog embryos have revealed an important role for RA signaling in patterning the neuroectoderm along its anterior-posterior axis (Blumberg et al., 1997; Durston et al., 1989; Sive and Cheng, 1991). Initial RA production, however, depends on the suppression of anterior genes by early signals that already act at late blastula stages, when the neural plate is still forming, such as, for example, different WNT and FGF proteins (Kudoh et al., 2002; Pera et al., 2014).

WNTs are secreted by non-axial mesoderm progenitors at the posterior neural plate (Bang et al., 1999; Green et al., 2014). From there, they diffuse anteriorly, generating a linear caudal-to-rostral WNT gradient that induces dose-dependent gene expression within the neural plate area, which ultimately leads to the segmentation of the forebrain, midbrain and hindbrain primordia (Bang et al., 1999; Green et al., 2014). The major function of WNT signaling during this process is probably to counter TCF3-dependent transcriptional repression, which is essential for vertebrate head formation (Dorsky et al., 2003; Green et al., 2014; Kim et al., 2000). In addition, FGFs are expressed in a similar caudal-to-rostral gradient during neural plate posteriorization and several studies have revealed permissive interactions and interdependences between these two signaling pathways, which probably reflect alternative modes of signal integration that have yet to be elucidated (del Corral and Morales, 2014; Green et al., 2014; Pera et al., 2014).

The RA synthesizing enzyme RALDH2 is first expressed during early gastrula stages in the paraxial mesoderm of various vertebrate embryos, including zebrafish, African clawed frog, chicken and mouse (Grandel et al., 2002). In the caudal epiblast (tail end), high levels of FGFs antagonize RA signaling (Figure 3A) by promoting *cyp26* and attenuating

raldh2 expression (del Corral and Morales, 2014; Green et al., 2014; Pera et al., 2014). This is necessary for maintaining a pool of axial stem cells that later on give rise to the spinal cord and somites (Green et al., 2014; Pera et al., 2014). However, FGFs also stimulate WNT signaling, which promotes *raldh2* expression in the paraxial mesoderm (del Corral and Morales, 2014; Green et al., 2014; Pera et al., 2014). In the rostral ectoderm, *cyp26* continues to be expressed, due to low levels or the complete absence of WNT and FGF signals (Kudoh et al., 2002). This protection of the anterior domain against the posteriorizing activities of RA signals is fundamental for enabling forebrain and midbrain development (Kudoh et al., 2002). Accordingly, the combinatorial effects of FGF and WNT signals are required for suppressing anterior fates and for modulating RA signaling, which has the distinct role of activating the expression of early posterior genes, such as homeodomain proteins (Grandel et al., 2002; Kudoh et al., 2002). For instance, work on zebrafish embryos with a mutated *raldh2* gene suggested that the loss of RA signaling most severely affects the general expansion of the hindbrain as well as *hox* gene expression patterns prior to somitogenesis (Grandel et al., 2002). Therefore, RA signaling acts already on the developing neural plate to establish the gross regionalization of the central nervous system (CNS).

6.2 Setting up the Retinoic Acid Signaling System and the Boundaries of the Neural Tube

Suppression of WNT and FGF signals in the anterior neural plate by various factors secreted from the pharyngeal endomesoderm, e.g. Cerberus, Dickkopf, FRZB and IGF, allows the expression of a cascade of transcription factors, including *otx*, *gbx*, *pax2/5/8* and *en1/2*, which establish the forebrain-midbrain and the midbrain-hindbrain boundaries

(Dworkin and Jane, 2013; Green et al., 2014; Nakamura et al., 2005; Pera et al., 2001). In the developing neural tube, the midbrain-hindbrain boundary acts as an important signaling center, also known as the isthmus organizer, which produces an anterior-high WNT and FGF signaling gradient throughout the midbrain and anterior hindbrain territory that mirrors signaling from the caudal end of the embryo (Dworkin and Jane, 2013; Nakamura et al., 2005; Pera et al., 2001).

At this point in development, RALDH2 is present in the paraxial/somitic mesoderm along the presumptive spinal cord domain, up to and including the first somite (Glover et al., 2006). Accordingly, the source of endogenous RA is located at the caudal limit of the presumptive hindbrain domain (Glover et al., 2006). From here, RA diffuses anteriorly, generating a posterior-high concentration gradient across the hindbrain territory that is patterned by localized CYP26 expression domains and opposed by the anterior-high WNT and FGF gradients emanating from the midbrain-hindbrain boundary (Figure 3B) (Cunningham and Duester, 2015; Glover et al., 2006; Hernandez et al., 2007; Shimozone et al., 2013; White and Schilling, 2008). The resulting interactions of RA, WNT and FGF signaling with CDX, LIM and TGIF transcription factors as well as their modulation of CYP26 activity in this area define the boundary between the presumptive hindbrain and spinal cord domains (Castillo et al., 2010; J. Chang et al., 2016; Cunningham and Duester, 2015; Lara-Ramírez et al., 2013). Among other things, this leads to the down-regulation of *cyp26* expression in the anterior spinal cord territory and to the onset of restricted *raldh2* expression within specific areas of the spinal cord territory, including the roof plate, the ventro-lateral motor neuron domains and, transiently, dorsal interneurons (Castillo et al., 2010; Cunningham and Duester, 2015; Lara-Ramírez et al., 2013; Wilson et al., 2004). As in

the hindbrain, RA signaling in the developing spinal cord is restricted through region-specific CYP26 expression domains and through an opposing posterior-high FGF gradient produced by the tail bud (Cunningham and Duester, 2015; Lara-Ramírez et al., 2013). In summary, a complex source and sink system of RA signals is created in the prospective hindbrain and spinal cord during neurulation that is subsequently required to regulate multiple neurogenic processes.

6.3 Patterning the Neural Tube along the anterior-posterior Axis

6.3.1 Hindbrain and anterior Spinal Cord

As described above, the combinatorial actions of WNTs, FGFs, CYP26 enzymes and other factors restrict RA signaling to somitic levels, where it inhibits both FGF and WNT signaling, in order to drive somitogenesis, neural fate specification and neuronal differentiation (Figure 3B) (del Corral and Morales, 2014; Green et al., 2014; Pera et al., 2014). Diffusion and localized degradation of the lipophilic RA molecule results in a linear two-tailed morphogen gradient, based on which the different cellular identities along the anterior-posterior axis of the developing neural tube are patterned (Blumberg et al., 1997; Cai et al., 2012; Dupé and Lumsden, 2001; Grandel et al., 2002; Lara-Ramírez et al., 2013; Shimozone et al., 2013).

Vertebrate hindbrain development is characterized by the generation of transient, lineage-restricted compartments called rhombomeres that can be identified through their expression of distinct sets of genes as well as through differences in their neuronal organization (Clarke and Lumsden, 1993; Lumsden and Krumlauf, 1996; Vaage, 1969). It has been demonstrated in several animal models, e.g. in lampreys (Murakami, 2004), chicken

(Dupé and Lumsden, 2001) and rats (White et al., 2000), that more posterior rhombomeres require progressively higher amounts of RA for their proper specification. This RA-induced patterning is mainly achieved through the regulation of nested, region specific expression patterns of homeobox genes, which produce a combinatorial “*hox*-code” capable of assigning distinct positional identities (Glover et al., 2006; Kessel and Gruss, 1991).

Typically, vertebrate *hox* genes are organized into chromosomal clusters that are characterized by temporal and spatial co-linearity, which means that a gene located in a more 3' position within its cluster generally becomes activated earlier and has a more anterior expression limit than the gene in the next more 5' position (Duboule and Dollé, 1989; Gaunt et al., 1989; Izpisua-Belmonte et al., 1991). Several *hox* genes are directly regulated by RA signaling through RAREs in their regulatory sequences and, intriguingly, *hox* gene co-linearity appears to be linked to RA signaling, since genes at the 3' end of a *hox* complex respond more rapidly to RA signals than genes positioned in more 5' positions (Alexander et al., 2009; Oosterveen et al., 2003; Simeone et al., 1991, 1990; Zhang et al., 2000). Moreover, 3' *hox* genes tend to become activated in response to RA signals, while 5' *hox* are more likely to be repressed (Liu et al., 2001). These differences might well be associated with the ability of RA signaling to remove repressive modifications from the chromatin domains of the 3' *hox* genes *hox1-hox5* (Mazzoni et al., 2013).

Generally, the developing hindbrain expresses *hox1-hox5* paralogs, while *hox4-hox11* are present in the spinal cord territory (Philippidou and Dasen, 2013). However, this expression is spatially and temporally dynamic, with some *hox* genes being expressed over a narrow time window during early development and others persisting into postnatal stages (Philippidou and Dasen, 2013). Thus, not only the concentration but also the timing

of RA exposure is an important factor in *hox* gene regulation, which further requires interactions with several competing signals. Simply put, FGFs alone promote a midbrain character, FGFs plus WNTs a rostral hindbrain character and RA plus WNTs a caudal hindbrain and anterior spinal cord character (del Corral and Morales, 2014).

Initially, RA signals directly activate *hoxa1* and *hoxb1* expression throughout the hindbrain and towards the midbrain, but induction of *cyp26* by FGF signaling from the anterior midbrain-hindbrain region subsequently limits this expression to the prospective boundary between rhombomeres 4 and 5 (Duester, 2008; Sirbu et al., 2005). FGF8 from the midbrain-hindbrain boundary defines the extension of rhombomere 1 by suppressing *hox* gene expression, while RA signals activate the transcription factor VHN1 in the posterior hindbrain (Alexander et al., 2009; Terriente and Pujades, 2015). FGF3 and FGF8, which are released from the area of the rhombomeres 3 to 5, cooperate with VHN1 to regulate the expression of *krox20* in rhombomere 5 and of *mafb/kreisler* in the rhombomeres 5 and 6 (Alexander et al., 2009; Terriente and Pujades, 2015). The three *cis*-regulatory elements of *krox20* integrate inputs from RA, WNT, FGF, and several transcription factors to specifically induce transcription in the rhombomeres 3 and 5, where *krox20* is needed for repressing *hoxb1* by binding its activator PISxβ (Alexander et al., 2009). Interestingly, this repression also requires RA signaling via a RARE in the *hoxb1* repressor (Studer et al., 1994).

In contrast, levels of *hoxb1* expression in rhombomere 4 are stimulated by HOXA1 as well as by HOXB1 itself, via an upstream HOX response element and in conjunction with the co-factors PBX and MEIS (Moens and Selleri, 2006; Tvrdik and Capecchi, 2006). In order to prevent disruptive fluctuations in RA availability, HOXA1-PBX-MEIS also binds directly to an enhancer that is required for maintaining normal *raldh2* expression levels in the

mesoderm (Vitobello et al., 2011). In addition, CRABPs have been shown to provide a key feedback mechanism, which compensates for changes in RA production and thus contributes to signaling robustness during hindbrain patterning (Cai et al., 2012). In the region of rhombomere 6, *kreisler*-dependent induction of *hoxa3* and *hoxb3* represses *hoxb1* through its HOX response element (Wong et al., 2011). As the somites differentiate, they cease RA production and thus stimulation of *hoxa1* and *hoxb1* expression fades throughout the hindbrain region (Alexander et al., 2009; Glover et al., 2006). Since *hoxa1* does not possess an auto-regulatory element, its expression rapidly declines, whereas HOXB1 maintains its own expression in rhombomere 4 and activates *hoxa2* and *hoxb2* expression, which provide positive feedback to *hoxb1* expression (Studer et al., 1998; Tümpel et al., 2007; Tvrdik and Capecchi, 2006).

Multiple feedback loops and the gradual retreat of *raldh2* expression from anterior maturing somites cause shifting differences in RA, WNT and FGF signaling levels along the developing hindbrain and anterior spinal cord, which are required for the generation of nested *hox* gene expression domains, together with the combinatorial activities of various transcription factors and the direct auto- and cross-regulatory interactions between the *hox* genes themselves (Glover et al., 2006; Sheth et al., 2014; Terriente and Pujades, 2015). Moreover, recent studies on lampreys show that this *hox* regulatory network is largely conserved to the base of vertebrates (Parker et al., 2014).

6.3.2 Spinal Cord

The posterior part of the nervous system has a separate developmental origin than the anterior part and thus the majority of spinal cord progenitors receive different signals

and undergo a different transcriptional program than the cells contributing to the fore-, mid- and hindbrain territories (Gouti et al., 2015). Most of the cells that make up the developing spinal cord originate from a pool of axial stem cells, which is maintained by the anti-differentiation effects of FGF signaling at the caudal epiblast (del Corral and Morales, 2014; Gouti et al., 2015; Pera et al., 2014). These stem cells give rise to neuromesodermal progenitors that express both mesoderm-inducing and neural transcription factors, including Brachyury (BRA), SOX2, SOX3, NKX1-2 and CDX (Gouti et al., 2015). Accordingly, neuromesodermal progenitors can become either presomitic mesoderm precursors, expressing MSGN1 and TBX6, or pre-neural tube cells expressing NKX1-2 and SOX2 (Gouti et al., 2015). However, positive feedback between CDX/BRA and WNT/FGF signaling from the caudal epiblast is thought to maintain their neuromesodermal progenitor state (Figure 3B) (Gouti et al., 2015).

