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Chapter 1: Introduction

1.1. Wnt Signaling through the Ror Receptor in the Nervous System

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Wnt Signaling through the Ror Receptor in the Nervous System

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Abstract The receptor tyrosine kinase-like orphan receptor (Ror) proteins are conserved tyrosine kinase receptors that play roles in a variety of cellular processes that pattern tissues and organs during vertebrate and invertebrate development. Ror signaling is required for skeleton and neuronal development and modulates cell migration, cell polarity, and convergent extension. Ror has also been implicated in two human skeletal disorders, brachydactyly type B and Robinow syndrome. Rors are widely expressed during metazoan development including domains in the nervous system. Here, we review recent progress in understanding the roles of the Ror receptors in neuronal migration, axonal pruning, axon guidance, and synaptic plasticity. The processes by which Ror signaling execute these diverse roles are still largely unknown, but they likely converge on cytoskeletal remodeling. In multiple species, Rors have been shown to act as Wnt receptors signaling via novel non-canonical Wnt pathways mediated in some tissues by the adapter protein disheveled and the non-receptor tyrosine kinase Src. Rors can either activate or repress Wnt target expression depending on the cellular context and can also modulate signal transduction by sequestering Wnt ligands away from their signaling receptors. Future challenges include the identification of signaling components of the Ror pathways and bettering our understanding of the roles of these pleiotropic receptors in patterning the nervous system.

Keywords Ror · Wnt · CNS · Neuronal Migration · Axonal Branching and Pruning · Synapse

Introduction

The receptor tyrosine kinase (RTK) superfamily consists of multiple diverse cell surface receptor proteins required for essential cellular processes such as cell survival and metabolism, cell cycle control, cell migration, proliferation, and differentiation [1]. Despite strong overall structural conservation of RTKs in evolution from Caenorhabditis elegans to humans, significant diversity exists within the RTK super family. In humans, for example, there are 58 distinct RTKs that can be divided into 20 subfamilies [2]. All RTKs have similar constellations of predicted protein domains: an extracellular domain with ligand-binding properties, a single transmembrane (TM) domain and an intracellular domain with a putative tyrosine kinase (TK) domain. Generally, activation of RTKs is initiated by binding of a ligand which leads to receptor dimerization or oligomerization. Subsequent autophosphorylation activates the kinase to phosphorylate substrates on specific tyrosines and is followed by recruitment of downstream pathway members [3, 4, 2].

The focus of this review is one subfamily of the RTKs, the receptor tyrosine kinase-like orphan receptors (Rors), and their roles in the development and function of the nervous system. Similar to most RTKs, the Rors are highly conserved during evolution. The Ror receptors were first identified in a human neuroblastoma cell line by their homology to the tropomyosin receptor kinase (Trk) receptor [5]. Orthologs were later found to exist in Aplysia [6], Torpedo [7], Drosophila (Ror [8] and neurospecific receptor kinase (Nrk) [9]),

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Xenopus [10], mouse [11], rat [12], and C . elegans [13, 14]. We will begin by discussing the predicted structures of the Ror orthologs and recent reports on their mechanisms of action in non-neural tissues.

Ror family proteins exhibit a high degree of similarity in their amino acid sequences and putative domain structures, however, some deviate from the norm as detailed below. The consensus Ror protein structure consists of an extracellular cysteine rich ligand-binding domain (CRD), an immunoglobulin (Ig) domain, a kringle domain, a TM domain, and an intracellular TK-homologous domain (Fig. 1). The CRD domain of Rors is similar to that in the Frizzled (Fz) receptors [15, 16, 17], which were found to act as receptors for Wnt proteins [18]. Recently, there have been a number of studies showing that in various species, Wnt signaling can also be mediated by the Ror receptor family, likely by Wnt binding to its CRD domain [10, 19–27]. The Ig domain [28], which is predicted to facilitate the interaction of Ror with other cell-surface proteins, is present in one or two copies in all Ror family members except those in Drosophila . In addition, some of the Ror receptors, are predicted to have a constellation of one or two serine/threonine- (S/TRD) and/or proline-rich (PRD)-domains [29–32].

The kringle domain, present in all Rors, was first identified as a domain present in proteases of the blood-clotting cascade [33, 34]. It forms a large protein loop stabilized by disulfide bonds and is thought to mediate its association to other proteins, membranes, and/or phospholipids. An analysis of the role of the kringle domain in Ror function has not yet been reported.

All Rors possess a putative TK domain, however, catalytic activity has been demonstrated for only some of them, i.e., for mRor2 [21, 23, 35, 36] and Drosophila Nrk [9]. The Ror TK domain contains the YXXDYY motif, also common in other RTKs like Trk and muscle-specific kinase (MuSK) and specifically required for kinase activation [37, 38]. In addition, the TK domain of mammalian and Drosophila Rors have a conserved sequence (YALM in mammalian Rors and Drosophila Nrk and YSLM in Drosophila Ror), which is predicted to bind the Src homology 2 (SH2) region of the c-Src non-receptor tyrosine kinase [39].

We start by briefly reviewing important insights into Ror function gleaned from studies outside of the nervous system. An important in vivo role for mRor2 receptor tyrosine kinase activity was demonstrated by Mikels and colleagues who implicated its' requirement for Wnt5a induced inhibition of β-catenin signaling in transgenic mice bearing the Wnt reporter Axin2 [40]. They reported that the Ig domain, CRD, TK, S/TRD, and PRD are required for mRor2 to transduce the Wnt5a signal. Furthermore, they showed that Wnt5a protein can enhance mRor2 tyrosine kinase activity in an in vitro assay [40]. In other cellular contexts, it was found that c-SRC is required for mRor2 receptor activation [41, 42]. Native mRor2 phosphorylation is induced by binding of Wnt5a and is blocked by pharmacological inhibition of c-Src kinase activity. In osteosarcoma cell lines, Wnt5a-Ror2 signaling activates c-Src, thereby inducing the expression of the matrix metalloprotease MMP-13, that stimulates the formation of invadopodia correlated with tumor cell invasion [41]. The PRD of mRor2 plays a crucial role in recruiting c-Src and subsequent full activation of mRor2 via phosphorylation by c-Src [42]. In this study, an "ignition trigger" model of Ror2 activation was proposed, whereby low levels of intrinsic Ror2 kinase activity are sufficient to recruit and activate c-Src which amplifies Ror2 receptor phosphorylation to the full active state.

