PERSPECTIVE

Is retinoic acid a signal for nerve regeneration in insects?

Until quite recently the retinoic acid (RA) signaling pathway was believed to occur only in vertebrates, but in the last decade, several ground-breaking studies have shown that it also exists in invertebrates. These include the discovery of endogenous retinoids (Nowickyj et al., 2008), of retinoid receptor orthologs in insects (Hayward et al., 1999) and the very specific effect of RA on axonal guidance in the fresh water snail *Lymnaea stagnalis* (Dmetrichuk et al., 2008). Despite some earlier indications of RA-dependent gene activation in insects, these papers really started retinoid research in invertebrates.

Vitamin A and retinoic acid signaling in the animal kingdom: The nuclear receptors (NR) form a large superfamily of ligand-activated transcription factors that occur in all animal phyla, but only in the animal kingdom. It seems likely that the NR have their evolutionary origin in an ancestral fatty acid-activated receptor at the basis of metazoan phylogeny. Among the NRs, the RA receptors (RAR) and the structurally similar retinoid X receptors (RXR) form a small but functionally important subgroup. The RARs and several other NR families form obligatory heterodimers with an RXR. In vertebrates, the natural ligand of RAR is all-*trans* RA, whose local enzymatic synthesis constitutes the most important regulatory step of the signaling pathway. Although almost all RA research focuses on mammals, there is genetic evidence that the enzymatic machinery of the RA pathway came with the evolution of bilateria: orthologous enzymes of Bco/Rpe65 (carotenoid cleavage, isomerase activity), MDR-Adh and SDR-Rdh (retinol oxidation), Raldh (retinoic acid synthesis), Cyp26 (retinoic acid oxidation) and retinoid receptors were found in protostomes and deuterostomes (Albalat, 2009).

Evidence for retinoid signaling in insects: With respect to the receptors, RXR orthologs were found in insects, but since these form dimers with a variety of other NRs, this does not by itself imply RA signaling. There is no evidence for storage and transport systems of retinoids, and Cyp26 orthologs may

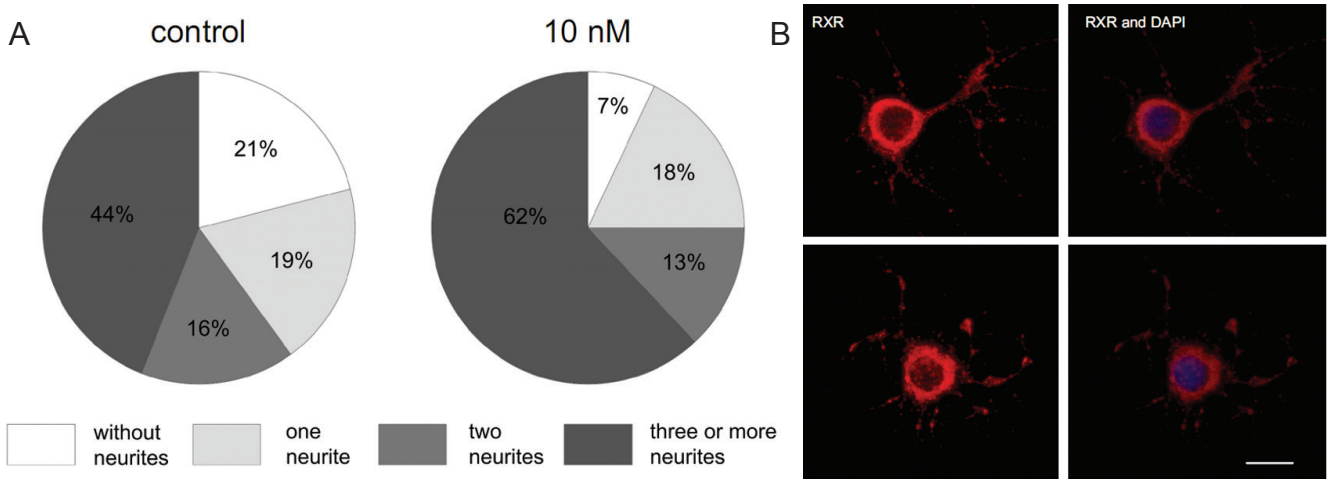

Figure 1 Retinoic acid influences neuritogenesis of insect neurons.
(A) Retinoic acid treatment of primary cultures from the brain and optic lobes of locust embryos at 50% developmental stage promotes neurite outgrowth. Pie segments indicate percentage of cells with 0, 1, 2 or more neurites after 3 days *in vitro*. The statistical analysis revealed a significant difference between the groups (contingency test; $df = 3$, $\chi^2 = 13.2$, $P < 0.01$). These data result from an independent replication of the experiments published by Sukiban et al. (2014). (B) Cytoplasmatic localization of *Lm*RXR (red) in cultivated neurons from a locust embryo at 90% developmental stage and in the first nymphal instar. Scale bar: 10 µm.