As cells migrate out from the caudal lateral epiblast, they are exposed to decreasing levels of WNT and FGF signaling, but increasing levels of RA signals, which emanate from the somites that are differentiating in an anterior-to-posterior wave along the forming CNS (Gouti et al., 2015; Lara-Ramírez et al., 2013). Due to their ability to attenuate WNT and FGF signaling and to stimulate the expression of *hox* genes, RA signals have been implicated in inducing the developmental progression of neuromesodermal progenitors (Gouti et al., 2015). From *in vitro* experimentation, it has been proposed that a prolonged exposure to WNT and FGF signaling versus RA signaling biases neuromesodermal progenitors to adopt a mesodermal rather than a neural fate (Gouti et al., 2015, 2014). This idea is supported by the fact that mutant mice lacking a functional *raldh2* gene for RA synthesis exhibit an expansion of mesodermal markers at the expense of markers of the

prospective posterior neuroepithelium (Ribes et al., 2009). However, the molecular mechanisms underlying the neural-mesoderm switch at the caudal epiblast remain to be characterized.

Cells assuming a pre-neural tube fate join the caudal end of the neural plate, which borders at the posterior-most portion of the developing neural tube, and follow the morphogenetic movements involved in neural tube closure (Gouti et al., 2015). Upon entering the spinal cord, pre-neural tube cells are instructed by increasing levels of RA signaling to transition to a neural progenitor fate, which is marked by a down-regulation of NKX1-2 and an up-regulation of PAX6 and IRX3 (Gouti et al., 2015). In addition, RA signals have been shown to stimulate neural progenitor proliferation within the forming spinal cord, which initially consists of a single layer of pseudo-stratified epithelium that expands rapidly so that the subsequent differentiation of neural cells can take place within the lateral mantle layer, while undifferentiated progenitors remain in the ventricular layer that lines the lumen of the neural canal (del Corral and Morales, 2014; England et al., 2011; Mich and Chen, 2011; Wilson et al., 2003). This function of RA signaling is believed to be particularly important at the brachial and lumbar levels of the spinal cord, to increase the number of progenitors within the prospective lateral motor columns, from where the limbs will be innervated (del Corral and Morales, 2014; Sockanathan and Jessell, 1998). These lateral motor columns are absent from other thoracic levels of the spinal cord, where instead two distinct columns of lateral motor neurons are generated: the pre-ganglionic motor column contains visceral motor neurons, which innervate the sympathetic ganglia and glandular targets, and the hypaxial motor column contains motor neurons, which innervate the musculature of the body wall (Philippidou and Dasen, 2013). In addition, a

group of mammal-specific phrenic motor neurons is formed at rostral cervical levels to supply the diaphragm muscle and a median motor column, comprising motor neurons that project to the axial muscles, spans across the entire length of the spinal cord (Philippidou and Dasen, 2013).

While interneuron subclasses are typically generated along the full extent of the spinal cord, the expression of distinct LIM transcription factors and HOX proteins has been associated with the segregation of motor neurons into different columns as well as into different pools within these columns, each of which exhibiting a separate identity and axonal target (Dasen et al., 2008, 2005, 2003; Kania and Jessell, 2003; Landmesser, 2001; Liu et al., 2001). As in the hindbrain, the *hox* genes of the spinal cord display nested, co-linear expression patterns (Philippidou and Dasen, 2013). RA signaling is required to induce the expression of *hox5* at cervical levels and of *nolz1* at brachial levels (Ji et al., 2009; Lara-Ramírez et al., 2013; Philippidou and Dasen, 2013). The transcription factor NOLZ1 in turn activates *hoxc6* at the forelimb level, which is a post-mitotic determinant of the brachial lateral motor column (Ji et al., 2009; Lara-Ramírez et al., 2013). A particular importance of RA for the specification of the lateral motor columns is reflected by the strong expression of *raldh2* in brachial and lumbar, but not thoracic, motor neuron domains (Lara-Ramírez et al., 2013). Loss of this *raldh2* expression leads to the formation of reduced and disorganized lateral motor columns, while the retroviral miss-expression of *raldh2* at thoracic levels was shown to cause an ectopic induction of lateral motor column identities, as marked by the expression of typical transcription factors, such as *isl1*, *isl2* and *lim1* (Lara-Ramírez et al., 2013; Paschaki et al., 2013b; Sockanathan and Jessell, 1998; Vermot et al., 2005). Initially, ISL1 is present in all lateral motor column neurons, but RA signals

subsequently restrict *is/1* expression to more medial areas, while instead promoting *lim1* expression, thus favoring lateral rather than medial motor neuron fates (Ji et al., 2009, 2006; Paschaki et al., 2012; Sockanathan and Jessell, 1998). This is especially relevant for establishing the specific topography of the lateral motor column, since LIM homeodomain proteins control EphA receptors and ephrin-A ligands that coordinate cell body positioning and axonal trajectory development (Kania and Jessell, 2003).

Conversely, RA appears to be dispensable for the induction of more posterior spinal cord *hox* genes, which instead rely on other graded caudal signals, including WNTs, FGFs and TGF β proteins, such as GDF11 (del Corral and Morales, 2014; Liu et al., 2001; Philippidou and Dasen, 2013). However, RA signals are needed for the refinement of the anterior borders of *hox* gene expression domains along the spinal cord, a process during which they exert rostralizing rather than caudalizing influences (del Corral and Morales, 2014; Liu et al., 2001; Philippidou and Dasen, 2013). This strictly context-dependent manner of RA signaling action is probably achieved through the modulation of neural progenitor responsiveness to WNT/FGF versus RA signals by CDX transcription factors. CDX removes repressive chromatin modifications from posterior 5' *hox* genes in order to enable their transcriptional activation via FGF/WNT signaling in the caudal spinal cord (Bel-Vialar et al., 2002; Mazzoni et al., 2013; Philippidou and Dasen, 2013). In contrast, RA signaling modifies the chromatin domains of anterior 3' *hox* genes, in order to induce their expression within the hindbrain (Mazzoni et al., 2013; Philippidou and Dasen, 2013). Interestingly, RA signaling is able to exclude CDX from the prospective hindbrain, but up-regulates it in the spinal cord, where *cdx* expression is further induced and maintained by

caudal WNT and FGF signals (J. Chang et al., 2016; Houle et al., 2003, 2000; Lee and Skromne, 2014; Philippidou and Dasen, 2013; Skromne et al., 2007).

Moreover, CDX appears to stimulate the RA signaling pathway in the hindbrain, but antagonizes it in the posterior spinal cord. For instance, blocking CDX causes a posterior shift of mesodermal *raldh2* expression, which creates an overlap of high FGF and RA signaling levels within caudal neural tissues that leads to the ectopic formation of hindbrain and anterior spinal cord structures in the posterior-most neural tube territory (Shimizu et al., 2006; Skromne et al., 2007). Conversely, CDX represses *cyp26* expression in the prospective hindbrain domain, allowing for the proper positioning of the boundary between the hindbrain and spinal cord territories (J. Chang et al., 2016). Accordingly, RA signals and CDX trigger the activation of *hox* genes from opposite sites of the cluster and seem to cross-regulate each other in an opposite manner in the hindbrain and in the posterior spinal cord. This might be due to the fact that the FGF/WNT signaling center at the midbrain-hindbrain boundary arises much later in development than the one at the caudal epiblast, so that a robust positive feedback loop between FGF/WNT and CDX cannot be established, before high levels of RA signals clear CDX from the hindbrain. Alternatively, early activities of RA signaling at the future hindbrain territory of the neural plate, such as the induction of early genes like *hox1* and *meis3* in zebrafish (Kudoh et al., 2002), might initiate downstream events that prevent positive feedback interactions between FGF/WNT signaling and CDX. In any case, this mirrored use of the same interacting signaling gradients for the formation of two very different parts of the nervous system provides a striking example for the multi-functionality of developmental signaling pathways and highlights the importance of their dynamic integration into a specific temporal and spatial context.

6.4 Patterning the Neural Tube along the dorsal-ventral Axis

The embryonic dorsal-ventral axis is initially defined by a signaling gradient of BMPs, which are inhibited dorsally by secreted factors such as Chordin, Noggin and Follistatin (Langdon and Mullins, 2011). Low BMP signaling levels allow neuroectodermal tissues to develop from the dorsal ectoderm, while moderate and high BMP levels specify lateral and ventral cell types, respectively (Langdon and Mullins, 2011). In gastrulating embryos of the African clawed frog, RA is synthesized in the dorsal mesoderm and degraded in the adjacent dorsal ectoderm and ventral mesoderm (Pera et al., 2014). Morpholino oligonucleotide-mediated inhibition of RA synthesis in these embryos causes a ventralized phenotype, indicating that RA signaling exerts dorsalizing functions during early embryogenesis (Pera et al., 2014; Strate et al., 2009). Indeed, reduced levels of RA signaling were shown to down-regulate the dorsalizing BMP antagonist Chordin, but up-regulate ADMP (anti-dorsalizing morphogenetic protein), an activator of the ventralizing BMP signaling pathway, whereas an increase in RA signaling levels had the opposite effect (Pera et al., 2014; Strate et al., 2009). Thus, early interactions of RA signaling with the BMP pathway likely contribute to setting up the dorsal-ventral axis in embryos of the African clawed frog.

As neurulation proceeds, previously lateral epidermis comes to lie dorsally above the closing neural tube (Liu and Niswander, 2005). Paracrine secretion of TGF β proteins (e.g. BMPs, Dorsalin, Activin) from this new dorsal epidermis specifies the dorsal roof plate of the neural tube, while Sonic hedgehog protein (SHH) from the underlying notochord induces the ventral floor plate (Liu and Niswander, 2005). Subsequently, the dorsal roof plate cells induce a cascade of dorsalizing TGF β protein signals throughout the dorsal

neural tube, whereas the ventral floor plate acts as a ventralizing SHH secreting signaling center (Liu and Niswander, 2005). As a result, two opposing signaling gradients are established that provide positional cues to the cells found along the dorsal-ventral axis of the neural tube (Figure 3C) (Liu and Niswander, 2005). Nevertheless, in order for early dorsal-ventral patterning and neural differentiation to occur, high levels of RA signaling from the paraxial mesoderm first have to extinguish FGF signaling from somitic levels and thus terminate the FGF-mediated repression of homeodomain-containing transcription factors (del Corral and Morales, 2014; Mazzone et al., 2013; Novitsch et al., 2003; Patel et al., 2013). Accordingly, RA signals temporally coordinate dorsal-ventral with anterior-posterior patterning (Hashiguchi and Mullins, 2013). Moreover, a decrease of RA signaling in the developing hindbrain and spinal cord has been shown to cause a dorsal expansion of ventral patterning genes and progenitor domains, whereas an increase in RA signaling triggers a rapid ventral shift of both (Glover et al., 2006; Wilson et al., 2004). Importantly, these effects can be observed during early as well as during late stages of neurogenesis, suggesting that RA signals not only contribute to establishing the dorsal-ventral axis, but also actively maintain it throughout a protracted period of CNS development (Glover et al., 2006; Wilson et al., 2004).

The actions and interactions of BMP/SHH, WNT/FGF and RA signaling with CDX, LIM and TGIF transcription factors are necessary for the down-regulation of *cyp26* expression in the anterior spinal cord territory as well as for the generation of restricted domains of *raldh2* expression in the roof plate, in the ventro-lateral motor neuron domains and, transiently, in dorsal interneurons throughout the developing spinal cord (Castillo et al., 2010; Cunningham and Duester, 2015; Lara-Ramírez et al., 2013; Wilson et al., 2004).

Conversely, transcriptomic analyses of mutant mice with impaired RA signaling and studies on retinoid-free VAD (vitamin A deficient) quails have revealed a miss-regulation of several components of the BMP signaling pathway and of other dorsal-specific factors, including PAX3, PAX7, MSX2 and WNTs (Martinez-Morales et al., 2011; Paschaki et al., 2013b; Ribes et al., 2009; Wilson et al., 2004). In particular, MSX2 and WNTs are activated in a rostral to caudal progression in normal embryos, but depleted in VAD quails, indicating that their induction might depend on somite-derived RA (del Corral and Morales, 2014).

Furthermore, SHH signaling appears to be reduced by both the blocking and the over-activation of the RA signaling pathway (del Corral and Morales, 2014). These results suggest that RA signaling exerts its dorsalizing effects, at least in part, through crosstalk with the signaling gradients present along the dorsal-ventral axis of the neural tube.

From dorsal to ventral, the spinal cord comprises the dorsal roof plate cells, the dorsal interneurons DI1, DI2, DI3, DI4, DI5 and DI6, the ventral interneurons V0, V1 and V2, motor neurons, the ventral interneurons V3 and the ventral floor plate cells, all of which express unique combinations of transcription factors (Catela et al., 2015). Low levels or the absence of RA signaling have been associated with the specification of more ventral interneurons, such as V3, while higher levels of RA signaling are thought to promote more dorsal fates, such as V0, V1 and motor neurons (England et al., 2011; Lara-Ramírez et al., 2013; Lupo et al., 2006). A lack of RA signals was thus shown to diminish V0, V1 and V2 interneuron populations and reduce the expression of *dbx1* and *dbx2* in progenitors of V0 and V1 interneurons as well as of *nkx6.2* and *irx3* in progenitors of V1 and V2 interneurons (del Corral et al., 2003; Novitch et al., 2003; Pierani et al., 1999; Wilson et al., 2004).