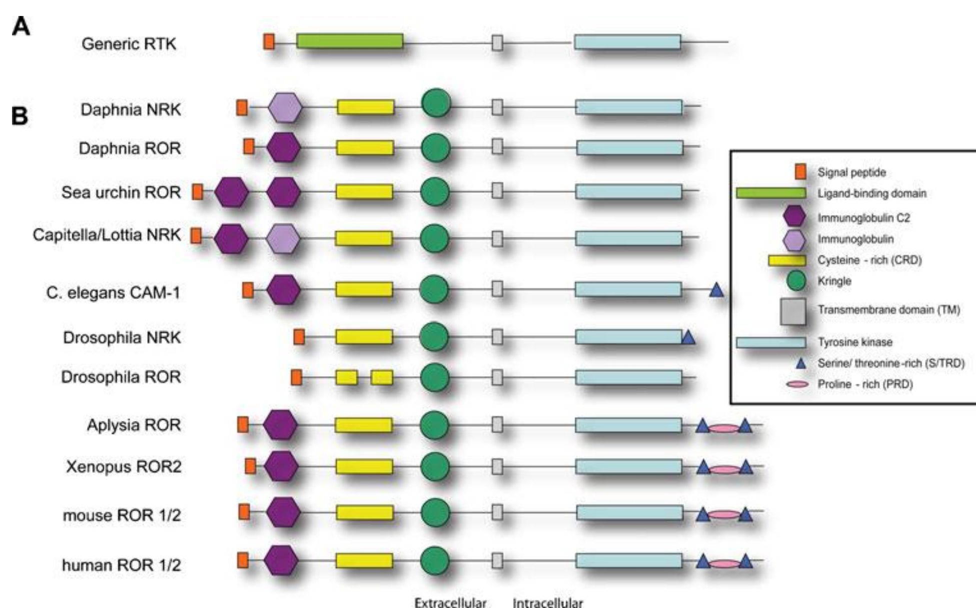

Fig. 1 Schematic domain structures of a generic RTK receptor (a) and of the Ror receptors in different species (b). The domains are identified in the box on the right hand side and are not presented on relative scale

It is much less clear whether Ror1 has kinase activity: there are six deviations from the canonical tyrosine kinase consensus sequence and three of these alternative amino acids are in parts predicted to be essential for the catalytic activity, C482G, K614R, and L634F [32, 43, 44]. The intrinsic catalytic activity of hRor1 was examined by testing its ability to auto phosphorylate or to phosphorylate exogenous substrates. The amount of hRor1 autophosphorylation was insignificant in comparison with a reference tyrosine kinase (ErbB2). Similarly, hRor1 failed to phosphorylate exogenous peptides like catalytically inactive ErbB3 [45]. The authors conclude that hRor1 likely is a pseudokinase. Drosophila Ror also has a number of amino acid substitutions in its kinase domain that would likely cause it to be inactive as a kinase, for example, it does not contain the conserved tyrosine that is expected to be the target for autophosphorylation [8].

Ror expression during development has been studied in a number of organisms, e.g., C . elegans , Drosophila melanogaster, Aplysia californica , Xenopus laevis , and the mouse. In all these species, Ror RTKs are found to be highly abundant in the nervous system, suggesting potential roles of these receptors in the development and maintenance of this tissue. An important step towards a better understanding of Ror function was the discovery that Rors can act in Wnt-dependent pathways [10, 19–27]. Wnts are secreted intracellular signaling proteins acting in many tissues during development [46]. They have roles, among others, in axon guidance, nervous system cell fate determination, and in the formation and maintenance of synapses (reviewed in [47–51]). Wnts can act via a number of distinct signaling pathways, five of which have been described to date. The most studied is the so-called canonical Wnt pathway (reviewed in [52]). Canonical Wnt signaling is activated by Wnt binding to the Fz and LDL-receptor-related protein (LRP) families of co-receptors, resulting in the cytosolic stabilization and nuclear translocation of β-catenin. T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, together with β-catenin, regulate transcription of specific target genes. Wnt binding to Fz receptors can also activate a non-canonical pathway involving small Rho family GTPases and the c-Jun N-terminal kinase which regulates planar cell polarity (PCP) [53].

A third Wnt/Fz pathway controls heteromeric G-proteins to induce the release of intracellular Ca^{2+}, acting via Ca^{2+}/calmodulin regulated kinase and protein kinase C, resulting in the nuclear accumulation of the transcription factor NF-AT [54]. A fourth Wnt pathway involves another family of Wnt receptors, the related to tyrosine kinase (Ryks) (reviewed in [55]). While Ryks are distinct from the Rors, they also belong to the RTK superfamily [2]. However, Ryk catalytic activity has not yet been demonstrated and little is known about Ryk's downstream effectors. The Ryks, although not functioning exclusively in the nervous system [56], have been shown to play important roles there (reviewed in [55]).

The signaling components of the Wnt/Ror pathways are still largely unknown. However, in some cellular contexts Rors activate the non-canonical Wnt/Jun N-terminal kinase (JNK) PCP pathway and in other contexts, inhibit the canonical β-catenin/TCF-LEF pathway. The non-canonical Wnt ligand Wnt5a appears to be the predominant ligand for Ror receptor signaling in vertebrates. Specifically, Wnt5a has been demonstrated to bind Ror2 resulting in its heterodimerization with Fz2 via its CRD domain and has been shown to activate the non-canonical JNK pathway [19]. Wnt5a binding to Ror2 can also inhibit contemporaneous Wnt3a/Fz canonical signaling in a dose-dependent manner [23]. The canonical pathway target of this inhibition is not known but it does not appear to be β-catenin but a still unknown pathway member downstream of it. Supporting these observations, it has been shown that suppressing the levels of Ror2 or Wnt5a in human osteosarcoma SaOS-2 cells results in an enhancement of TCF / LEF –mediated transcription without apparent affect upon the stabilization of β-catenin [41]. Disheveled (Dvl) is a common downstream pathway member of all Wnt pathways known to date and, perhaps not surprisingly, Wnt5a-Ror signaling has recently been shown to also affect the level of Dvl2 phosphorylation [57].

Two human skeletal disorders have been linked to mutation of Ror2. Homozygous mutations in hRor2 causes Robinow syndrome, a skeletal dysplasia syndrome characterized by limb shortening, segmental defects of the spine and dysmorphic facial appearance; heterozygous mutation in hRor2 causes brachydactyly type B1 (BDB1), a terminal deficiency of fingers and toes [58–66]. Interestingly, the PRD of hRor2 is deleted in BDB1, suggesting that in the patients suffering from this disorder, Ror mutant receptors are defective in kinase activation as a result of a failure to recruit Src. Rors have also been shown to play essential roles in a variety of other developmental processes. In mice, mRor2 is essential for cardiac septal formation; development of limbs and tail; ossification of limbs, tails, vertebrae, and ribs, proliferation; and maturation and motility of chondrocytes [67–71]. Furthermore, abnormally high expression of hRor1 , but not hRor2 , has been correlated with a number of hematological malignancies [72]. To date, no human neurological disorders have been linked with deficiency in Ror signaling.