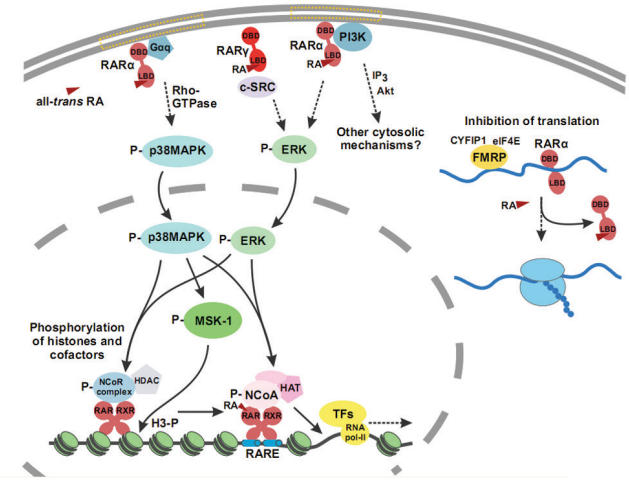

Figure 2 Non-classical effects of retinoic acid (RA) signaling.
In mammalian cells, RARα was found in lipid rafts of the plasmalemma, where RA binding leads to association of a Gαq protein or of PI3K. Both processes cause activation of MAPK enzymes in the cytosol. RARα *via* cSRC can also lead to MAPK phosphorylation. After nuclear translocation these enzymes phosphorylate nuclear cofactors and, converging on MSK-1, of histone 3, eventually affecting the regulation of gene transcription [after Rochette-Egly (2014), Biochim Biophys Acta 1851:66-75]. In dendrites of hippocampal neurons, unliganded RARα inhibits translation of mRNA, requiring another translational regulator, FMRP. Protein synthesis is enhanced by RA binding to the RAR [after Chen et al. (2014), Neuropharmacology 78:3-12].
c-Src: Cellular sarcoma tyrosine kinase; CYFIP1: cytoplasmic FMR1 interacting protein 1; elF4E: eukaryotic initiation factor 4E; ERK: extracellular-signal regulated kinase; FMRP: fragile X mental retardation protein; HAT: histone acetyl transferase; MAPK: mitogen-activated protein kinase; MSK: mitogen and stress-activated protein kinase; NCoA: nuclear receptor co-activator; PI3K: phosphatidylinositol-4,5-bisphosphate-3-kinase; RAR: retinoic acid receptor; RARE: retinoic acid response elements; TFs: transcription factors.

have been lost in the ecdysozoan lineage, which seems also to be the case for the RAR family (Albalat, 2009). Some arthropods, which lack RARs, do show a physiological response to RA (Němec et al., 1993; Sukiban et al., 2014), however, and endogenous retinoids were detected in several insect species (Nowickyi et al., 2008). Thus, if RA has a physiological role in insects, one possibility how this may be realized on the molecular level is that RA exerts its transcriptional activity by binding to the RXRs. These NRs would then mediate transcriptional activity of RA *via* the classical mechanism. Retinoid-dependent RXR activation does not exclude other modes of action or, indeed, non-transcriptional roles of the receptors (see below).

So far, physiological roles of RA in insects have only been associated with developmental processes. It was found to inhibit metamorphosis and affect organogenesis (Němec et al., 1993). In general, invertebrate RA signaling plays a role in neuronal differentiation, cell viability and chemotaxis (Albalat, 2009). In contrast to RXR, the related receptor *ultraspiracle* (USP) is not able to bind RA and, therefore seems to be solely a heterodimeric partner for EcR (Nowickyj et al., 2008). As in vertebrates, some teratogenic effects of RA were found also in invertebrates. For example, 9-*cis* RA causes abnormal larval development in *Rhodnius prolixus* (Nakamura et al., 2007). In Lymnaea embryos, disturbed eye development and shell formation under influence of 9-*cis* RA was shown (Carter et al., 2010).