Multiple roles of RA signaling have further been described during the specification of motor neurons, which become dramatically decreased under RA-deprived conditions (Novitch et al., 2003; Wilson et al., 2004). Emanating from the paraxial mesoderm, RA signals promote class I homeodomain gene expression (e.g. *pax7*, *pax6*, *dbx1/2* and *irx3*), while coincident SHH signaling from the notochord and floor plate counteracts RA signaling activity and induces class II homeodomain gene expression (e.g. *nkx6* and *nkx2*) (Novitch et al., 2003). Class I and class II homeodomain proteins exhibit mutual cross-repressive interactions and define individual progenitor domains in a combinatorial fashion by regulating the expression of neuronal subtype determinants (Novitch et al., 2003). Thus, the motor neuron lineage, becomes specified by PAX6 (class I homeodomain) and NKX6 (class II homeodomain) proteins, which down-regulate repressors of OLIG2, a known determinant of motor neuron and oligodendrocyte fates, while RA signaling mediates the transcriptional activation of the *olig2* gene (Novitch et al., 2003). This requirement of RA signaling for *pax6* and *olig2* expression seems to be a general feature of vertebrates, as it has been reported in at least zebrafish, quail and mice (del Corral et al., 2003; England et al., 2011; Paschaki et al., 2013b; Ribes et al., 2009; Wilson et al., 2004). In addition, RA signaling also acts downstream of *olig2* expression to induce pan-neuronal differentiation genes and homeodomain protein effectors, such as HB9, involved in motor neuron maturation (Lee et al., 2009; Novitch et al., 2003).

Finally, even in the ventral-most progenitors of spinal cord V3 interneurons, RA signals are needed for the stimulation of Notch receptor activity to decrease, but not eliminate, *ascl1* expression, thus favoring V3 interneuron identity over serotonin-expressing neurons, which are characteristic of hindbrain levels (Jacob et al., 2013).

Accordingly, multiple sources of RA within the spinal cord and from the somitic mesoderm, together with restricted domains of degradation by CYP26, provide crucial cues for the specification of different neural subtypes throughout the neural tube. While some of these effects might be the result of a direct regulation by RA signaling of ventral patterning genes, it is likely that a large part of RA signaling functions during the dorsal-ventral patterning of the spinal cord is achieved indirectly, through the modulation of SOXB1 transcription factors and other components involved in the cross-regulatory networks of ventral patterning genes (del Corral and Morales, 2014; Ribes et al., 2009).

6.5 Orchestrating the Proliferation, Differentiation and Survival of Neural Progenitors

Apart from conferring cellular identities during developmental patterning, RA signaling also plays a central part in the decision between stem cell maintenance and the timing of neural progenitor maturation. In anamniote vertebrates (i.e. in cyclostomes, cartilaginous and bony fish and amphibians), primary neurogenesis begins within the deep layer of the open neural plate, with superficial cells maintaining their proliferative state (Chalmers et al., 2002). However, these primary neurons are thought to be mostly transient and have probably been lost secondarily in amniotes (i.e. in reptiles, birds and mammals) as a consequence of their direct development, which does not require an early sensory-motor system to guide simple larval behaviors (Wullimann, 2009; Wullimann et al., 2005). In amniotes, the first neurons appear in the rostral brain and give rise to an early axonal scaffold, which is comparable to the fiber tracts generated by the earliest formed anamniote neurons that arise in the cortex during secondary neurogenesis (Ware et al., 2015). Moreover, initial neurogenesis in the amniote and secondary neurogenesis in the anamniote brain are characterized by a fairly similar deployment of neural transcription

factors (e.g. NGN1, NEUROD, MASH1/ZASHLA and PAX6), LIM and HOX proteins (e.g. LHX6/7, TBR2 and DLX2a) as well as GABA/GAD positive cells (Wullimann, 2009; Wullimann et al., 2005). It thus seems likely that the developmental mechanisms governing the differentiation of the first cerebral neurons are conserved across vertebrates.

While it is well established that RA signals promote the differentiation of anamniote primary neurons by inhibiting key proliferation factors, such as *zic*, *geminin*, *notch* and *foxd4l1*, as well as by stimulating pro-neural and neurogenic gene expression, studies addressing the potential involvement of RA signaling in anamniote secondary neurogenesis or amniote primary neurogenesis, are relatively scarce (Janesick et al., 2015). So far, RA signaling has been implicated in the differentiation of mammalian photoreceptors, hippocampus and cortical neurons (Janesick et al., 2015). For the latter, RA secreted from the meninges, which comprises several membranes ensheathing the brain, appears to be a crucial cue that controls cell cycle exit in the cortex (Siegenthaler et al., 2009; Siegenthaler and Pleasure, 2011). Interestingly, RA signals derived from radial glia cells have recently been shown to drive the formation of the blood brain barrier, which constitutes the innermost layer of the meninges (Mizee et al., 2013). Astroglia cells are known to produce RA in order to maintain retinoid homeostasis in the brain and it has further been suggested that astroglia-derived RA might stimulate neurogenesis within the neurogenic niches of the post-natal brain (Haskell and LaMantia, 2005; Környei et al., 2007; Shearer et al., 2012; Wang et al., 2011). However, these findings remain to be corroborated by *in vivo* analyses, which should consider the embryonic brain as well (Shearer et al., 2012).

RA generated by RALDH3 in the subventricular zone of the basal ganglia is required for ventral forebrain development, in particular for the correct expression of the dopamine

auto-receptor D2 and GABAergic differentiation (Chatzi et al., 2011; Molotkova et al., 2007). In addition, the cerebrospinal fluid has been identified as an important source of RA signals, which were shown to promote neuroepithelial cell survival and neurogenesis (Alonso et al., 2014, 2011; J. T. Chang et al., 2016; Parada et al., 2008). RA signals that originate from the paraxial mesoderm and from within the posterior neural tube also drive neurogenesis, once the neural identities of the prospective hindbrain and spinal cord have been established (del Corral et al., 2003; Duester, 2013; Janesick et al., 2015). During this process, RA simultaneously acts as a permissive differentiation signal, by repressing the FGF-dependent maintenance of progenitor cell function, and as an instructive signal that induces neuronal differentiation (Duester, 2013).

A direct target of RA signaling, which is needed for inducing embryonic stem cells to differentiate into neurons, is *hoxa1* (Janesick et al., 2015; Makki and Capecchi, 2011). Furthermore, *btg2* is believed to contribute to RA-induced differentiation and was shown to enhance RAR activity by modulating histone H4 methylation and acetylation (Janesick et al., 2015). Many other genes that facilitate cell cycle exit or inhibit the proliferation of neural progenitors can be regulated through either direct or indirect mechanisms of RA signaling, but often require specific developmental conditions (Janesick et al., 2015). Highly cell- and gene-specific transcriptional responses to RA are mediated by numerous co-regulators, by the placement or removal of epigenetic marks as well as through a number of alternative RAR-independent signaling pathways (Carvalho and Schubert, 2013; Gudas, 2013; Urvalek and Gudas, 2014; Wei, 2015). For instance, RA signals can determine the precise timing of stem cell differentiation by generating heritable epigenetic changes in chromatin responsiveness to both retinoid and other signaling pathways (Gudas, 2013) and

exert pleiotropic effects on cell survival and cell cycle, depending on the presence of specific binding proteins (Schug et al., 2007; Wolf, 2008). Accordingly, the blocking of RA signaling was shown to reduce neural progenitor proliferation in the telencephalon of mice (Rajaii et al., 2008) as well as in the neural tube of quail and zebrafish embryos (England et al., 2011; Mich and Chen, 2011; Wilson et al., 2003), while RA treatment increases the number of proliferating cells in chicken ventral neural tube explants (Sockanathan and Jessell, 1998). Intriguingly, RA signals seem to attenuate, but not extinguish, the proliferation of precursor cells in the olfactory epithelium, in order to prevent a depletion of the progenitor pool (Paschaki et al., 2013a). A similar mode of action has also been described for a distinct population of slowly dividing precursors in the developing and adult forebrain of mice (Haskell and LaMantia, 2005). Taken together, current evidence indicates that RA signals can modulate cell cycle progression as well as cell cycle speed in a precisely timed and context-dependent manner. However, the involvement of RA signaling in amniote neurogenesis, in particular in the anterior the CNS, still remains largely unexplored.

6.6 Inducing and coordinating Neural Crest Development

In the dorsal ectoderm of blastula stage embryos, FGF signals together with antagonists of WNT and BMP signaling activate early neural genes, such as *erni*, *otx2* and *sox1/3*, which specify the neural plate (Streit et al., 2000). However, this dorsal neural plate territory is surrounded by a zone, where BMPs from ventral and WNTs from posterior lateral levels of the embryo are only partially inhibited (Groves and LaBonne, 2014). The resulting medial-low, lateral-high gradient of WNT/BMP activity defines the neural plate border and, probably in conjunction with FGF, RA and Notch signaling from underlying

tissues, induces the expression of a distinct set of transcription factors, known as neural plate border specifiers (Groves and LaBonne, 2014; Martinez-Morales et al., 2011; Milet and Monsoro-Burq, 2012; Yardley and García-Castro, 2012). Subsequently, mutual cross-regulatory interactions between these neural plate border specifiers, which include *distalless5*, *pax3*, *pax7* and *zic1/3*, as well as between the neural and non-neural transcription factors activated in adjacent tissues stabilize and sharpen the neural plate border domain by suppressing neural as well as epidermal fates (Simões-Costa and Bronner, 2015).

Within the neural folds that arise from this neural plate border, a transient population of multipotent migratory cells becomes specified during gastrulation, the so-called neural crest. Neural crest cells give rise to various structures, such as the craniofacial skeleton, the peripheral nervous system and melanocytes (Marchant et al., 1998; Weinstein and Hemmati-Brivanlou, 1999; Wilson and Hemmati-Brivanlou, 1997). Neural crest cells are, however, absent from the anterior neural folds, which differentiate into the forebrain, suggesting that the specification of the neural crest requires additional posteriorizing signals (Knecht and Bronner-Fraser, 2002; Villanueva et al., 2002). Exposure to FGF, WNT or RA has been shown to transform the anterior neural folds into neural crest, while blocking of these signals inhibits the expression of neural crest marker genes in the medial and posterior neural folds (Knecht and Bronner-Fraser, 2002; Martinez-Morales et al., 2011; Monsoro-Burq et al., 2003; Villanueva et al., 2002). Thus, the induction of neural crest is restricted to the medial and posterior portions of the neural folds and requires low levels of BMP signaling as well as the posteriorizing activities of FGF, WNT and RA signals

(Knecht and Bronner-Fraser, 2002; Martinez-Morales et al., 2011; Monsoro-Burq et al., 2003; Villanueva et al., 2002).

WNTs secreted from caudal non-axial mesoderm progenitors diffuse anteriorly to activate *gbx1/2* transcription throughout the posterior domain of the neural plate (Green et al., 2014; Milet and Monsoro-Burq, 2012). Next, mutual antagonism between GBX1/2 and the initially widely expressed neural plate marker OTX2 divides the neural plate at the mid-hindbrain boundary (Milet and Monsoro-Burq, 2012). In the anterior section, OTX2 enables the expression of the WNT antagonist DKK1, which represses posterior fates within the rostral neural plate border (Figure 4A). This promotes placode instead of neural crest formation, which is crucial for vertebrate head development (Milet and Monsoro-Burq, 2012). However, the distinction between the future placodal and neural crest cells is already pre-figured in the neural plate border, where neural crest regulators are located more medially than placodal regulators (Simões-Costa and Bronner, 2015). As the neural plate folds, medial neural plate border specifiers thus induce a suite of genes called neural crest specifiers, which include *foxd3*, *sox9*, *id*, *twist* and *snail*, while lateral neural plate border specifiers induce key placodal regulators such as *six1*, *eya1/2* and *irx1* (Simões-Costa and Bronner, 2015). This process is thought to require an increase in BMP signaling levels, which is caused by the elevation of the neural folds (Milet and Monsoro-Burq, 2012; Simões-Costa and Bronner, 2015). In addition, neural border and neural crest specifier induction is modulated by WNT, FGF and RA signals that function posterior to the midbrain-hindbrain boundary to activate early posterior neural plate markers, such as *gbx2* (Martinez-Morales et al., 2011; Milet and Monsoro-Burq, 2012).

The neural crest specifiers induce various neural crest effector genes that provide unique properties to the neural crest cells and allow them to undergo an epithelial-to-mesenchymal transition, during which they detach themselves from the dorsal neural tube, migrate along characteristic pathways through the body and differentiate into a variety of different cell types within their respective target tissues (Martinez-Morales et al., 2011; Sauka-Spengler and Bronner-Fraser, 2008; Simões-Costa and Bronner, 2015). Moreover, neural crest specifiers engage in positive cross-regulatory interactions that stabilize the regulatory state of the neural crest and ensure the maintenance of a number of specifier genes within migrating neural crest cells, which is thought to be crucial for preserving their plasticity and developmental potential (Groves and LaBonne, 2014; Plouhinec et al., 2014; Simões-Costa and Bronner, 2015). For instance, the progeny of a single neural crest cell can include sensory neurons, melanocytes, adrenomedullary cells and glia cells (Gilbert, 2010). The generation of these diverse neural crest cell fates might well be controlled through their progressive specification and emigration from the developing neural tube, which occurs in an anterior to posterior sequence that is highly coordinated with somitogenesis in the adjacent mesoderm (del Corral and Morales, 2014; Martinez-Morales et al., 2011; Sauka-Spengler and Bronner-Fraser, 2008). Thus, the formation of the peripheral nervous system (PNS) and other neural crest derivatives is directly linked to both CNS development and mesodermal segmentation (del Corral and Morales, 2014; Martinez-Morales et al., 2011).