Ror Protein Structure and Expression Patterns in the Nervous System of Diverse Species

An overview of the Ror expression domains and functional data gained from studies using different model organisms is presented in Table 1.

Table 1 reported expression domains and roles for Ror family members in the nervous system

Ror	Species	Expression	Reported function	References
CAM-1 (KIN-8)	C. elegans	CNS; intestinal hypodermal and body wall muscle; pharynx	Neuronal migration and neurite outgrowth; axon guidance; axonal pruning; synaptic transmission/function; Wnt receptor	[13, 14, 25, 73, 83, 84, 88, 103]
Ror	Drosophila	CNS; PNS	Not reported	[8, 110]
Nrk	Drosophila	CNS; PNS	Not reported	[9]
mRor1	Mouse	Developing CNS (see Box 1); heart; kidney	Neurite extension; synapse formation; neurogenesis; axon branching; heterodimerization with mRor2; Wnt5a/Dvl signaling pathway	[11, 12, 26, 57, 80, 82, 89]
mRor2	Mouse	Developing CNS (see Box 1); sympathetic neurons; osteoclast precursor cells	Neurite extension; synapse formation; neurogenesis; axon branching; heterodimerization with mRor1; Wnt5a receptor; Wnt5a/Dvl signaling pathway	[11, 12, 19, 26, 57, 67, 71, 80, 82, 89, 102]
Xror2	Xenopus	Dorsal marginal zone of the mesoderm, notochord, and neuroectoderm	Convergent extension; neural plate closure; interacts with XWnt5a, XWnt11, Xfz7	[10, 19, 111]
Apror	Aplysia	Developing neurons; peripheral neuronal processes and ganglionic neuropil	Neuropeptide release	[6]
hRor1	Human	Brain, heart, prostate, kidney, lung	Originally isolated from human neuroblastoma cell line; high expression in patients with myeloid malignancies	[5, 72]
hRor2	Human	Brain, thymus, heart, pancreas, prostate, kidney, uterus, ovary, intestine	Originally isolated from human neuroblastoma cell line; BDB1; Robinow syndrome	[5, 58–66].

Caenorhabditis elegans

The *C. elegans* genome encodes one member of the Ror family of receptors, *cam-1* [13], also called *kin-8* [14]. It contains the prototypic predicted domains of the Ror protein family but lacks the PRD and the second S/TRD, present in mammalian, Aplysia and Xenopus Rors (Fig. 1). CAM-1 is expressed in the nervous system; in the intestinal, hypodermal, and body wall muscles; and in parts of the pharynx. In neurons, CAM-1 is predominantly detected in axons and dendrites [13]. The protein has been localized to central synapses and to the postsynaptic side of the neuromuscular junction (NMJ) [13, 27, 73].

Drosophila melanogaster

Two Ror orthologs exist in *Drosophila*, *Ror* and *Nrk*, also called *Dror* and *Dnrk*. Both predicted proteins have most structural features of typical Ror receptors, such as extracellular CRD and kringle domains, a TM domain, and an intracellular TK domain, highly similar to the TK domain of the vertebrate Trk proteins [3, 74]. However, they do not have the Ig domain that is predicted to be present in all other Rors (Fig. 1). Ror shares 61 % identity with the TK domain of human Ror1 and 54 % with that of human Ror2 [8]. Nrk is somewhat more related to human Ror2 than to Ror1, with overall identities of 45 and 34 %, respectively. In addition, Nrk has considerable overall sequence similarity to MuSK; therefore, Nrk may be evolutionarily distinct from the other Ror family members [75]. *Drosophila* Nrk is considered to be a member of the MuSK family based on the strong homology of the Nrk kinase domain to that of chordate MuSKs; the homology to chordate or other bilaterian Ror kinase domains is considerably lower [76]. However, *Drosophila* Nrk has a kringle domain that is absent from mammalian and sea urchin MuSKs, but present in all Rors [76]. Moreover, mammalian and zebra fish MuSKs possess a signaling domain containing an NPxY consensus site that is not present in *Drosophila* Nrk or any of the Rors [76]. In this review, we include *Drosophila* Nrk as a member of the Ror family based on these last two properties, but it should be noted that its kinase domain has diverged evolutionary from that of most Ror family members.

Striking features of *Drosophila* Ror are that its C-terminus does not contain the typical tyrosine for potential autophosphorylation and its CRD domain is interrupted by a 55-amino acid insertion between the fifth and sixth cysteines (Fig. 1) [8]. Nrk kinase activity has been demonstrated [9]. Ror and Nrk are predominantly expressed throughout the embryonic central and peripheral nervous systems in overlapping domains [8, 9] and are also present in larval muscle fibers and motoneurons (unpublished data, IMP, LGF, and JNN).

Aplysia californica

The genome of the marine mollusk Aplysia californica encodes one Ror protein (ApRor) with an overall structure similar to both human Rors. ApRor also has several non-conserved sites: a potential SH2-binding motif (YSEM) in the kinase domain, several glutamine rich regions in the C-terminal portion and a putative PDZ domain-binding site at the C-terminus [6]. During development, ApRor is expressed in most neurons and later in some adult neuronal populations, including the neuroendocrine-secreting bag-cell neurons, in peripheral neuronal processes and in the ganglionic neuropil [6]. In cultured bag-cell neurons, most of the ApRor protein is present in intracellular organelles with only a small fraction expressed at the cell surface. Cell surface protein is clustered on neurites. ApRor protein co-localizes with the P-type calcium channel BC-alpha1A at bag-cell neuron varicosities, suggesting a possible role for ApRor in stabilizing neuropeptide release sites there [6].

Xenopus laevis and Zebra fish

The Xenopus laevis genome encodes one Ror, Xror2 , a putative ortholog of mammalian Ror2 . Xror2 is expressed in the dorsal marginal zone, the notochord and the neuroectoderm posterior to the midbrain–hindbrain boundary [10]. It contains an Ig domain, a CRD domain, a kringle domain and a TM and a TK domain. The TK domain includes a predicted ATP-binding motif (GXDXXG-AIK), present in all Ror2 receptors [11]. The spatio-temporal expression pattern of Xror2 suggests a role in the development of the embryonic nervous system and indeed such a role has been described in convergent extension of the dorsal neuroectoderm [10].