A question of isomers – specific roles of 9-*cis* and all-*trans* RA: As mentioned above, in vertebrates the all-*trans* RA isomer is the natural ligand of RARs in RAR/RXR heterodimers. The three RAR isotypes (α, β, γ) show no differences with regard to ligand binding. While 9-*cis* RA also binds to RARs with high affinity and, in addition, can induce transcriptional activity of RXR homodimers, its natural occurrence has long been disputed. At present, the only recognized source of endogenous 9-*cis* RA in mammals is the pancreas, where it participates in the regulation of glucose metabolism (Kane, 2012). Other isomers, specifically 13-*cis* and 9,13-*di-cis* RA, were detected, but these possess only low affinities to the NRs. Although very few HPLC analyses of retinoids have been performed with tissues from invertebrates, it is already clear that in arthropods (Nowickyj et al., 2008) and mollusks (Dmetrichuk et al., 2008) 9-*cis* RA occurs as a natural signaling molecule. Another phylogenetic difference appears to be the role of RXRs, which in vertebrates mainly act as heterodimeric partners of other NR families, effects of polyunsaturated fatty acids notwithstanding. In contrast, the insect orthologs of RXR are directly activated by ligand binding (Nowickyj et al., 2008).

Phylogenetic evolution of insect retinoid X receptors: In the grasshopper *Locusta migratoria* two isoforms of RXR were identified (*Lm*RXR-S and -L) that are considered to be homologs of USP of other insect orders (Hayward et al., 2003). Similar to RXRs in vertebrates *Lm*RXR-S might act as heterodimer partner of the EcR and play a role in ecdysone signaling of insects. The structure of *Lm*RXR-L shows characteristics of a ligand-modulated NR. In contrast to USP of more advanced insects (flies and moths) *Lm*RXR was found during early embryogenesis despite the absence of EcR (Nowickyj et al., 2008). While the DNA binding domain of these NRs is highly conserved, the ligand binding domains are much more diverse. Genetic comparisons led to the suggestion that the ligand binding domain altered its function during the course of insect evolution (Hayward et al., 1999). Insects convert vitamin A to various retinoids. Importantly, all-*trans* and 9-*cis* RA were detected in locust embryos, and both isomers bind to *Lm*RXR with high affinity (Nowickyj et al., 2008). These findings strongly support a transcriptional role of RA signaling in development in some insect orders such as Orthoptera which possess RXRs.

The role of RA and its receptors for neurite outgrowth in invertebrates: Recently, we reported a positive effect of 9-*cis* RA on cell survival and neurite outgrowth in cultures of cells dissociated from the central nervous system of locust embryos (Sukiban et al., 2014). Low concentration of 9-*cis* RA caused the growth of more neurites compared to control cultures when prepared from 50% stage embryos but not from later stages (**Figure 1A**). In the central nervous system (CNS) of 50–60% stage embryos we found expression of *Lm*RXR, although the neuritogenic effect of RA declined between these stages. Also in the snail *Lymnaea stagnalis*, RA (9-*cis* RA and all-*trans* RA) had a positive effect on the number and lengths of growing neurites in culture (Dmetrichuk et al., 2008; Farrar et al., 2009). In this species the local application of RA induced neuronal growth cone turning. This effect was found to be based on a non-genomic mechanism which cannot be blocked with the transcriptional inhibitor actinomycin D and works in growth cones that have been severed from their cell body (Farrar et al., 2009). The growth cone turning response to RA seems to require Ca^{2+} influx and *de novo* local protein synthesis. The observation that application of an RXR agonist could induce growth cone turning in isolated neurites and the cytoplasmic localization of adult *Lym*RXR with localization in neurites and growth cones suggest a non-genomic mechanism (Carter et al., 2010). This may also be the case in insects because a vertebrate RXR-antibody showed non-nuclear, cytoplasmic staining and localization in neurites similar to the distribution shown for *Lymnaea* neurons (**Figure 1B**). In the embryonic CNS of *Locusta*, we found prominent RXR labeling in neurites of specific cells of the optic lobes and suboesophageal ganglia (manuscript in preparation).