The orderly onset of neural crest induction, specification and emigration along the rostral-caudal axis of the developing neural tube, is ensured by several signals that are integrated in a complex and dynamic manner (Figure 4B). RA signaling, for instance, is first

required for expanding the expression of early dorsal patterning genes, such as the key neural border specifier *pax7*, towards the caudal neural plate (Martinez-Morales et al., 2011). Conversely, a posterior-high FGF gradient prevents the neural border specifiers from inducing early neural crest markers, such as *snail2*, and maintains high levels of the BMP-antagonist Noggin (Martinez-Morales et al., 2011). While FGF signaling and BMP inhibition are necessary for the initial specification of the neural plate border and neural crest domain, further neural crest development depends on an increase of BMP levels and on a decrease of FGF levels during neurulation (Martinez-Morales et al., 2011; Milet and Monsoro-Burq, 2012). Thus, FGFs delay neural crest specification in caudal regions and prevent BMP signals from stimulating *wnt1* expression, which initiates the emigration of specified neural crest cells (Martinez-Morales et al., 2011). Anteriorly, RA signaling from the maturing somites antagonizes FGF signaling. Lower levels of FGFs permit *snail2* expression and the down-regulation of Noggin by signals from the paraxial mesoderm (Martinez-Morales et al., 2011; Sela-Donenfeld and Kalcheim, 2000). This enables various positive regulators to augment BMP signaling levels, which drive neural crest specification as well as the WNT1-mediated induction of neural crest emigration (Martinez-Morales et al., 2011; Milet and Monsoro-Burq, 2012). In addition, RA signaling also triggers *wnt1* expression, albeit only in specified neural crest cells that already express *snail2* (Martinez-Morales et al., 2011). Accordingly, the antagonism between FGF and RA signaling appears to govern the rostral-to-caudal progression of neural crest development in concert with WNT, Noggin/BMP and various other signals, although their specific roles and interactions have not been defined in detail.

6.7 Inducing and coordinating Cranial Placode Development

The cranial placodes arise from a common precursor field in the anterior neural plate border, termed the pre-placodal region (Steventon et al., 2014). As described above, the neural plate border is induced by a combination of intersecting signals, including FGFs from the dorsal ectoderm and intermediate levels of WNTs and BMPs from lateral and ventral regions of the embryo, that activate the expression of neural plate border specifiers such as *distalless5*, *pax3*, *pax7* and *zic1/3* (Groves and LaBonne, 2014; Milet and Monsoro-Burq, 2012). However, some of these genes are expressed more medially within the neural plate border than others (Simões-Costa and Bronner, 2015). Subsequent cross-regulatory interactions between the neural plate border specifiers and neural as well as non-neural transcription factors from adjacent tissues thus divide the cells of the neural plate border into a medial pre-migratory neural crest population and a more lateral pre-placodal population (Simões-Costa and Bronner, 2015). In addition, the secretion of DKK1 from the anterior pharyngeal endoderm inhibits WNT signaling anterior to where the midbrain-hindbrain boundary will be established, thus suppressing posterior fates and promoting placode formation (Figure 4A) (Milet and Monsoro-Burq, 2012).

As the neural plate folds, the more lateral neural plate border specifiers induce key placodal regulators, such as *six1*, *eya1/2* and *irx1* (Simões-Costa and Bronner, 2015). One of the earliest neural border specifiers expressed at the anterior neural plate is the zinc-finger transcription factor ZIC1, which is necessary for promoting placodal fate and regulating *six1* and *eya1/2* expression (Hong and Saint-Jeannet, 2007). Since *zic1* expression does not overlap with the prospective pre-placodal region, it affects placode formation in a non-cell autonomous manner (Jaurena et al., 2015). Recently, it has been reported that ZIC1 up-

regulates *raldh2* expression in a discrete U-shaped ectodermal domain around the anterior neural plate and that it further induces LPGDS (lipocalin-type prostaglandin D2 synthase), which serves as a secreted lipophilic carrier for RA (Jaurena et al., 2015). This function of ZIC1 was shown to be crucial for the induction of placodal fate markers by RA (Jaurena et al., 2015). In the anterior neural plate, ZIC1 thus activates RA signaling, which acts rostrally to specify the pre-placodal region, with the neural plate itself being protected from RA by the expression of CYP26 enzymes (Figure 4A) (Jaurena et al., 2015). Interestingly, RA signaling appears to exert this role in a RAR-independent manner (Jaurena et al., 2015), while its effects on the anterior-posterior patterning of the pre-placodal territory are RAR-dependent (Janesick et al., 2012).

RA signaling via RAR initially up-regulates the TBX1 transcription factor (Figure 4A), which is required for *fgf8* expression and engages in a positive feedback loop with this key pre-placodal marker gene (Janesick et al., 2012). However, RAR activation also induces *ripple3* expression lateral to the pre-placodal region marked by *fgf8* expression (Janesick et al., 2012). Together with its co-repressor GROUCHO, RIPPLEY3 represses the ability of TBX1 to maintain *fgf8* expression in adjacent tissues, thus setting the posterior-lateral border of the pre-placodal region (Janesick et al., 2012). In addition, TBX1 further enhances this process by positively regulating *ripple3* (Janesick et al., 2012). Accordingly, antagonism between FGF and RA signals is deployed for developmental patterning, even in this anterior-most part of the developing nervous system.

As development proceeds, the pre-placodal region becomes sub-divided into separate territories with distinct identities that can be perceived as transient local thickenings of the embryonic head and neck ectoderm (Steventon et al., 2014). These

placodes give rise to a multitude of different cell types and are generally referred to as the olfactory, lens, trigeminal, epibranchial and otic placodes (Steventon et al., 2014). The proper positioning of the pre-placodal territory via RA signaling might be relevant for the subsequent separation of the different placodes, since this process is strongly influenced by the interactions and cellular movements between pre-placodal and adjacent tissues (Janesick et al., 2012; Saint-Jeannet and Moody, 2014). However, current evidence does not support a directly involvement of RA signals during the regionalization of the pre-placodal region, which is mainly achieved through transcription factor interactions and the dynamic integration of FGF, WNT and BMP signaling (Saint-Jeannet and Moody, 2014).

6.8 Regulating the Formation of Neural Crest and Cranial Placode Derivatives

The placodes and neural crest give rise to a great variety of different structures, including sensory organs and the PNS (Steventon et al., 2014). RA signals have been shown to affect the behavior of migrating neural crest cells, for instance, through the direct transcriptional regulation of *nedd9*, a scaffolding protein that assembles complexes involved in cell adhesion, migration, division and survival, via a complex upstream RARE (Aquino et al., 2009; Knutson and Clagett-Dame, 2015). This interaction likely contributes to at least some of the diverse functions RA signals exert during the morphogenesis of neural crest derivatives. Especially the neural crest cells that give rise to craniofacial structures and originate mostly from caudal forebrain, midbrain and anterior hindbrain levels are well known to be under the control of RA, which at later developmental stages is synthesized locally in the forebrain and facial ectoderm by RALDH2 as well as RALDH3 enzymes (Lee et al., 1995; Minoux and Rijli, 2010). These RA signals shape the facial area together with FGF8 and BMP4 signaling, through the coordination of local cell survival

around the nasal placode and patterning of the pre-nasal and pre-maxillary skeletal elements derived from of the frontal nasal process (Minoux and Rijli, 2010).

By modulating the migration, proliferation, survival, patterning and/or differentiation of neural crest cells as well as their interaction with target tissues, RA signals further contribute to the development of many other neural crest derivatives, including, for example, periocular tissues of the eye (Bohnsack et al., 2012; Kumar and Duester, 2010; Matt et al., 2008, 2005), cartilage structures of the posterior pharyngeal arches (Minoux and Rijli, 2010; Niederreither et al., 2003), components of the cardiac outflow tract and heart septum (El Robrini et al., 2015; Zaffran and Niederreither, 2015) as well as teeth (Samarut et al., 2015).

In addition, mutant mice lacking a functional RALDH2 enzyme for RA synthesis typically exhibit an aganglionic bowel phenotype (Niederreither et al., 2003, 2001). Therefore, RA signaling is thought to be crucial for the morphogenesis of the enteric nervous system from vagal neural crest cells, which exit the neural tube at the level of the hindbrain and migrate past the somites towards the foregut (Simkin et al., 2013). During this process, RA exposure was shown to stimulate the expression of *ret*, a receptor for glial-derived-neurotrophic-factor (GDNF), within the vagal neural crest cells (Simkin et al., 2013). GDNF is secreted by the gut mesenchyme and can promote survival, migration, proliferation and differentiation of its target cells (Simkin et al., 2013). Furthermore, RA was shown to stimulate cell interactions that are involved in organizing migrating vagal neural crest cells into chains (Simkin et al., 2013). Thus, RA signaling enables vagal neural crest cells to efficiently invade and colonize the foregut and regulates their subsequent differentiation into enteric nervous system (Simkin et al., 2013).

The peripheral components of the sensory nervous system often arise from both neural crest and placode progenitors, which tend to interact to drive the complex morphogenetic events that underlie PNS development (Steventon et al., 2014). For instance, neural crest cells constitute a major part of the frontal-nasal mesenchyme that surrounds and patterns the olfactory placode by releasing RA, which further regulates neural progenitor fate and differentiation within the olfactory epithelium (Bhasin et al., 2003; Paschaki et al., 2013a). Moreover, antagonism between FGF and RA signaling in the olfactory placode was shown to be responsible for the specification of a small population of neuroendocrine cells, which are located in the rostral hypothalamus and basal forebrain, where they integrate inputs from multiple brain areas and secrete gonadotropin-releasing hormone, a key regulator of gonad maturation, puberty and sexual behavior (Sabado et al., 2012). RA signaling has further been implicated in the development of the adenohypophyseal (Maden et al., 2007) and lateral line (Gibbs and Northcutt, 2004) placodes as well as in the morphogenesis and patterning of the otocyst, which is formed through the invagination of the otic placode and gives rise to components of the inner ear (Hans et al., 2007; Romand et al., 2006; Thiede et al., 2014).

Taken together, RA signals are used throughout the embryo and affect the formation of very different neural crest and placode derivatives, often by antagonizing FGF and other signaling pathways. Nonetheless, the precise roles of RA signaling as well as the underlying mechanisms of its actions during neural crest and placode development remain largely unknown, in particular regarding the potential contribution of RA to the formation of the PNS, which is hinted at, for example, by the importance of RA signals during

peripheral nerve regeneration (Mey, 2006; Monaghan and Maden, 2012; Zhelyaznik et al., 2003).

7. Retinoic Acid Signaling and the Establishment of Neurotransmitter System

7.1 Natural Neuroactive Substances: Neurotransmitters, Neuromodulators and Neurohormones

Any chemical agent that is synthesized by a neuron and affects the properties of other neurons or effector cells (e.g. muscle cells and gland cells) can broadly be defined as a natural neuroactive substance (Hoyle, 1985). Three main types of natural neuroactive substances are generally distinguished: neurotransmitters, which are released at a synapse and alter ion permeability of the post-synaptic membrane, neuromodulators, which are released in the vicinity of synapses and affect transmission at the pre- and/or post-synaptic membrane, and neurohormones, which are released into the extracellular space or general circulation and may affect proximal as well as distant targets (Hoyle, 1985). However, for most neuroactive substances these functions overlap to variable degrees (Hoyle, 1985) and often neurons use co-transmission of several neuroactive substances to generate complex disparate outputs (Nusbaum, 2001; Powis and Bunn, 1995). Since every neurotransmitter system usually comprises specific synthesis enzymes as well as a carefully regulated molecular machinery responsible for the transport, packing, storage, release, uptake, activation, inactivation and reception of a neuroactive substance, it is not surprising that neurons are among the most transcriptionally active cells (Moroz et al., 2006; Nelson et al., 2006).

Most hormones are short polypeptides, but can also be derived from amino acids or lipids, such as steroid hormones like cortisone or estrogen in vertebrates and ecdysone in arthropods that are synthesized from the lipid cholesterol (Hartenstein, 2006). Important vertebrate neurohormones are the releasing hormones of the hypothalamus, which mainly regulate hormone secretion in the pituitary gland and thus control a multitude of physiological processes (e.g. thyrotropin-, corticotropin-, gonadotropin- and growth-hormone-releasing hormone, somatostatin, dopamine and neurotensin) (Besser and Mortimer, 1974; Guillemin, 2005), the neurohormones of the pituitary gland, which are involved in smooth muscle contraction, homeostasis, neuromodulation and the mediation of various social behaviors (oxytocin and vasopressin) (Stoop, 2012), the catecholamines of the chromaffin cells of the adrenal medulla, which act, for instance, on the cardiovascular system, muscle contraction and general metabolism (dopamine, epinephrine and norepinephrine) and the indolamines of the enterochromaffin-like cells in the gastrointestinal tract, which regulate the secretion of hydrochloric acid, digestive enzymes and various other compounds (histamine and serotonin) (Westheide and Rieger, 2015). A great diversity of both peptidic and lipidic neurohormones can also be found in invertebrates, such as FMRFamides that can control heartbeat, respiration and egg laying, myomodulins that affect muscle contraction, adipokinetic hormones that stimulate fat metabolism, pigment dispersing, timeless and period hormones that are involved in generating a circadian rhythm, allatostatins and allatotropins that regulate food intake and inhibit the synthesis of juvenile hormone, tachykinin-related peptides that have diuretic functions and influence endocrine secretion, prothoracicotropic hormones and molt-inhibiting hormones that influence ecdysteroid synthesis, which is crucial for molting and maturation, and many others (Alzugaray et al., 2013; Bendena et al., 1999;

Grimmelikhuijzen et al., 1999; Nässel, 2002, 1996; Smith and Sedlmeier, 1990; Veenstra, 2010).