Comparative genomic analyses also identified Ror1 and Ror2 orthologs in the zebra fish species Danio rerio [77]. The ror2 gene consists of nine exons and is predicted to encode a 939-aa transmembrane protein. It bears 71.7 and 56.2 % total amino acid identity with human Ror2 and Ror1, respectively. No studies of the expression or function of the zebra fish Rors have been reported to date.

Mice

Two mouse Ror receptor RTKs exist, mRor1 and mRor2. Besides the prototypical domains present in all Ror RTKs, mRors also contain a single Ig domain and a PRD at their cytoplasmic C-terminus. Interestingly, mRor1 (and hRor1) also possesses a consensus motif XPPXY within its PRD, that is predicted to bind WW domain containing proteins [78]. The same motif can also bind SH2-containing proteins upon phosphorylation of the tyrosine residues within the motif [78]. Additionally, mRor2 (and hRor2 and Drosophila Nrk) harbor the motif YALM, another potential target for binding of proteins with SH2 domains, subsequent to phosphorylation on tyrosine.

Both mouse Ror genes are expressed during prenatal development, but while mRor1 expression is particularly high in the neural crest, mRor2 is widely expressed in both neuronal and non-neuronal tissues. After birth, mRor2 expression declines and can only be detected in a limited domain in the cerebellum; mRor1 expression persists postnatally not only in the brain but also in non-neuronal tissues such as heart and lungs. The specificity of the spatial and temporal expression patterns of the two mouse Rors suggest their differential roles in the development of the nervous system and other organs such as heart and lungs [11]. A detailed description of the complex expression domains of the mouse Rors is presented in Box 1.

Box1: Temporal and Spatial mRor1 and mRor2 Expression Domains During Mouse Development [79] [11].

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mRor expression during prenatal development is firstly detected at stage E7.5, when mRor2 is expressed in the primitive streak and mRor1 in a domain in the anterior part of the embryo. At stage E8.5 there are high levels of mRor1 in neural crest cells and mRor2 is more widely expressed in non-neuronal and neuronal tissues, including the prosencephalon, mesencephalon, and the neural tube. At E9.5 through E10.5, both mouse Rors have overlapping expression domains in a number of tissues originating, in part, from neural crest cells. mRor2 is present in the forebrain and midbrain, while mRor1 is expressed in the dorsal part of the diencephalon and mid-hindbrain boundary. During E12.5 and 13.5, mRor1 and mRor2 are detected in the perichondrium of the digits and the marginal regions of the limbs. In the developing brain at stage 13.5, mRor2 is predominantly expressed in the limbic neocortex, the hippocampal neuroepithelium, and caudate putamen. At this stage, mRor1 is not detected in the brain, but accumulates in the lens epithelium of the developing eye.

After birth at postnatal day p6 and p8, both genes are expressed in the medulla oblongata. After p23 up to adult stages mRor2 can no longer be detected in any tissue except for certain domains in the cerebellum, whereas mRor1 expression is sustained in the heart, lungs, kidney, thymus, and in the brain. Specifically, in the developing cerebellum after birth, mRor1 is mainly expressed in the external granular layer and weakly in the Purkinje cell layer, whilst mRor2 is expressed exclusively in the Purkinje cell layer. mRor1 is also expressed pre- and postnatally in the heart, while mRor2 expression in the heart is only detected prenatally. In conclusion, the expression of mRor1 increases during prenatal development and is sustained in many domains in the nervous system and in non-neuronal tissues after birth. In contrast, mRor2 is widely expressed prenatally in neuronal and non-neuronal tissues, but its expression becomes more confined after birth, eventually restricted to a subdomain of the cerebellum.

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mRor1 and mRor2 are expressed in cultured hippocampal neurons and associated with sites of neurite elongation and synapse formation, suggesting roles for mRors in these processes [80]. mRors are localized along the neurite processes extended by hippocampal neurons before their differentiation into axons and dendrites and their levels increase as the neurons develop; expression subsequently declines when neurons mature [80]. Both mouse Rors are also detected in glial

cells [81] and in cultured astrocytes [12]. On a subcellular level, they are associated with components of the cytoskeleton. In particular, mRor1 protein co-localizes with F-actin along the stress fibers and mRor2 partially co-localizes with tubulin [12]. Expression of both mRors is also detected in the neural progenitor cells (NPC) in the developing mouse neocortex [82]. Interestingly, Wnt5a is similarly highly expressed in NPCs [82].

Human

The human Rors, hRor1 and hRor2 , were originally identified as Trk homologues present in a cDNA library derived from human neuroblastoma cells [5]. Human Ror expression profiles show overlapping expression in brain, heart, prostate, and kidney and additional expression of hRor2 in thymus, pancreas, uterus, ovary, and intestine (www.genecards.org/cgi-bin/carddisp.pl?gene = ROR1&search = ror and www.genecards.org/cgi-bin/carddisp.pl?gene=ROR2&search = ror).

Roles of the Rors in the Nervous System

Neuronal Migration, Neurite Outgrowth, Convergent Extension, and Axonal Pruning and Branching

Neuronal Migration

A first indication for an important role for the Rors in the nervous system came from studies using *C. elegans* as a model to identify genes involved in cellular migration [13]. A number of neuronal cell lineages undergo stereotypic long-range directed migrations along the anterior-posterior axis during worm development. These cells provide an excellent model to study the molecular mechanisms that direct cellular migrations in a living organism. The *C. elegans* Ror ortholog, *cam-1*, was found to be essential for neuronal migration and orientation after the asymmetric cell division of multiple neuronal cell lineages. In the initial study, it was shown that canal-associated (CAN) and anterior lateral microtubule neurons that normally migrate posteriorly stop prematurely, while the hermaphrodite-specific (HSN) and BDU neurons migrate anteriorly beyond their normal locations in these mutant animals [13]. Furthermore, *cam-1* mutants show a failure to properly orient the polarity of the V cell and Pn.aap neuroblast divisions. Surprisingly, while *cam-1* acts cell autonomously in the migrating neurons, its tyrosine kinase activity is not required for proper migration. Overexpression or loss of expression of CAM-1 have opposite, reciprocal effects on neuronal migration patterns indicating that the levels of the receptor determine their final position [13].