Molecular mechanisms of RA-dependent axonal regeneration: By inducing cell differentiation RA causes neurite outgrowth in a number of neuroblastoma cell lines and primary cell cultures (Mey, 2006: Tables 1 and 3), and several corresponding results were obtained *in vivo*. Axonal elongation results from the interaction of several components of the cytoskeleton. In some instances, the molecular mechanisms of RA-dependent regeneration have been resolved. RA responsive genes involved in neurite outgrowth, *e.g.*, the human Neuron Navigator 2 (*Nav2*) and the neural precursor cell-expressed, developmentally down-regulated gene 9 (*NEDD9*) encode for proteins, which contain cytoskeletal interacting domains and are thought to mediate the effects of RA in this manner. Some mechanisms may be vertebrate specific. For instance, ligand-dependent RAR dimerization has been suggested to reduce expression of a component of the NogoR complex, which is responsible for the growth-inhibitory effect of myelin components from the mammalian CNS (Puttagunta et al., 2012). This effect is not likely to play a role in invertebrates. On the other hand, *Nav2* and 14-3-3ε, an interacting partner of *NAV2*, seem to be conserved across

different species (Marzinke et al., 2013). Regarding axonal regeneration in protostomes, the most convincing experiments so far point to non-genomic actions of RA on growth cone guidance in *L. stagnalis*, mentioned above (Dmetrichuk et al., 2008; Carter et al., 2010).

Cytoplasmic RA signaling in vertebrates and invertebrates: All animals share retinaldehyde as part of the light-sensitive pigment in the phototransduction process. Beyond that, retinoid signaling may have diverged between protostomes and chordates. So far, experiments in arthropods and mollusks point at non-genomic signaling mechanisms of RA, whereas in vertebrates its major function is transcriptional control. On the other hand, there are indications of non-transcriptional functions in vertebrates as well. For instance, in the fish retina RA causes electrical uncoupling of horizontal cells and spinule formation of photoreceptors. Rapid and transient interactions with second messenger pathways were reported involving phosphatidyl inositol-3-kinase, protein kinase-C, extracellular signal regulated kinase, p38 mitogen-activated protein kinase and the serine/threonine kinase mTOR (Al Tanoury et al., 2013). All these effects occur too fast to be attributed to genomic signaling. It remains to be seen whether similar mechanisms occur in invertebrates (**Figure 2**). Interactions of RAR with the Rho/Rac-GTPase system would be obvious candidates to account for axonal guidance processes because these mechanisms are implicated in remodeling of the cytoskeleton during growth cone guidance. We also see parallels with respect to the intracellular localization of retinoid receptors because, contrary to the expectations at the time, several laboratories found immunoreactive signals of RAR and RXR in the cytosol of mammalian cells. It is now accepted that a pool of RAR is designated for specific functions in the cytoskeleton. In the nervous system of *L. migratoria* we made the striking observation of RXR immune reactivity in neurites far away from the cell nucleus (**Figure 1A, B**). Our data indicate that only a subtype of neurons in locust embryos expresses *Lm*RXR. It needs to be tested whether 9-*cis* or all-*trans* RA is the natural ligand for this receptor and if so, whether ligand binding induces neuritogenesis. Since *Lm*RXR immunoreactivity was also seen on differentiated neurons in the CNS, another intriguing question is whether *Lm*RXR influences synaptic activity. This was reported for RARα in pyramidal neurons of the murine hippocampus, where the receptor associates with RNA granules and blocks translation of the glutamine receptor-1 (GluR1) and the calcium calmodulin-dependent protein kinase IIa. Since RA synthesis increased when synaptic activity was blocked, and since ligand binding relieved the inhibitory effect, this may represent a mechanism of synaptic homeostasis (**Figure 2**). Recent electrophysiological experiments with *L. stagnalis* demonstrate acute effects of the firing properties of neurons by RA in gastropods (Vesprini and Spencer, 2014).

The demonstration of RA-promoted neurite outgrowth of insect neurons *in vitro* and of RXR-like immunoreactivity in the cytoplasm of neurons support the hypothesis that RA and *Lm*RXR act as neuronal differentiation factors in early insect CNS development. In contrast to many studies in mammals, the retinoid signaling pathways of insects remain unclear so far.

The project was funded by the Excellence Initiative of the German federal and state governments.