Classical neurotransmitter systems include aminoacidergic (e.g. aspartate, GABA, glutamate, glycine and homocysteine), cholinergic (e.g. acetylcholine), monoaminergic (e.g. catecholaminergic, like dopamine, norepinephrine and epinephrine, indolaminergic, like tryptamine and serotonin, and others, such as histamine and taurine) and purinergic (e.g. adenosine, AMP, ADP and ATP) messengers as well as a plethora of neuropeptides and gaseous transmitters (e.g. nitric oxide and carbon monoxide) (Foye et al., 2008; Von Bohlen und Halbach and Dermietzel, 2006). These neurotransmitter systems are widely distributed throughout the animal kingdom and a surprisingly large set of their molecular components has been detected in the seemingly simple cnidarians as well as in nerveless animals, such as sponges and placozoans (Figure 5) (Grimmelikhuijzen et al., 1996; Jékely, 2013; Kass-Simon and Pierobon, 2007; Richards et al., 2008; Riesgo et al., 2014; Smith et al., 2014; Watanabe et al., 2009). Intriguingly, many amino acid-derived neurotransmitters, including catecholamines (epinephrine, norepinephrine and dopamine), indoles (serotonin and melatonin), histamine, acetylcholine and nitric oxide, are also present in microorganisms, which exploit them to communicate between themselves as well as with their respective hosts (Hughes and Sperandio, 2008; Roshchina, 2010). This finding has prompted the hypothesis that the acquisition of bacterial genes via horizontal gene transfer played an essential role in the evolutionary elaboration of the existing biochemical pathways involved in animal neurotransmission (Iyer et al., 2004).

RA signaling acts as a major regulatory factor during vertebrate neurogenesis (Duester, 2013; Janesick et al., 2015). Treatments with RA have been shown to induce the

differentiation of stem cells into neurons with different neural identities in a concentration-dependent manner (Okada et al., 2004). RA can thus be used to generate, for instance, dopaminergic (Cooper et al., 2010; Korecka et al., 2013; Li et al., 2012), cholinergic (Berrard et al., 1993; Handler et al., 2000; Szutowicz et al., 2015), glutamatergic (Urban et al., 2015; Yu et al., 2015) or GABAergic neurons (Addae et al., 2012; Chatzi et al., 2009; Shan et al., 2011). While progress has been made towards uncovering the mechanisms, through which RA signals modulate the cell cycle and promote neural fate (Duester, 2013; Ikami et al., 2015; Janesick et al., 2015; Rochette-Egly, 2015), relatively little is known about how RA signaling might contribute to the establishment of distinct neurotransmitter phenotypes (Table I).

7.2 Catecholaminergic Neurons

Common catecholamine neurotransmitters include dopamine, norepinephrine, epinephrine and octopamine. While dopaminergic neurotransmission is widespread in all bilaterian animals, norepinephrine and epinephrine are mainly present in vertebrates and octopamine is typical for protostomes, but can also be found as trace amine in vertebrates (Figure 5) (Barron et al., 2010; Moret et al., 2004). Dopamine has been shown to modulate motor circuits in representatives of the cnidarians, nematodes, platyhelminthes, annelids, mollusks, arthropods and chordates (Barron et al., 2010). In addition, dopamine plays a well conserved role in the regulation of feeding-related behaviors, learning, intrinsic reward and reinforcement in diverse animal phyla (Barron et al., 2010).

In the CNS of vertebrates, dopaminergic neurons can be found throughout the forebrain, the midbrain, in several “dopaminergic nuclei” of the caudal hindbrain and in the

spinal cord (Smeets and González, 2000). A loss of dopamine-secreting neurons in the substantia nigra (midbrain area), is known to cause Parkinson's disease (Gröger et al., 2014) and altered levels of dopamine neurotransmitter activity have been associated with schizophrenia (Kuepper et al., 2012), attention deficit hyperactivity disorder (del Campo et al., 2011) and restless legs syndrome (Trenkwalder and Paulus, 2010). In the periphery, dopamine is further released by sympathetic nerves (Goldstein and Holmes, 2008), chromaffin cells in the adrenal medulla, neuroendocrine cells in the kidney and pancreas, retinal cells and peripheral leukocytes (Rubí and Maechler, 2010). Within the bloodstream dopamine inhibits norepinephrine release and acts as a vasodilator, whereas in other regions of the body it is secreted in a paracrine manner to regulate local processes, including sodium excretion, urine output, pancreatic insulin production, gastrointestinal motility, intestinal mucosal tissue oxygenation and the activity of lymphocytes (Rubí and Maechler, 2010; Yamamoto and Vernier, 2011).

In contrast to dopamine, norepinephrine and epinephrine are mainly found in vertebrates (and in some protozoan, placozoan, cnidarian and cephalopod species). Protostomes, in contrast, generally use the closely related neurotransmitter octopamine (Kass-Simon and Pierobon, 2007; Moret et al., 2004; Pflüger and Stevenson, 2005). Within the CNS of vertebrates, norepinephrine is present in only six nuclei named A1, A2, A5, A7, locus coeruleus and subcoeruleus, which originate from different rhombomers and show distinct subtype identities (Robertson et al., 2013). The most prominent norepinephrine-containing cell cluster of the brain is the locus coeruleus, which sends projections to all cortical regions as well as to thalamic nuclei, septum, hippocampus and basal lateral amygdala (Loughlin et al., 1986). In comparison, epinephrine is found in three main clusters

of neurons known as nuclei C1, C2 and C3 as well as in the rostral medulla of the brainstem (Ziegler et al., 2002). The C1 neurons innervate brain areas that are strongly linked with anxiety and stress responses and provide major adrenergic input to the locus coeruleus. C2 neurons innervate the amygdala, which is an integrative center for emotion, emotional behavior and motivation, and C3 neurons also innervate the locus coeruleus (Ziegler et al., 2002). Norepinephrine and epinephrine neurotransmission in the CNS have been implicated in the stimulation of attention, arousal, alertness, restlessness and anxiety as well as in memory formation and retrieval (Berridge et al., 2012; Sara, 2015; Tank and Lee Wong, 2014). Furthermore, norepinephrine-positive neurons are the principal neuronal phenotype found in peripheral sympathetic ganglia, which target smooth and cardiac muscle, cutaneous and glandular structures as well as parenchymal organs (such as liver, kidney, bladder and reproductive organs) (Robertson, 2004). Upon stimulation by sympathetic pre-ganglionic neurons, sympathetic nerve terminals and most importantly the adrenal medulla, which is located at the center of the adrenal gland, release norepinephrine as well as adrenaline/epinephrine and small amounts of dopamine into the bloodstream (Robertson, 2004). This triggers a multitude of physiological responses, also known as fight or flight response, which include increased blood pressure and cardiac output, relaxation of bronchial, intestinal and many other smooth muscles, mydriasis and metabolic changes that increase levels of blood glucose and free fatty acids (Tank and Lee Wong, 2014).

Octopamine has first been discovered in the posterior salivary glands of the cephalopod, *Octopus vulgaris* (Erspamer and Boretti, 1951) and octopamine-dependent neurotransmission is typically found in protostomes, but may also occur in vertebrates,

where octopamine is used as a trace amine (Jagiełło-Wójtowicz, 1978). In most invertebrates, high concentrations of octopamine are present both within neuronal and non-neuronal tissues and modulate nearly all physiological processes by targeting peripheral organs as well as sense organs and processes within the CNS (Roeder, 2005).

Catecholamines such as dopamine, norepinephrine and epinephrine are synthesized from the amino acid tyrosine within the cytosol. The rate-limiting enzyme for catecholamine synthesis is tyrosine hydroxylase (TH), which catalyzes the hydroxylation of tyrosine to L-DOPA that is then decarboxylated to dopamine by aromatic amino acid decarboxylase (AADC) (Daubner et al., 2011). Both L-DOPA and dopamine can be used as precursors for melanin pigment synthesis and have been shown to play a role in immunity and cuticle hardening in insects (Tang, 2009) as well as in the production of neuromelanin in the substantia nigra of humans (Fedorow et al., 2005; Solano, 2014). Moreover, in some neurons L-DOPA itself acts as a neurotransmitter or neuromodulator, without being transformed into dopamine. This has been reported, for example, for neurons in the hypothalamus and the dorsal vagal complex of vertebrates (Björklund and Dunnett, 2007; Misu et al., 2003; Misu and Goshima, 2006).

Dopamine can further be transformed into norepinephrine by dopamine- β -hydroxylase (DBH) and, through an additional step catalyzed by phenylethanolamine-N-methyl transferase (PNMT), into epinephrine. The DBH enzyme is homologous to tyramine- β -hydroxylase (TBH), which synthesizes the neurotransmitter octopamine from tyramine (Monastirioti et al., 1996). Close relationships of protostome octopamine receptors and vertebrate α -adrenergic receptors suggest that the two neurotransmitter systems have diverged from a common evolutionary origin, with some evidence pointing towards the

octopaminergic system being younger than the vertebrate adrenergic system (Barron et al., 2010; Pflüger and Stevenson, 2005).

The TH enzyme catalyzes the rate-limiting step in dopamine synthesis (Daubner et al., 2011) and it has been suggested that the gene encoding TH might be a direct downstream target of the RA signaling pathway, since RAR can robustly activate the TH promoter in a ligand-dependent manner (Jeong et al., 2006). However, RA signaling appears to exert opposite effects on TH expression, depending on the cell type (Jeong et al., 2006). For instance, all-*trans* RA treatment decreases TH expression levels as well as the dopamine content in dopaminergic MN9D cells, but increases *th* mRNA levels in human neuroblastoma SK-N-BE(2)C cells and induces transcription of the *th* gene in human SMS-KCNR cells (Jeong et al., 2006). RA signals also directly regulate the expression of the dopamine autoreceptor D2 (Samad et al., 1997; Valdenaire et al., 1998) and the loss of RA synthesis enzymes or receptors causes a specific reduction or elimination of D2 expression, which is associated with impaired locomotion and depressive-like behaviors (Baik et al., 1995; Krezel et al., 1998; Krzyzosiak et al., 2010; Molotkova et al., 2007; Samad et al., 1997; Wolf, 1998).

The development of midbrain dopaminergic neurons has been particularly well investigated, since their dysfunction can be linked to movement disorders, such as Parkinson's disease, as well as to drug abuse and addiction (Gröger et al., 2014; Höglinger et al., 2004; Volkow et al., 2009; Whalley, 2014). During the development of meso-diencephalic dopamine neurons, the orphan NR NURR1 is responsible for the activation of TH expression and the induction of dopaminergic fate (Jacobs et al., 2011). In addition, the paired-like homeobox transcription factor PITX3 is required for the formation of a specific

subpopulation of meso-diencephalic dopamine neurons in the substantia nigra, which selectively express the RA-generating enzyme ADH2 (Jacobs et al., 2007). Both ADH2 and TH expression are lost in response to the mutation of PITX3, which prevents the final differentiation of substantia nigra dopaminergic neurons, but can be rescued by RA treatment (Jacobs et al., 2007). Apart from TH, expression of DELTA-like 1 and D2 can be restored in PITX3 deficient substantia nigra dopaminergic neurons through embryonic RA treatment, whereas a number of other genes appear to be specifically modulated by PITX3 (Jacobs et al., 2011). Moreover, RA signaling was shown to represses NURR1-mediated *dlk1* expression in precursors of substantia nigra dopaminergic neurons, which is thought to further promote terminal differentiation (Jacobs et al., 2011). These data demonstrate that, in contrast to other meso-diencephalic regions, the dopaminergic differentiation of neural precursors in the substantia nigra is specifically controlled through RA-dependent as well as RA-independent functions of PITX3 (Jacobs et al., 2011, 2007). Interestingly, dopamine neurons of the substantia nigra and the ventral tegmental area of the midbrain further express high levels of the enzyme RALDH1, which was shown to synthesize GABA for the co-release with dopamine, hence substituting the conventional GABA-synthesis enzymes GAD65 and GAD67 (Kim et al., 2015). Since RALDH1 is also capable of RA synthesis (Molotkov and Duester, 2003) and since RA is known to promote GABAergic neurotransmitter phenotypes (Addae et al., 2012; Chatzi et al., 2011), it might be valuable to investigate, whether RA signaling contributes to the specification and/or maintenance of both neurotransmitter phenotypes in neurons of the substantia nigra.