A breakthrough in understanding the molecular mechanisms by which Ror directs neural migration in *C. elegans* came when it was found that in this process, *cam-1* genetically interacts with members of the canonical Wnt signaling pathway [20, 25]. These studies revealed a competition between a Wnt/Fz-mediated pathway that promotes and a Wnt/Ror pathway that inhibits the migration of a subset of neurons, the HSN lineage, in the anterior direction. This was based on the following observations: mutations in *cam-1* cause the HSN neurons to migrate beyond their normal final anterior positions [20, 83], while mutations in *egl-20*/Wnt and *mig-1*/Fz suppress this phenotype. Similarly, excess *egl-20* causes an anterior displacement of HSN neurons in a similar fashion than that observed in the *cam-1* mutants [20, 25]. The CRD domain of CAM-1 was required to rescue the defect in HSN migration in the *cam-1* mutant, whereas the intracellular region was not [84]. The finding that solely the CRD domain of CAM-1 is sufficient for rescue, strongly suggests that CAM-1 acts to sequester Egl-20/Wnt away from MIG-1/Fz receptor signaling complex (Fig. 2).

This mode of action was also reported in a study which describes CAM-1's roles during vulva development [24]. Here, the CAM-1 extracellular domain is shown to be sufficient to antagonize in a non-cell autonomous manner the vulva-promoting action of multiple Wnts, among them, EGL-20 and CWN-1. A recent study has extended these findings and showed that CAM-1 protein on two posteriorly directed CAN neurons can modulate the location and strength of Wnt signaling along the worm's anterior-posterior axis by sequestering the posteriorly derived Wnts EGL-20 and CWN-1 [85]. In this way, neuronal CAM-1 controls the location and morphology of the vulva at mid-body position [85]. The centrally produced Wnts (MOM-2 and LIN-44) are required for the correct symmetry within the primary vulva. CAM-1-dependent localization of EGL-20 to the posterior CAN axon, its ability to bind EGL-20 in vitro [24], and the requirement for only the CAM-1 Wnt-binding extracellular domain to inhibit Wnt signaling further confirms that CAM-1 mediated Wnt sequestration is the mode of action in this process.

Interestingly, this role of CAM-1 is separate from that in establishing the orientation of the vulval precursor cells (VPCs) during asymmetric cell divisions, since there, CAM-1 mediates an instructive EGL-20 activity by a signaling, cell-autonomous pathway that requires VANG-1, a component of the planar cell polarity pathway [86]. While CAM-1/EGL-20/VANG-1 establishes ground polarity, an opposing Wnt pathway mediated by the WNTs MOM-2 and LIN-44 acting via FZ/LIN-17 and Ryk/LIN-18 orient the VPCs towards the center. In summary, the integration of multiple diverse Wnt pathways acting via distinct receptors is responsible for the refinement of the polarity of the *C. elegans* body plan.

Neurite Outgrowth

In contrast to CAM-1's domain requirements in neuronal migration, CAM-1 does need its intracellular domain for its function in neurite extension of RME neurons, a set of four

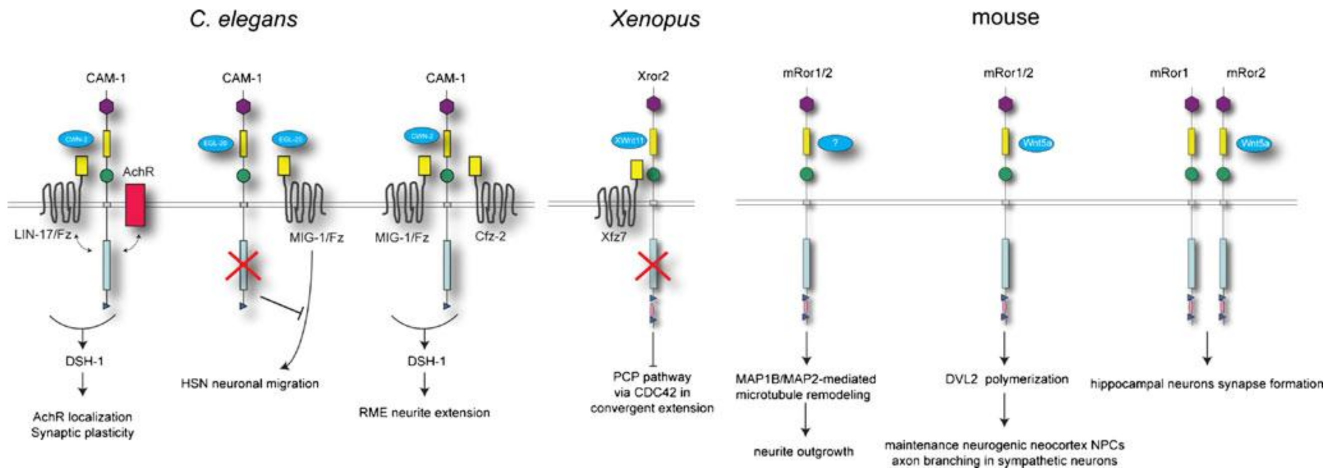

Fig. 2 Schematic Representations of the reported Ror signaling pathways in the nervous system of diverse species. In *C. elegans*, *cam-1* interacts with *cwn-2* and *lin-17* to localize pre- and postsynaptic proteins and direct transport of ACR-16/alpha-7 receptors to the NMJ [27, 73]. In another context, CAM-1 likely sequesters EGL-20/Wnt thereby inhibiting canonical EGL-20/MIG-1 signaling to promote HSN anterior migration; the CRD domain of CAM-1 is sufficient for this function [13, 20, 25]. In RME neurons, CAM-1 acts as a signaling receptor for CWN-2 and co-receptor of CFZ-2 and MIG-1 regulating neurite outgrowth. Furthermore, *cam-1* interacts with *dsh-1* in these neurons [88]. In *Xenopus*, Xror2 binds XWnt11 and act synergistically with Xfz7 to inhibit convergent extension via CDC42 [10]; the kinase domain of Xror2 is dispensable for this function. In mice, mRor signaling affects neurite outgrowth possibly via MAP mediated microtubule remodeling (Paganoni and Ferreira 2005). It is not yet clear what the ligand is of mRor there, but Wnt5a acts as the ligand for mRor2 in multiple other cellular contexts. In the neocortex, Wnt5a/Ror signaling results in DVL2 phosphorylation and polymerization thereby controlling NPC self- renewal and differentiation. It is also required for branching and peripheral target innervation of sympathetic neurons [57, 82, 102]. mRor1/mRor2 can form dimers, mRor2 directly binding Wnt5a, and the complex modulates synapse formation in hippocampal neurons [26]. The domains of the different Ror orthologs are the same as shown in Fig. 1

GABAergic motor neurons that innervate head muscles and regulate foraging movements [87, 88]. CAM-1 acts in these neurons as a receptor for the Wnt ligand, CWN-2, while two Fz family receptors, *Cfz-2* and *MIG-1* genetically interact with *cam-1* and possibly act as Ror co-receptors [88]. The kinase activity of CAM-1 is important for its role in neurite extension, as is the CRD domain. Interestingly, the intracellular portion of CAM-1 can physically interact with Dsh-1 by binding to Dsh-1's PDZ and DEP domains [88]. The *dsh-1*/*cam-1* double mutant phenotype is similar to that of the *dsh-1* only mutant, suggesting that these two genes act in the same signaling pathway. Together, these data indicate that Ror acts as a Wnt signaling receptor for RME neurite extension (Fig. 2).