Katrin Bui-Göbbels*, Renata Medinaceli Quintela, Peter Bräunig, Jörg Mey*

Institut für Biologie II, RWTH Aachen University, Germany (Bui-Göbbels K, Quintela RM, Bräunig P)
Hospital Nacional de Parapléjicos, Toledo, Spain (Mey J)
Euron Graduate School of Neuroscience, Maastricht, Netherlands (Mey J)

***Correspondence to:** Katrin Bui-Göbbels, Ph.D. or Jörg Mey, Ph.D., bui-goebbels@bio2.rwth-aachen.de or jmey@sescam.jccm.es.*
***Accepted:** 2015-04-18*
***doi:**10.4103/1673-5374.158349* ***http**://www.nrronline.org/*
Bui-Göbbels K, Quintela RM, Bräunig P, Mey J (2015) Is retinoic acid a signal for nerve regeneration in insects? Neural Regen Res 10(6):901-903.

References

Al Tanoury Z, Piskunov A, Rochette-Egly C (2013) Vitamin A and retinoid signaling: genomic and nongenomic effects. J Lipid Res 54:1761-1775.
Albalat R (2009) The retinoic acid machinery in invertebrates: Ancestral elements and vertebrate innovations. Mol Cell Endocrinol 313:23-35.
Carter CJ, Farrar N, Carlone RL, Spencer GE (2010) Developmental expression of a molluscan RXR and evidence for its novel, nongenomic role in growth cone guidance. Dev Biol 343:124-137.
Dmetrichuk JM, Carlone RL, Jones TR, Vesprini ND, Spencer GE (2008) Detection of endogenous retinoids in the molluscan CNS and characterization of the trophic and tropic actions of 9-cis retinoic acid on isolated neurons. J Neurosci 28:13014-13024.
Farrar NR, Dmetrichuk JM, Carlone RL, Spencer GE (2009) A novel, nongenomic mechanism underlies retinoic acid-induced growth cone turning. J Neurosci 29:14136-14142.
Hayward DC, Bastiani MJ, Trueman JW, Truman JW, Riddiford LM, Ball EE (1999) The sequence of Locusta RXR, homologous to Drosophila Ultraspiracle, and its evolutionary implications. Dev Genes Evol 209:564-571.
Hayward DC, Dhadialla TS, Zhou S, Kuiper MJ, Ball EE, Wyatt GR, Walker VK (2003) Ligand specificity and developmental expression of RXR and ecdysone receptor in the migratory locust. J Insect Physiol 49:1135-1144.
Kane MA (2012) Analysis, occurrence and functions of 9-cis retinoic acid. Biochim Biophys Acta 1821:10-20.
Marzinke MA, Mavencamp T, Duratinsky J, Clagett-Dame M (2013) 14-3-3′ and NAV2 interact to regulate neurite outgrowth and axon elongation. Arch Biochem Biophys 540:94-100.
Mey J (2006) New therapeutic target for CNS injury? The role of retinoic acid signaling after nerve lesions. J Neurobiol 66:757-797.
Nakamura A, Stiebler R, Fantappie MR, Fialho E, Masuda H, Oliveira MF (2007) Effects of retinoids and juvenoids on moult and on phenoloxidase activity in the blood-sucking insect Rhodnius prolixus. Acta Trop 103:222-230.
Němec V, Kodrík D, Matolín S, Laufer H (1993) Juvenile hormone-like effects of retinoic acid in insect metamorphosis, embryogenesis and reproduction. J Insect Physiol 93:1083-1093.
Nowickyj SM, Chithalen JV, Cameron D, Tyshenko MG, Petkovich M, Wyatt GR, Jones G, Walker VK (2008) Locust retinoid X receptors: 9-Cis-retinoic acid in embryos from a primitive insect. Proc Natl Acad Sci USA 105:9540-9545.
Puttagunta R, Di Giovanni S (2012) Retinoic acid signaling in axonal regeneration. Front Neurosci 4:1-9.
Sarti F, Zhang Z, Schroeder J, Chen L (2013) Rapid suppression of inhibitory synaptic transmission by retinoic acid. J Neurosci 33:11440-11450.
Sukiban J, Bräunig P, Mey J, Bui-Göbbels K (2014) Retinoic acid as a survival factor in neuronal development of the grasshopper, Locusta migratoria. Cell Tissue Res 358:303-312.
Vesprini ND, Spencer GE (2014) Retinoic acid induces changes in electrical properties of adult neurons in a dose- and isomer-dependent manner. J Neurophysiol 111:1318-1330.