It has further been postulated that RA signaling is involved in the specification and differentiation of late born norepinephrineric nuclei in the hindbrain, including the locus

coeruleus, and potentiates norepinephrine production by inducing AP-2, which regulates the transcription of both TH and DBH (Holzschuh et al., 2003; Kim et al., 2001; Wilson et al., 2007). In fact, regulation by AP-2 has been proposed for a multitude of neural genes, such as acetylcholinesterase, choline acetyltransferase, neuron-specific enolase, synapsin II, presenilin-2 and pro-enkephalin genes (Kim et al., 2001). It is thus not too surprising that RA treatments can switch neuroblastoma cell lines from a norepinephrinergic to a cholinergic phenotype by reducing TH activity, norepinephrine levels and the calcium-dependent release of norepinephrine, while enhancing the expression of choline acetyltransferase and inducing the calcium-dependent release of acetylcholine in conjunction with changes in the cellular distribution of both vesicular monoamine transporter 2 and vesicular acetylcholine transporter (Handler et al., 2000). In addition, RA was shown to induce the expression of a sodium-dependent norepinephrine transporter (NET) in the adrenergic cells of the superior cervical ganglia of newborn rats, while decreasing *th* mRNA levels (Matsuoka et al., 1997b), and has further been reported to promote the proliferation of TH-expressing cells in quail neural crest cultures, causing a dose-dependent increase in the number of adrenergic cells (Dupin and Le Douarin, 1995b; Rockwood, 1996). From these studies it is apparent that RA signals can modulate various components of catecholaminergic as well as other neurotransmitter systems, through both direct and indirect mechanisms, to instruct differentiating neurons to adopt a specific neurochemical identity in a highly context-dependent manner (Table I).

7.3 Serotonergic Neurons

Predating the evolutionary origin of neurons, the indolamine serotonin (or 5-hydroxytryptamin, 5-HT) functions as a signaling molecule in both prokaryotic and

eukaryotic microorganisms (Lyte and Freestone, 2010; Tsavkelova et al., 2006), plants (Murch et al., 2000; Murch and Saxena, 2002), protozoans (Csaba et al., 2010) and sponges (Nickel, 2010; Weyrer et al., 1999). Moreover, serotonin neurons that arise early during development have been described in almost all animal groups (Figure 5), prompting the hypothesis that the role of serotonin as a neurotransmitter might have been a direct consequence of the evolution of synapses (Hay-Schmidt, 2000; Ryan and Grant, 2009). In vertebrates, serotonin modulates a wide range of physiological processes in all major organ systems, including the cardiovascular, pulmonary, gastrointestinal and genitourinary systems as well as the CNS (Berger et al., 2009). Most serotonin neurons are found in the hypothalamus and hindbrain of vertebrates, where they contribute to the regulation of locomotion, mood, sleep, anxiety, drug abuse, food intake and sexual behavior (Veenstra-VanderWeele et al., 2000).

The first rate-limiting step of serotonin synthesis is the hydroxylation of L-tryptophan to L-5-hydroxytryptophan by tryptophan hydroxylase (TPH). Vertebrates possess at least two different TPH proteins, with TPH1 being found in several tissues and TPH2 being neuron-specific (Côté et al., 2003; Gutknecht et al., 2009; Savelieva et al., 2008; Walther, 2003). Subsequently, AAAD and its co-enzyme pyridoxal 5'-phosphate decarboxylate L-5-hydroxytryptophan to serotonin (Lovenberg et al., 1963).

In the hindbrain of African clawed frog larvae, exogenous RA induces dose-dependent changes in the number, distribution and projection patterns of serotonin-positive neurons (Ruiz i Altaba and Jessell, 1991). Moreover, in cells derived from rat embryonic raphe nuclei RA treatments have been shown to increase intracellular levels of serotonin, of the 5-HT_{1A} receptor as well as of the re-uptake transporter SERT (Neumeister

et al., 2004; O'Reilly et al., 2007). Since both the 5-HT_{1A} receptor and SERT are important negative regulators of serotonin levels within the extracellular cleft, their increased expression probably causes an inhibition of serotonin neurotransmission and might thus contribute to RA-inducible depression-related behaviors in mice (Neumeister et al., 2004; O'Reilly et al., 2007). Consistently, it has been postulated that brain serotonin concentrations can be lowered through the oral administration of RA (Smazal and Schalinske, 2013). These data indicate an influence of RA signaling on several components of the serotonin neurotransmitter system.

In vertebrates, TGF β , together with FGFs and SHH, are crucial for establishing the molecular signaling pathways leading to serotonergic differentiation (Farkas et al., 2003; Ye et al., 1998) and the formation of serotonin-producing cells has been shown to depend on a specific transcription factor program involving at least four genes: *ascl1*, *nkx2.2*, *lmx1b* and *pet-1* (Pattyn, 2003; Pattyn et al., 2004). Together, *ascl1* and *nkx2.2* activate the expression of *lmx1b*, *pet-1*, *isnm1* and *gata3*, which induces the serotonin transmitter phenotype (Jacob et al., 2009; Pattyn et al., 2004). LMX1B controls major components of serotonin metabolism, including TPH and SERT. Notably, besides working together with PET-1 and NKX2.2 to specify the vast majority of serotonin-positive neurons, LMX1B may act autonomously or with other factors to control the formation of specific subsets of serotonergic neurons, especially in rostral brain regions (Cheng et al., 2003). Moreover, LMX1B is also necessary for the development and maintenance of dopaminergic neurons (Nakatani et al., 2010; Yan et al., 2011). PET-1, on the other hand, is a precise marker of developing and adult serotonergic neurons and is mainly involved in serotonergic neuron maturation, regulating axonal innervation of the somatosensory cortex, expression of

appropriate firing properties and the expression of the 5-HT_{1A} and 5-HT_{1B} receptors (Hendricks et al., 2003). PET-1 is further required in adult serotonin neurons to preserve normal anxiety-related behaviors through direct auto-regulated control of serotonergic gene expression (Liu et al., 2010).

However, the transcriptional code of serotonin progenitors in the ventral hindbrain is largely shared by the topographically related progenitors of V3 neurons in the ventral spinal cord, which also express NKX2.2, ASCL1 and FOXA2, but acquire a glutamatergic neurotransmitter phenotype (Carcagno et al., 2014; Jacob et al., 2013). Recent studies in mice have revealed that different levels of RA signaling are responsible for the serotonin versus V3 fate decision (Carcagno et al., 2014; Jacob et al., 2013). Around the time neurogenesis commences, RA signaling is weak or absent in the ventral hindbrain domain of serotonin progenitors and *ascl1* expression levels are markedly higher than in the ventral spinal cord domain of V3 progenitors, where RA signaling is strong (Jacob et al., 2013). In order to achieve this dose-dependent attenuation of *ascl1* expression, RA signals activate the Notch pathway, directly as well as through the induction of *neurog3* expression in the spinal cord, which ultimately leads to the repression of *ascl1* by HES proteins (Carcagno et al., 2014; Jacob et al., 2013). Thus, since high levels of ASCL1 are sufficient for committing neural tube progenitors to a serotonergic fate, RA signals allocate serotonergic and glutamatergic V3 identities in an ASCL1-dependent manner by inducing quantitative changes in the transcriptional code, via NEUROG3 and Notch function (Carcagno et al., 2014; Jacob et al., 2013).

Hindbrain serotonergic neurons are subdivided into a rostral group near the mid-hindbrain boundary and a caudal group in the prospective myelencephalon, with a

serotonin neuron-free zone located in between, in rhombomere 4 (Smidt and Hooft, 2013). Due to the dynamic auto- and cross-regulatory interactions of RA, FGF and WNT signals as well as of various transcription factors and *hox* genes, the expression of HOXB1 is induced and selectively maintained in rhombomere 4, where it activates the transcription factor PHOX2 that represses the serotonergic developmental program (Glover et al., 2006; Philippidou and Dasen, 2013; Smidt and Hooft, 2013; Terriente and Pujades, 2015). Therefore, RA signaling is crucial for determining the proper distribution of serotonin-positive neurons during development and potentially affects serotonin signaling directly through the modulation of the 5-HT_{1A} receptor and SERT (Table I).

7.4 Glutamate and GABAergic Neurons

As for serotonin, glutamate and GABA (γ -aminobutyric acid) are ancient chemical messengers that fulfill a variety of functions in plants, protists and metazoans (Bouché et al., 2003; Bucci et al., 2005; Elliott and Leys, 2010; Ramoino et al., 2014, 2010; Van Houten et al., 2000). Glutamate is the most abundant excitatory amino acid in the vertebrate brain, which participates in most nervous system functions and is crucial for synaptic plasticity, learning and memory (McEntee and Crook, 1993). GABA, on the other hand, reduces neuronal excitability, which is especially important for the control of motor function (Draper et al., 2014; Wong et al., 1991) and the regulation of anxiety (Lydiard, 2003). In addition, GABA is involved in vision (Herrmann et al., 2011) and, during nervous system development, influences neural migration as well as neurite extension and acts as a neurotrophic factor (Watanabe et al., 2002). Outside the nervous system, glutamate and GABA signaling have further been implicated in the control of numerous physiological

processes, ranging from insulin release and bone formation to gastrointestinal motility and hormone secretion (Nedergaard et al., 2002; Watanabe et al., 2002).

Glutamate is a non-essential amino acid and thus, for metabolic purposes, found in most cells, albeit at variable concentrations. Since glutamate cannot cross the blood/haemolymph brain barrier that is present in numerous taxa, including vertebrates, arthropods and cephalopods (Figure 5), it has to be synthesized within the CNS from local precursors (Hawkins, 2009; Parpura and Verkhratsky, 2012). However, neurons are not capable of producing glutamate *de novo* from glucose through the tricarboxylic acid cycle, because they lack the enzyme pyruvate carboxylase, which, in vertebrates, is specifically expressed in astrocytes and, in insects, is found in glia cells (Bak et al., 2006; Freeman et al., 2003; Shank et al., 1985). Instead, neuronal glutamate production is based on a metabolite trade known as the glutamate/glutamine cycle, during which the glutamate precursor glutamine is released from glial cells and subsequently metabolized to glutamate within the pre-synaptic vesicles of neurons by the mitochondrial enzyme glutaminase (Bak et al., 2006). Astrocytes, in turn, take up glutamate released into the synaptic cleft by neurons and convert it back to glutamine, a reaction catalyzed by the glial glutamine synthetase enzyme (Bak et al., 2006; Schousboe et al., 2013).

GABA is synthesized from glutamate by glutamate decarboxylase (GAD). In mammals, there are two GAD enzymes named GAD65 and GAD67 (Erlander et al., 1991). While GAD67 is an active holoenzyme that is present throughout the cytosol of GABAergic neurons, GAD65 is enriched in nerve endings and functions predominantly as a dormant apoenzyme, which can rapidly be activated through binding of its co-enzyme, pyridoxal phosphate (Walls et al., 2011). In addition, a second evolutionary conserved mechanism for

GABA synthesis exists, which involves the oxidative deamination of the precursor putrescine to γ -aminobutyraldehyde and the subsequent conversion into GABA by an aldehyde dehydrogenase (Kim et al., 2015; Seiler and Eichenkopf, 1975). Metabolization of GABA to succinic semialdehyde by GABA transaminase occurs only in the presence of α -ketoglutarate, which by accepting the amino group removed from GABA reforms glutamate, while succinic semialdehyde is oxidized to succinic acid that enters the tricarboxylic acid cycle thus completing a metabolic glutamate/GABA loop known as the GABA shunt (Richard and DeLorey, 1999; Schousboe et al., 2013).

As described above, RA signals are crucial for the specification and differentiation of glutamatergic V3 neurons in the ventral spinal cord, where they suppress serotonin neurotransmitter phenotypes by up-regulation of *neurog3* and Notch-mediated inhibition of *ascl1* (Carcagno et al., 2014; Jacob et al., 2013). In cell culture studies, RA was further shown to promote glutamatergic sensory cell fates (Martinez-Monedero et al., 2008; Urban et al., 2015; Yu et al., 2015), which is particularly interesting, since RA signaling has been implicated in the development and regeneration of sensory hair cells in the inner ear that use glutamate as a major neurotransmitter (Lee et al., 2015; Romand et al., 2006; Rubbini et al., 2015; Thiede et al., 2014). However, it is currently unknown if RA signaling can also affect the induction and/or functioning of glutamatergic neurotransmitter systems in a non-permissive manner. In the adult CNS, RA has been reported to decrease levels of astrocytic glutamate transporter-1 (GLT1) through RXR-mediated transcriptional inhibition and the activation of protein kinase C activity, which leads to GLT-1 internalization (Chan et al., 2012). Since GLT-1 is responsible for about 90% of glutamate uptake in the forebrain, its deletion from astrocytes causes excess mortality, lower body weight and spontaneous

seizures, due to the defective clearance and neurotoxicity of extracellular glutamate (Petr et al., 2015). Furthermore, GLT-1 deficiency has been linked to dicarboxylic aminoaciduria and obsessive-compulsive disorders in humans as well as neuro-degeneration in mice (Petr et al., 2015). Conversely, over-expression of GLT-1 reduces glutamate activity and impairs prepulse inhibition of the startle reflex, a simple form of information processing that is reduced in schizophrenia (Bellesi et al., 2009; Bellesi and Conti, 2010). Thus, RA signaling might contribute to the regulation of extracellular glutamate levels, which is essential for CNS function and homeostasis.

RA treatment of embryonic stem cells can be used for the efficient and scalable generation of functional GABAergic interneurons in culture (Addae et al., 2012; Chatzi et al., 2009; Shan et al., 2011). In the subventricular zone of mice basal ganglia, RA generated by RADLH3 is required for the specification and differentiation of both GABAergic striatal projection neurons and GABAergic interneurons that migrate to the olfactory bulb and cortex (Chatzi et al., 2011). In this context, RA signaling was shown to activate the expression of the GABA-synthesizing enzyme GAD67, albeit through an unidentified mechanism, since a RARE has not been identified in the promoter region of the mouse *gad67* gene (Chatzi et al., 2011; Goodman and Kline, 1996).