Ror receptors have also been reported to mediate neurite extension in the vertebrate nervous system. mRor1 and mRor2 are important for neurite elongation and branching of cultured hippocampal neurons [89] [90] and astrocytes [12]. mRor1 and mRor2 are associated with different components of the cytoskeleton in astrocytes: while mRor1 co-localizes with F-actin along stress fibers, mRor2 partially co-localizes with microtubules. RNAi mediated knock down of either mRor1 or mRor2 in cultured hippocampal neurons results in shorter minor processes of neurons, elongated axons and reduced neurite branching [89].

Neurite outgrowth involves dynamic changes in microtubule polymerization and stabilization, mediated via the tightly regulated expression of a number of microtubule-associated proteins (MAPs) such as MAP1B, MAP2, and Tau [91–96]. When expression levels of mRor1 and 2 are knocked down in cultured hippocampal neurons via RNA-interference, a significant decrease in the levels of MAP1B and MAP2 is observed [89]. Overexpression of mRor1 and mRor2 also affected the levels of these MAPs: MAP1B expression decreased, whereas MAP2 expression increased. These data suggest that the Ror receptors exert their function in neurite elongation by mediating microtubule remodeling of the neurite cytoskeleton. Consistent with such a role is the observation that Ror expression coincides with periods of active neurite extension both in vivo and in vitro and that Ror protein directly associated with the cytoskeleton [6, 9, 11, 80, 81, 97] and that mRor1 and mRor2 are highly concentrated at the growth cones of immature neurons and present throughout the somatodendritic compartment of mature hippocampal neurons [80].

Convergent Extension

Convergent extension is the process by which embryonic tissue is restructured to converge along one axis and extend along a perpendicular axis by cellular migration. This process plays a crucial role in shaping the vertebrate body plan during gastrulation, neurulation, axis elongation, and organogenesis. In *Xenopus*, convergent extension patterns the dorsal mesoderm and the neural ectoderm and the process is mediated by Xror2 via Wnt signaling [10, 98–100]. Overexpression of

Xror2 in embryos inhibits convergent extension of the dorsal mesoderm and neuroectoderm; its kinase domain is dispensable for this gain-of-function effect, while the CRD is required [10]. Interestingly, coexpression of Xror2 with XWnt11 , Xfz7 or both synergistically inhibits convergent extension. Xror2 binds XWnt11 and XWnt11 and Xfz7 exhibit significant overlapping expression with Xror2 in the dorsal marginal zone [10]. Hikasa and coauthors further report that the Xror2 -XWnt11 -mediated signal leads to the activation of a member of the Rho GTPase, Cdc42 , a known member of the PCP Wnt pathway. In summary, Xror2 acts in non-canonical PCP Wnt signaling via small GTPases leading to the inhibition of convergent extension thereby establishing mediolateral polarity in the vertebrate nervous system (Fig. 2).

Axonal Pruning

The mechanisms by which neurites are selectively eliminated from or stabilized into mature circuits during development are largely unknown. The trophic theory postulates that the fate of a neurite depends on its ability to receive and interpret local survival cues emanating from surrounding cells that inhibit neurite elimination.

Recently, it has been shown that Wnt–Ror signaling plays such a tropic role in the C . elegans ' nervous system [101]. They found that the rate of neurite elimination in AIM neurons is enhanced in cam -1 loss-of-function mutants, without affecting the number of neurons, while cam -1 overexpression inhibits neurite elimination. CAM-1 protein localizes to the proximal regions of neurites destined to be eliminated. These data indicate that cam -1 is not involved in the cell type specification of the AIMs, but is specifically active as an inhibitor of neurite pruning, thereby promoting neurite survival. cam -1 's role in neurite pruning is a Wnt-dependent process; CWN-1 and CWN-2 secreted from nearby neurons located on the ventral and dorsal sides of the AIM neurons control the rates of neurite elimination [101]. Interestingly, this Wnt/Ror pathway acts to counteract the activity of MRB-1, a transcription factor that promotes neurite elimination [101]. The cellular processes by which Wnt/Ror regulates developmental pruning remain to be resolved, but it likely involves the modification of local cytoskeleton stabilization in the neurite.

Axonal Branching

Mice that lack both the mRor1 and mRor2 proteins show phenotypes that mirror those of Wnt5a KO mice, such as tissue elongation defects resulting in severe truncations of the caudal axis, the limbs and facial structures and deficits in sympathetic axon innervation patterns [57]. These data suggest that Rors are important mediators of Wnt5a signaling during vertebrate development. The deficits in the sympathetic nervous system of mice lacking all Ror signaling are clearly visible at embryonic stage E17.5, the time point when sympathetic axons are beginning to innervate peripheral targets. It appears that Rors specifically promote axon branching of sympathetic neurons when they innervate their targets, but they are not needed for neuronal specification [57]. This study also shows that Dvl2 phosphorylation is an important physiological target of the Wnt5a-Ror signaling pathway in primary MEFs, while neither β-catenin-dependent Wnt signaling, or c-JUN phosphorylation are affected by Wnt5a-Ror signaling in these cells. Thus, these data reveal a novel Ror mediated Wnt pathway that is distinct from the canonical and PCP Wnt pathways [57].

Neurogenesis

A Wnt/Ror pathway operating in the neocortex has also been recently uncovered. Specifically, the two mouse Ror orthologs, mRor1 and mRor2, and their ligand Wnt5a are expressed in the neuronal progenitor cells (NPCs) of the developing neocortex [82]. NPCs self-renew and differentiate simultaneously during neurogenesis to generate large numbers of differentiated neurons. How the balance between proliferation and differentiation of NPCs is regulated, is not well understood, but its outcome is crucial for the formation of a functional neocortex. Endo and colleagues present evidence to suggest that the Wnt5a-mRor1 and Wnt5a-mRor2 signaling pathways regulate neurogenesis though the maintenance of populations of proliferative and neurogenic NPCs in cultured cells and in vivo [82]. Apparently, Wnt5a activates these Ror-dependent pathways via phosphorylation and polymerization of Dvl2 in a non-canonical manner without effecting canonical β-catenin-TCF/LEF signaling [102]. It is however not yet clear via which cellular mechanisms Ror plays a role in this process.