As previously mentioned, RA signaling is involved in the specification of a population of neurons located in the substantia nigra that co-release dopamine dopamine and GABA (Jacobs et al., 2011; Kim et al., 2015). In these cells, GABA is synthesized by RALDH1, a typical RA synthesizing enzyme, instead of by GAD (Jacobs et al., 2011; Kim et al., 2015). In addition, neurons of the ventral tegmental area, which is located in the vicinity of the substantia nigra, also express RALDH1 and have recently been reported to

co-release GABA with glutamate (Kim et al., 2015; Root et al., 2014). From these findings it can be hypothesized that RA signaling might be involved in the induction and/or the maintenance of a GABA co-transmitter phenotype.

Apart from influencing neurotransmitter identity, RA is capable of rapidly changing excitatory as well as inhibitory synaptic strength, by acting through a very distinct non-genomic mechanism requiring the binding of RA to RAR, which subsequently acts as RNA-binding protein to regulate translation (Arendt et al., 2015a,b). RA synthesis is induced in response to prolonged neural inactivity, due to a lack of calcium influx in the absence of spontaneous neurotransmission (Arendt et al., 2015a,b). In excitatory synapses, RA subsequently enhances synaptic strength by increasing the abundance of postsynaptic ionotropic glutamate receptors (AMPA_Rs) (Arendt et al., 2015a,b; Chen et al., 2014). In inhibitory synapses, on the other hand, RA induces the rapid internalization of GABA_A receptors from the synaptic membrane, thus attenuating inhibitory neurotransmission (Arendt et al., 2015a,b; Sarti et al., 2013). In sum, while the transcriptional regulation by RA signaling can promote distinct neurotransmitter identities in a context dependent manner, RA activity on the translational level directly interferes with synaptic plasticity and neurotransmission (Table I).

8. Retinoic Acid Signaling outside Vertebrates - Evolutionary Considerations

Recent estimates suggest that the total number of animal species on earth ranges from 7.400.000 to 10.000.000, albeit it has also been proposed that there might be 5.000.000 to 6.000.000 species of insects alone (Pimm et al., 2014). Thus far, approximately 1.900.000 extant animal species have been described, but the great majority is still

unknown (Pimm et al., 2014). The phylogenetic relationships between these species hold the key to understanding how different body plans and characters have emerged throughout the course of evolution and what fundamental properties animal life forms have in common. However, many critical relationships, especially at the root of the phylogenetic tree, between the sponge, ctenophore, cnidarian and bilaterian lineages, are still uncertain (Figure 5) (Borowiec et al., 2015; Dunn et al., 2014; Schierwater et al., 2016; Telford and Copley, 2011).

RA signaling was initially described in mammals and, over the past few decades, has been thought to be first vertebrate-, then chordate- and finally deuterostome-specific, with putative roles in protostomes, where incomplete complements of the classical signaling pathway had been identified (Figure 6) (Campo-Paysaa et al., 2008). While the vertebrate RAR binds both all-*trans* and 9-*cis* RA, RXR is thought to bind only 9-*cis* RA (Gutierrez-Mazariegos et al., 2014b). However, endogenous levels of both isoforms have been measured in a variety of animals, including species that lack an unequivocal RAR ortholog (André et al., 2014; Gutierrez-Mazariegos et al., 2014b). Studying how the RA system is deployed in other animal taxa is thus likely to reveal not only the conserved properties of this impotent vertebrate morphogen, but also new functions, signaling cascades and regulatory mechanisms. This might further allow the identification of new medical targets or strategies to manipulate RA-dependent processes either during disease progression or in regenerative therapy (Camacho, 2003; Collins and Mao, 1999; Dollé and Niederreither, 2015).

ADH and RALDH enzymes for the synthesis of RA are ubiquitously distributed across the animal kingdom (Figure 6) (André et al., 2014). Even the demosponge *Suberites*

domuncula possesses a β -carotene dioxygenase and a retinal dehydrogenase/reductase and it was further suggested that various retinoid precursors are generated by bacteria encapsulated in the bacteriocytes of this animal (Müller et al., 2011; Wang et al., 2013; Wiens et al., 2003). Treatment with exogenous RA was shown to up-regulate the gene expression of a RXR-like protein, a BMP and a LIM protein in primmorphs of *Suberites domuncula*, while also stimulating the formation of canal-like structures and siliceous spicules (Larroux et al., 2006; Müller et al., 2011; Wang et al., 2013; Wiens et al., 2003). Additional evidence for the RA-mediated regulation of a homeobox-containing gene comes from the freshwater sponge *Ephydatia muelleri* (Nikko et al., 2001). Furthermore, three aldehyde dehydrogenases, a single CYP26 and a single RXR have been identified in the placozoan *Trichoplax adhaerens* (Albalat and Cañestro, 2009; Reitzel et al., 2011). Together, these data indicate an ancient origin of RA signaling at the base of metazoans, which might have involved microorganismic contributions. However, the presence of other pathway components, such as retinoid binding proteins and true RAR receptors, as well as the structural and functional properties of sponge RXR-like proteins remain to be investigated.

Comparable to the situation in sponges, RARs are probably absent from ctenophores and cnidarians (Figure 6) (Laudet et al., 2015). In fact, only three NRs have been identified in ctenophores so far, all of which belong to the subfamily NR2A (HNF4) and lack an otherwise well-conserved zinc-finger DNA-binding domain (Reitzel et al., 2011). Analyses of the genomes of two anthozoan and one hydrozoan species, all of which do not have a jellyfish stage in their life cycle, did not detect a bona fide RXR (Chapman et al., 2010; Fuchs et al., 2014; Putnam et al., 2007; Shinzato et al., 2011). In contrast, RXRs are present in species possessing a medusoid stage, such as the scyphozoan *Aurelia aurita*, the

hydrozoan *Clytia hemisphaerica* and the cubozoan *Tripedalia cystophora*, in which the RXR was further shown to bind 9-*cis* RA with high affinity and to interact as a monomer with the promoters of J1A and J1B crystallins (Fuchs et al., 2014; Kostrouch et al., 1998). In the scyphozoan *Aurelia aurita*, the transition from a sessile asexual polyp stage to a pelagic medusa via strobilation (spontaneous transverse body segmentation) coincides with the differential up-regulation of RA synthesizing enzymes and RXR (Fuchs et al., 2014). Moreover, pharmacological blocking of RXR function suppresses strobilation, while incubation in retinol or RA is sufficient to substitute for the temperature shift that normally induces this process (Fuchs et al., 2014). These findings prompted the hypothesis that RA signaling links putative temperature-sensing receptors to downstream genes responsible for the onset of metamorphosis in *Aurelia aurita* (Fuchs et al., 2014).

This notion is further supported by a recent transcriptomic analysis of the different life cycle stages of *Aurelia aurita*, showing that RXR up-regulation during strobilation is accompanied by the up-regulation of *klf13* (krüppel-like factor 13) and precedes the expression of the paired-like homeobox gene *drg* (Brekman et al., 2015). In vertebrates, KLFs are involved in various developmental processes, such as cell cycle regulation, and are known for their regulatory interactions with NRs, including RAR-dependent RA signaling (Knoedler and Denver, 2014; Shi et al., 2012; Zheng et al., 2009). DRG transcription factors, in turn, are known for controlling somatosensory functions in the PNS of a wide variety of animals, including both invertebrates and vertebrates (Brekman et al., 2015; Nomaksteinsky et al., 2013). Several other studies provide evidence for an involvement of RA signaling in cnidarian development. For instance, in cell cultures of the anthozoan *Renilla koellikeri* RA positively regulates cell proliferation and differentiation into

epithelium-associated cells including sensory cells (Estephane and Anctil, 2010), in the planula larvae of the hydrozoan *Clava multicornis* RA signaling is required during nervous system patterning and neurogenesis (Pennati et al., 2013), and in two anthozoan species RXR-like immunoreactivity was localized in putative ectodermal sensory neurons (Bouzaïene et al., 2007). In sum, RA signaling functions appear to be well integrated into cnidarian development, with particularly important roles during metamorphosis and neural development. Yet, the classical RA signaling pathway, mediated by RAR/RXR heterodimers, is likely an innovation of bilaterian animals, although taxon sampling in non-bilaterian groups is still insufficient to reach a final conclusion (Laudet et al., 2015).

In the Ecdysozoa, RARs have most likely been lost secondarily (Figure 6) and the only documented case of a cellular retinoid binding protein, which recognizes both retinol and RA, comes from the shrimp *Metapenaeus ensis*, where it is expressed in the ovary (together with RXR), in the eyestalk, testis and epidermis (Cui et al., 2013; Gu et al., 2002; Laudet et al., 2015). However, the proposed phylogenetic affinity between this shrimp retinoid binding protein and the chordate CRBPs and CRABPs is highly controversial (Gu et al., 2002; Laudet et al., 2015) and it has thus been proposed that retinoid binding proteins evolved independently in the Ecdysozoa and Chordata (Albalat et al., 2011). Furthermore, a nematode-specific fatty acid- and retinoid-binding protein (FAR) has been described that is able to bind RA and is involved in nematode development, reproduction, and the infection of host plants (C. Zhang et al., 2015). Of note, the phylogenetic relationships between this nematode-specific protein and the chordate CRBPs and CRABPs still remain to be assessed.

RXRs, on the other hand, are well known to be widely distributed across all bilaterian phyla and have been cloned from various representatives of the Deuterostomia,

Ecdysozoa and Lophotrochozoa (Figure 6), including the platyhelminthes *Schistosoma mansoni* and *Schistosoma japonicum* (de Mendonça et al., 2000; Laudet et al., 2015; Qiu et al., 2013). While the two RXR homologues of *Schistosoma mansoni* are unlikely to bind RA and probably require specific heterodimeric partners to interact with DNA response elements, it was reported that the RXR of *Schistosoma japonicum* can bind 9-*cis* RA and is up-regulated by RA treatment (de Mendonça et al., 2000; Qiu et al., 2013). In nematodes, RXR antagonist as well as synthetic and natural retinoids have been shown to cause developmental defects and decrease animal viability (Hurst et al., 2014).

Arthropod RXRs typically form heterodimers with ecdysone receptors to mediate ecdysteroid hormone functions during development, reproduction, molting, metamorphosis and regeneration (André et al., 2014). In crustaceans, multiple RXR isoforms are differentially expressed (Cui et al., 2013; Li et al., 2014), but it is not clear whether they can also transduce RA signals, which have so far only been shown to affect glucose metabolism and negatively interfere with limb regeneration in decapods (Hopkins and Durica, 1995; Sreenivasula Reddy and Srilatha, 2015). In insects, however, the presence of endogenous 9-*cis* RA and all-*trans* RA as well as their ability to directly activate RXR orthologs has been confirmed (Nowickyj et al., 2008) and a number of physiological responses to RA signals have been described, such as teratogenic effects on larval development (Nakamura et al., 2007) and the inhibition of metamorphosis (Němec et al., 1993), but also positive effects on cell survival and neurite outgrowth in cultures of embryonic locust neurons (Sukiban et al., 2014). Interestingly, RXR immunoreactivity was detected in both the cytoplasm and the neurites of specific neuronal subsets in the embryonic and adult CNS of the locust *Locusta migratoria*, hinting at a potential role for RA

in insect neurogenesis and nervous system function (Bui-Göbbels et al., 2015; Sukiban et al., 2014). Moreover, RA signals have been reported to regulate neurohormonal gene expression in response to tissue damage in the fruit fly *Drosophila melanogaster*, thus generating a developmental checkpoint for regeneration (Halme et al., 2010). This is a surprising result considering that the RXR of *Drosophila melanogaster*, called Ultraspiracle (USP), is probably unable to bind RA (Iwema et al., 2007). Altogether, these findings strongly suggest that RA signaling is present in at least some ecdysozoan species, although the physiological relevance and underlying mechanisms are still largely unexplored.

In addition to RXRs, lophotrochozoans and deuterostomes also possess RARs and *cyp26* orthologs (Figure 6), suggesting an increased importance of RA signaling functions in these two clades (Laudet et al., 2015). In gastropod mollusks, *rxr* and *rar* are expressed starting from early developmental stages and treatment with either exogenous RA, RXR pan-agonist or vertebrate RAR antagonist disrupts embryogenesis causing, for example, abnormal eye and shell development (Carter et al., 2015, 2010; Creton et al., 1993). Similar to the locust *Locusta migratoria*, RXR, but also RAR, of the gastropod *Lymnaea stagnalis* are localized in the cytoplasm, neurites and growth cones of CNS neurons (Bui-Göbbels et al., 2015; Carter et al., 2015, 2010). While RAR antagonist blocks RA-dependent growth cone turning in regenerating neurons of *Lymnaea stagnalis*, exposure to exogenous RA or RXR agonist induces growth cone turning, even in isolated neurites without a cell body or nucleus (Carter et al., 2015, 2010; Farrar et al., 2009). This suggests that a non-genomic RA-dependent mechanism, mediated by both RAR and RXR, controls growth cone guidance in *Lymnaea stagnalis* (Carter et al., 2015, 2010).

Electrophysiological experiments have further revealed rapid dose- and isomer-dependent effects of RA on the firing properties of *Lymnaea stagnalis* neurons, which were significantly reduced in the presence of RXR antagonist and are likely mediated through the RA-dependent modulation of calcium signaling (Vesprini et al., 2015; Vesprini and Spencer, 2014). RA is also necessary for long-term memory formation in *Lymnaea stagnalis*, but not for learning or intermediate-term memory, and this function of the (light-sensitive) RA molecule can be promoted by constant darkness (Rothwell et al., 2014; Rothwell and Spencer, 2014). Altogether, these roles of RA signaling in gastropod mollusks are reminiscent of non-genomic RA actions during vertebrate homeostatic synaptic plasticity. For example, within neuronal dendrites RA has been shown to bind RAR that subsequently functions locally as a RNA-binding protein modulating protein translation (Chen et al., 2014). Concerning RA functions outside of the mollusk nervous system, injection of 9-*cis* RA into adult gastropod females was shown to trigger the development of male sexual organs, in a RXR-dependent, and likely RAR-independent, manner (Abidli et al., 2013; Castro et al., 2007; Stange et al., 2012). Intriguingly, this phenomenon, also known as imposex, seems to correlate with an inability to store retinoids in the form of retinyl esters in susceptible species (Gesto et al., 2013). Taken together, these studies illustrate multiple roles for both RXR- and RAR-dependent RA signals during snail development and neurogenesis. It is thus tempting to conclude that lophotrochozoan and vertebrate RA signaling systems are quite similar. Conversely, however, the RARs of at least two gastropod species, *Nucella lapillus* and *Thais clavigera*, are incapable of binding RA, albeit exhibiting the capacity to form heterodimers with RXRs and to recognize DNA response elements organized in direct repeats (Gutierrez-Mazariegos et al., 2014a; Urushitani et al., 2013). Accordingly, investigations of classical as well as alternative RA signaling pathway components and their

functional properties in basal lophotrochozoans, such as lophophorate annelids and platyhelminthes, are required to shed additional light on the mechanisms of action of this morphogen in lophotrochozoans.

In deuterostomes, all major components needed for classical RA signaling, i.e. RAR and RXR as well as RALDH and CYP26, appear to be present (Figure 6), although retinoid binding proteins, such as CRBPs and CRABPs, have so far only been identified and characterized in vertebrates, with the notable exception of one putative CRBP ortholog in cephalochordates (Laudet et al., 2015). At present, very little is known about the functions and mechanisms of RA signaling in non-chordate deuterostomes. For instance, *all-trans* RA might either disrupt or merely delay sea urchin embryonic development and probably interferes with the migration of micromere-derived cells (Laudet et al., 2015). In contrast, studies in invertebrate chordates, such as cephalochordates and ascidian tunicates, have revealed numerous well-conserved functions of RA signals during chordate development, for example in axial patterning, tissue specification and organogenesis (Campo-Paysaa et al., 2008; Carvalho and Schubert, 2013; Laudet et al., 2015). In tunicates, an independent loss of the RA machinery has occurred in certain groups, such as the larvaceans, and some studies have implicated RA and RAR in tissue regeneration and bud development (André et al., 2014). Conversely, it has been shown that the RXR of the ascidian *Polyandrocarpa misakiensis* induces transdifferentiation-related genes such as RXR, ERK and MYC in a RA-dependent manner without a major contribution of RAR (Kawamura et al., 2013). Remarkably, despite gene loss and fragmentation of the tunicate Hox cluster, RA-driven *hox1* expression in the epidermal ectoderm of the ascidian *Ciona intestinalis* was shown to be essential for organizing the atrial siphon placode, a homolog of the vertebrate otic

placode (Sasakura et al., 2012). This hints at an invertebrate chordate origin of RA signaling functions in vertebrate neural crest and placode development. In addition, interactions between the RA, FGF/MAPK and canonical Wnt pathways control anterior-posterior patterning of the tail epidermis and tail PNS in *Ciona intestinalis* in a manner that resembles mechanisms, which are typically deployed by vertebrates to coordinate embryonic patterning and body elongation (Pasini et al., 2012).

In contrast, RA and FGF signals probably act independently during posterior elongation in the cephalochordate amphioxus and, contrary to vertebrates, are not mandatory for posterior somite formation (Bertrand et al., 2015). Yet, cephalochordates are characterized by a particularly vertebrate-like, albeit less elaborate, retinoid signaling system (Laudet et al., 2015). Since this invertebrate chordates have not undergone the two rounds of whole genome duplications that are typical of vertebrates, *rar* and *rxr* are each encoded by a single genomic locus in amphioxus (André et al., 2014; Laudet et al., 2015). Importantly, it has been demonstrated that the amphioxus RAR/RXR heterodimer can be activated by binding RA, which is necessary, for instance, for establishing the collinear expression patterns of amphioxus *hox* genes as well as for the specification of different cellular identities throughout the amphioxus larva (Escriva et al., 2002; Schubert et al., 2006). Nevertheless, amphioxus seems to lack several genes encoding retinol storage, transport and cellular uptake proteins, which might thus be a vertebrate exclusivity, although storage of retinyl esters might also occur in gastropod mollusks (Albalat et al., 2011; André et al., 2014; Laudet et al., 2015). Moreover, RA signaling in amphioxus does not seem to engage in the mutual antagonistic crosstalk with FGF signaling that governs many developmental processes in vertebrates (Bertrand et al., 2015).

In conclusion, RA signaling exhibits a great diversity of biological functions (Figure 7) and molecular mechanisms that seem to be scattered across the animal kingdom. This impression can be attributed at least in part to the very scarce availability of reliable information from many invertebrate taxa. Future work will thus have to address this problem by focusing on the characterization of the retinoid system and its function in various organisms located at key positions in the animal tree of life, including, but not being limited to, sponges, ctenophores, cnidarians, xenacoelomorphs, basal ecdysozoans and lophotrochozoans, cephalopods, echinoderms, hemichordates as well as additional invertebrate chordate taxa.

9. Concluding Remarks

Throughout vertebrate development, opposing RA and FGF signaling gradients are fundamental for coordinating the formation and patterning of many different tissues and organ systems (Figure 2). During this process, RA signaling contributes to the proper specification of neural progenitor populations and controls their progression into differentiated neurons in a context-dependent manner, thus matching neurogenesis to other organogenetic processes occurring along the embryonic trunk. However, while considerable progress has been made towards elucidating the molecular framework underlying RA-driven neural progenitor specification and differentiation, much less is known about how and under which conditions other functions of RA are mediated. For instance, the role RA plays in promoting neural progenitor proliferation and maintenance, in determining the subtype identity and neurotransmitter phenotype of differentiating neurons and in modulating neurotransmission and synaptic plasticity are just beginning to be uncovered.

In order to better exploit RA for therapeutic purposes, it is thus urgent to expand our knowledge of its context-dependency and to characterize its alternate signaling mechanisms, functions and interactions. To this end, new techniques that allow the visualization of RA signaling activity *in vivo* should help to pinpoint regions of particular interest (Shimozono et al., 2013). Furthermore, it will be important to expand studies of RA signaling towards later developmental and adult stages and perform comprehensive stage-, tissue- and cell-specific assays of its genomic and non-genomic targets.

Moreover, it will be important to investigate the evolution of RA signaling systems, since most invertebrate species appear to deploy this molecule in different ways that could provide valuable clues for the identification of novel targets and tools for the pharmaceutical manipulation of RA-dependent processes in vertebrates. Also, exciting new insights into how a more complex RA signaling system might have contributed to the formation of a more elaborate nervous system could potentially be gained from taxons, such as cephalochordates, which possess a prototypical chordate RA signaling and nervous system, or cephalopods, which are characterized by a highly complex nervous system that has evolved independently from that in vertebrates (Figure 5).

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Figure and Table captions

Figure 1 (to be featured on page 10): Schematic representation of the structure, metabolism and canonical signaling pathway of retinoic acid (RA). Abbreviations: ADH, alcohol dehydrogenase; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; CYP26, cytochrome P450 subfamily 26 protein; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RBP, retinol binding protein; RXR, retinoid X receptor; SDR, short-chain dehydrogenase/reductase; STRA6, stimulated by retinoic acid protein 6; TTR, transthyretin. Modified from (Laudet et al., 2015).

Figure 2 (to be featured on page 15): Overview of vertebrate tissues and organ systems that require retinoic acid (RA) signaling for their proper development. Modified from (Carvalho and Schubert, 2013).

Figure 3 (to be featured on page 20): Networks of interacting signals that, in vertebrates, control (A) the anterior-posterior regionalization of the neural plate, (B) the anterior-posterior patterning of the hindbrain and spinal cord domains of the neural tube and (C) the dorsal-ventral patterning of the spinal cord domain. Abbreviations: BMP, bone morphogenetic protein; CDX, caudal-related homeobox protein; CER, cerberus protein; DI1-DI6, dorsal interneurons 1-6; DKK, dickkopf protein; FB, forebrain; FGF, fibroblast growth factor; FP, floor plate; FRZB, frizzled-related protein; HB, hindbrain; HOX, homeobox protein; IGF, insulin-like growth factor; MB, midbrain; MN, motorneurons; RA, retinoic acid; RP, roof plate; SC, spinal cord; SHH, Sonic hedgehog; V0-V3, ventral interneurons 0-3; WNT, wingless-related integration site protein.

Figure 4 (to be featured on page 40): Network of interacting signals that, in vertebrates, act along the developing central nervous system (CNS) to govern neural crest and cranial placode formation. A) Specification and regionalization of the pre-placodal region (PPR) at the anterior neural plate border. B) Coordination of neural crest cell (NCC) specification and emigration along the developing neural tube, posterior to the midbrain-hindbrain boundary (MHB). Abbreviations: BMP, bone morphogenetic protein; CYP26, cytochrome P450 subfamily 26 protein; DKK, dickkopf protein; FGF, fibroblast growth factor; MHB, midbrain-hindbrain boundary; NCC, neural crest cells; PPR, pre-placodal region; RA, retinoic acid; RIPPLY3, ripply transcriptional repressor 3; TBX1, t-box 1 protein; WNT, wntless-related integration site protein; ZIC1, zic family member 1.

Figure 5 (to be featured on page 50): Phylogenetic tree depicting a consensual view of metazoan relationships. Dotted lines indicate taxons with uncertain phylogenetic positions. Names of species discussed in this review are indicated. Data were retrieved from (Borowiec et al., 2015; Dunn et al., 2014; Telford and Copley, 2011; Westheide and Rieger, 2013).

Figure 6 (to be featured on page 66): Distribution of retinoic acid (RA) signaling pathway components across the animal kingdom. The colored bars indicate the presence of proteins required for canonical RA signaling in different taxa. Abbreviations: CYP26, cytochrome P450 subfamily 26 protein; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; RALDH, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid X receptor.

Figure 7 (to be featured on page 75): Summary of retinoic acid (RA)-dependent processes in different invertebrate taxa. Note that, except for cephalochordates, these functions are

likely mediated through alternative signaling mechanisms, i.e. not involving the activation of retinoic acid receptors (RARs) by RA.

Table I (to be featured on page 51): Overview of different neurotransmitter systems that, in vertebrates, can be induced (+) or suppressed (-) by retinoic acid (RA) signaling in specific neuronal subsets.

Effects of Retinoic Acid Signaling on Neurotransmitter Phenotype Differentiation

Neurotransmitter	Location	Affected genes	References
Dopamine	Substantia nigra	TH D1 and D2 receptor	(Jacobs et al., 2007; 2011)
Norepinephrine	Hindbrain	AP-2	(Holzschuh et al., 2003)
Serotonin	Hindbrain	Neurog3 SERT 5-HT _{1A} receptor	(Neumeister et al., 2004; O'Reilly et al., 2007; Jacob et al., 2013; Carcagno et al., 2014)
Glutamate	Spinal cord	Neurog3 Notch/Delta GLT1	(Chan et al., 2012; Jacob et al., 2013; Carcagno et al., 2014)
GABA	Basal ganglia of the subventricular zone Substantia nigra	GAD67 ALDH1a1	(Chatzi et al., 2011) (Jacobs et al., 2011; Kim et al., 2015)

Central nervous system
Blood-brain barrier

Neural plate

Neural tube

Neural crest

Cranial placodes

RA

**Anterior
endoderm**

**Trunk
mesoderm**

Germ cells

Craniofacial structures

Teeth

Eye

Inner ear

Olfactory epithelium

Enteric nervous system

Lung

Foregut

Liver

Pancreas

Skeleton

Musculature

Heart

Kidney

B	Neural tube
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C	Spinal cord
---	-------------

A

B

CRABP

CRBP

RAR

RXR

CYP26

RALDH

Porifera Cnidaria Placozoa Lophotrochozoa Ecdysozoa Ambulacraria Cephalochordata Tunicata Vertebrata

Chordata

Protostomia

Deuterostomia

Bilateria

Porifera

**Stimulation of canal and
spicule formation**

Cnidaria

**Strobilation, cell cycle control,
nervous system patterning**

Ecdysozoa

**Metamorphosis, regeneration,
neurite outgrowth**

Lophotrochozoa

**Imposex, eye and shell formation,
synaptic plasticity, Ca²⁺ signaling,
neurite outgrowth**

Chordata

**Axial patterning, tissue specification,
organogenesis, regeneration,
neurotransmission**