Axon Guidance

There is one report to date describing a role for a Ror family member in axon guidance. In this study, Wnt/Ror signaling is shown to be required for positioning the nerve ring, the most anterior neuropil in the worm [103]. The nerve ring comprises more than half the total number of neurons in the worm and is considered to be its brain [104]. Two sets of neurons are essential organizers of nerve ring development, the SIA and the SIB lineages. In cam -1 and cwnt -2 mutants similar defects in the guidance of the SIA and SIB neurons are observed: many axons do not exit the nerve ring, or when they do, they follow alternate routes resulting in an anterior shift of the nerve ring location. cwn -2 acts non-autonomously in nerve ring placement, while cam -1 acts cell autonomously, i.e., it is required for rescue within SIA and SIB neurons and it requires its intracellular domain for this function. CWN-2 acts as the ligand for CAM-1, probably with MIG-1/Fz acting as co-receptor. While cwn -2 is required in cells posterior to the

nerve ring at the time of nerve ring formation, this requirement is not limited to one particular posterior cell type, an observation in line with the expected non-cell autonomous nature of a secreted ligand [103]. In summary, CWN-2 apparently directly controls SIA and SIB axon guidance at a specific developmental stage via the CAM-1 receptor present on their cell surfaces.

Synapse Formation and Maintenance

Most studies discussed in this review have uncovered roles for the Rors in mediating aspects of neuronal development that likely reflect their importance for axon cytoskeleton remodeling. In 2005, it was reported that Ror can also act to regulate synaptic transmission via controlling the localization and/or stabilization of presynaptic release sites and postsynaptic acetylcholine receptors at the *C. elegans* NMJ [73]. At this moment, it is not yet clear whether this aspect of Ror function is also mediated by cytoskeleton remodeling.

CAM-1 is widely expressed in the worm's body wall musculature and accumulates on cholinergic motor neuron cell bodies and processes in a manner consistent with localization to the cell membrane [73]. CAM-1 protein is also visible in punctae at the distal tips of the muscle arms at the contact points between muscle and the ventral nerve cord [73]. Mutations in *cam-1* lead to changes in the localization of both pre- and postsynaptic proteins. In the *cam-1* mutant motoneuron, the distribution of cholinergic synaptic vesicles and the SYD-2 presynaptic marker are altered; in mutant muscle the postsynaptic acetylcholine receptor subunit ACR-16 is mislocalized [73]. Normally, ACR-16 also localizes to punctae at the tips of the muscle arms, but in *cam-1* mutants, the number of these punctae at proximal regions of the muscle arms dramatically increases. CAM-1's tyrosine kinase domain is not required for the proper localization of ACR-16, but the catalytically inactive protein had to be tethered to the membrane to fully rescue the mutant phenotype. In contrast, the localization of other postsynaptic receptors such as the gamma-aminobutyric acid (GABA) receptors and the UNC-29-containing acetylcholine receptors (AChRs) are not altered in the *cam-1* mutant and are thus independent of the presence of functional CAM-1. *cam-1* is also dispensable for muscle arm outgrowth, since no morphological differences are observed in the body wall muscle [73].

The nicotine-gated excitatory ACh-dependent currents are reduced in *cam-1* mutants, apparently as a consequence of the altered ACR-16 distribution observed [73]. There is a reduction in the amplitude, but not the frequency, of synaptic events. GABA neurotransmission is not affected in *cam-1* mutants, consistent with the normal distribution of GABA in *cam-1* mutants. The kinetics of the nerve-evoked responses was slower in the mutants, suggesting that presynaptic release is also impaired. At the mutant cholinergic synapses, there is an increase in the number of synaptic vesicles and their distribution is altered, i.e., vesicle abundance does not decrease as a function of the distance from the active zone, as it normally does. Instead, mutations in *cam-1* lead to the accumulation of synaptic vesicles at non-synaptic sites not associated with active zones [73].

In summary, CAM-1 is expressed both pre- and postsynaptically at the NMJ, has a role in muscle to maintain or scaffold the postsynaptic nicotinic receptors and it functions presynaptically to limit the size of the active zone neurotransmitter release sites. At present, it is not clear whether the presynaptic alterations are a consequence of the absence of postsynaptic Ror or whether post- and presynaptic Rors function independently in separate pathways to locate target proteins. Francis and coauthors proposed a model for pre- and postsynaptic CAM-1 proteins interacting homophilically at the NMJ for the precise alignment of the presynaptic release sites and the postsynaptic receptors [73].

A follow-up study by the same group showed that CAM-1's role in synaptic transmission at the NMJ is mediated by Wnt signaling [27]. A CWN-2/CAM-1/LIN-17(Fz) receptor complex regulates acetylcholine receptor translocation to the postsynaptic side of the NMJ. *lin-17*, *cwn-2*, and *dsh-1* mutants display similar phenotypes to those reported for *cam-1* mutants, i.e., abnormal subsynaptic localization of ACR-16/alpha7, a consequent reduction in synaptic currents, and behavioral defects [73] [27]. ACh-gated currents in *lin-17* or *cam-1* mutants and double *lin-17; cam-1* mutants are similarly impaired, suggesting that these proteins signal through the same pathway. As described above, CAM-1 is expressed in muscle cells [73] and the decreased ACh-gated currents in *cam-1* mutants appear to result from reduced delivery of ACR-16/alpha7 receptors to the cell surface. Muscle-specific, but not neuron-specific CAM-1 expression restores the ACR-16/alpha7-mediated currents to their normal levels [27]. CAM-1 requires its extracellular, but not its kinase domain, to rescue this phenotype. These results indicate that CAM-1 possibly acts via an interaction with another receptor, Lin-17, to affect downstream signaling.

CAM-1 and LIN-17 were found to partially co-localize in muscle arms: the extracellular domain of CAM-1 is required for complex formation with LIN-17. CWN-2 secretion by motoneurons was necessary and sufficient for rescue of the *cwn-2* mutant phenotype. In contrast, the phenotypes of *lin-17* and *dsh-1* were ameliorated by expression of their respective rescue constructs in muscle but not in neurons. Therefore, a model was presented for neuronal CWN-2 to signal via muscle-expressed CAM-1/LIN-17/DSH-1 to control ACR-16/alpha7 location and, therefore, the ion influx mediated through these receptors. In addition, it was shown that that Wnt/Ror signaling controls activity-dependent translocation of ACR-16/alpha7 receptors from subsynaptic pools to the NMJ; decreased Wnt signaling results in a large relatively

immobile pool of subsynaptic ACR-16/alpha7. These two studies clearly show that presynaptic Wnt signaling via postsynaptic Fz/Ror receptor complexes is critical for activity-dependent synaptic plasticity in the adult nervous system (Fig. 2).

CAM-1 activity at the NMJ is negatively regulated by the cell surface Ig superfamily member RIG-13 [105]. RIG-3 is a GPI-anchored protein with two Ig domains and a fibronectin type III domain and is expressed in cholinergic motor neurons. RIG-3 can be shed from the plasma membrane but the form active at the synapse is associated with the presynaptic plasma membrane. Rig -3 mutants do not present significant defects in synapse formation, maintenance, or baseline synaptic transmission, but do exhibit an enhanced sensitivity to the cholinesterase inhibitor aldicarb. The higher drug responsiveness in these mutants is caused by an increase in muscle ACR-16 abundance, suggesting that RIG-3 controls the number of mobile ACR-16 receptors available for synaptic recruitment. RIG-3 effects on cholinergic transmission are mediated by Wnt signaling, specifically by inhibiting the activity of CAM-1 on synaptic ARC-16 recruitment [73]. In summary, presynaptic RIG-3 constrains synaptic plasticity by inhibition of postsynaptic CAM-1 activity. The molecular mechanisms that mediate this inhibition are not yet clear.

The Rors have also been implicated in the establishment of synaptic contacts in the vertebrate central nervous system (CNS). Paganoni and colleagues have shown that down regulation of either mRor1 or mRor2 in cultured rat hippocampal neurons leads to a decrease in their synaptic contacts [26]. In addition, Ror-depleted axons generate fewer branches, but extend longer axons, presumably via a cellular mechanism employed to compensate for the lack of branches. However, not only the total number of synapses formed per neuron is decreased, but the synaptic density calculated as the mean number of synapses formed per dendritic length, is also reduced. In complementary experiments, exposure of cultured rat hippocampal neurons to Wnt5a increased not only the number of synapses per neuron and per average dendritic length, but also the average density of synapses in a Ror-dependent manner [26]. Therefore, it seems unlikely that the decrease in synaptic contacts after Ror depletion is only a secondary effect of the increase in axon elongation or branching. It is more likely that Wnt5a-Ror signaling enhances synaptogenesis independently of its roles in neurite extension.

Synapse density was equally reduced when either one or both mRors are down-regulated in these cultured cells [26]; therefore, mRors possibly function in a heterodimeric complex to regulate synaptogenesis. Consistent with this hypothesis, mRor1 and mRor2 form heterodimers in vivo and in vitro but only mRor2, and not mRor1, directly interacts with Wnt5a [26] (Fig. 2). Furthermore, mRor1 and mRor2 co-localize to the somatic dendritic compartment of neurons in the early mouse brain, an area that is substantially remodeled during synapse formation.

Concluding Remarks

An important step towards understanding the roles of the Ror family of tyrosine kinase receptors during development was the identification of their ligands, the members of the Wnt gene family. Notwithstanding a number of important advancements, two decades since their discovery in 1992, still little is known about the relevance of their potential kinase activity, their substrate targets and their downstream signaling pathways. Evidence has been presented indicating that Rors are highly pleiotropic receptors with diverse roles in essential processes during vertebrate and invertebrate development. Importantly, while Rors are differentially expressed in many tissues, all Rors studied so far are expressed in the nervous system; however, no human neurological disorders have been linked to a deficiency in Ror signaling. Wnt/ROR signaling may possibly contribute to poor post-trauma axonal regeneration in patients with brachial plexus injury [106]. Wnt5a is highly upregulated in the neuroma scar tissue that forms at the site of a brachial plexus injury, possibly preventing ROR-expressing peripheral nerves from regenerating across the site of injury (unpublished data, MJM and JV).

What are the cellular mechanisms via which Ror proteins act? Most phenotypic effects in animals with reduced Ror function likely reflect failures in proper cytoskeleton/ microtubuli remodeling. Ror signaling has also been implicated in Wnt5a/Ryk-driven Fz internalization via clathrin-mediated endocytosis [107]. Ror's involvement in this process is likely indirect given that Ror does not localize to the endosomes. Since there is no clear information about the cellular aspects of Ror's role in endocytosis and synapse stabilization, it is attractive to speculate that Ror's function involves microtubule remodeling and maturation also at these sites. Further studies are needed to elucidate its mode of action in these distinct cellular contexts.

Elegant experiments using the *C . elegans* NMJ as a model indicate a requirement for *cam -1* in transport of ACR-16/ alpha7 receptors from subsynaptic pools to the NMJ [27]. In unpublished experiments, we have found that the two Drosophila Ror orthologs, Ror and *Nrk* , are expressed in larval motoneurons and the muscle fibers they innervate and that synaptic transmission is impaired in these animals (unpublished data, IMP, LGF, and JNN). Moreover, Rors are highly homologous to MuSK, a muscle specific kinase that via Agrin promotes the scaffolding of a receptor complex with LRP and AChRs at the vertebrate NMJ in a Wnt-dependent manner [108, 109]. At central hippocampal synapses, Wnts are also essential for synaptic function: Wnt7a effects presynaptic clustering of α7 AChRs with APC to regulate synapse formation, number, and

strength. These aspects of Wnt signaling might illustrate an ancestral aspect of this signaling cascade that promotes the scaffolding of neurotransmitter receptors in an activity-dependent manner during synaptic plasticity.

How do Rors signal to their downstream targets? The finding that Rors mostly signal through the non-canonical Wnt pathway member Wnt5a in mice and that Rors in some contexts neither suppress or enhance canonical WNT signaling through β-catenin suggest that Rors can mediate a novel, non-canonical Wnt pathway. The non-receptor kinase Src can act downstream of Ror, as has been shown for another non-canonical Wnt receptor, Ryk. Rors share an additional interesting feature with RYKs. In some cellular contexts, these receptors do not require their tyrosine kinase domain for their function, but act to sequester Wnt ligands away from other Wnt receptors, such as Fz. It is unlikely that all Rors are pseudokinases since there is evidence for the requirement of catalytic kinase activity for some Ror family members' functions.

While during the 20 years since the discovery of the first Ror orthologs, much has been learned about their biological roles, many unresolved questions remain. Through which downstream targets does this pathway function? Do Rors act directly on microtubule remodeling? What are the targets of the crosstalk between canonical and Ror mediated non-canonical Wnt signaling? Moreover, what are the clinically relevant roles of Ror signaling in nervous system repair and synaptic plasticity in vertebrates? These are just some of the outstanding questions to be addressed in years to come.

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