Retinoic acid signaling in axonal regeneration

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INTRODUCTION
The limited regenerative capacity observed following an acute central nervous system (CNS) lesion, such as stroke or brain/spinal cord traumatic injuries, is due to both the presence of an extrinsic inhibitory growth environment and the lack of intrinsic growth factors. Interestingly, the peripheral nervous system (PNS) does regenerate to a certain extent following a lesion, which has led to further research to determine the distinction between the CNS and PNS. The inhibitory environment of the CNS is vastly different than that of the PNS (Richardson et al., 1980). Following PNS injury, an inflammatory response is activated, Schwann cells infiltrate the injured area, debris is removed, neurotrophic factors are released, regeneration is initiated, and sheathing of these growing axons occurs (George and Griffin, 1994). Conversely, the glia cells in the CNS, oligodendrocytes, and astrocytes, provide an inhibitory environment for growth (McKeon et al., 1991; Qiu et al., 2000; Domeniconi et al., 2002; Niederost et al., 2002; Yi and He, 2006).

Specifically, the neuronal insulating layer, myelin, is fragmented following a CNS lesion, releasing the extrinsic inhibitory molecules, myelin associated glycoprotein (MAG), Nogo, and Oligodendrocyte myelin glycoprotein (OMgp) (DeBellard et al., 1996; Huber and Schwab, 2000; Wang et al., 2002; He et al., 2003) that inhibit axonal outgrowth and functional recovery following injury. These myelin proteins signal through the neuronal membrane bound Nogo Receptor (NgR) complex, which includes NgR1 (Chen et al., 2000; GrandPre et al., 2000), Lingo-1 (Mi et al., 2004), and p75NTR (Domeniconi et al., 2002; Wong et al., 2002) or TROY (Park et al., 2005). Myelin protein engagement of the NgR complex activates RhoA, which induces ROCK-dependent phosphorylation of cofilin, thus actin depolymerization and growth cone collapse (He and Koprivica, 2004). When the RhoA pathway has been blocked following a CNS lesion, regeneration has been observed (Lehmann et al., 1999; Dergham et al., 2002; Fournier et al., 2003). The Paired immunoglobulin-like receptor B (PirB) is another receptor with high affinity for myelin inhibitory molecules that mediates outgrowth inhibition through dephosphorylation of tropomyosin receptor kinase (Trk) neurotrophin receptors (Atwal et al., 2008; Fujita et al., 2011).

Although following a severe CNS lesion a glial scar forms to repair the site of blood brain barrier disruption and limit inflammation (Rolls et al., 2009) it also represents a physical barrier to axonal growth (McKeon et al., 1991). The glial scar consists mainly of reactive astrocytes proteoglycans, and collagen, of which collagen IV provides the basement membrane scaffold for chondroitin sulphate proteoglycans (CSPGs) to bind (McKeon et al., 1991). It has been shown that CSPGs, of the glial scar, are inhibitory to axonal outgrowth in culture via binding to the recently discovered receptor PTPsigma (McKeon et al., 1991; Snow et al., 1996; Shen et al., 2009). Furthermore, removing CSPG glycosaminoglycan chains with chondroitinase ABC (ChABC) promotes functional recovery after spinal cord injury (SCI) (Bradbury et al., 2002).

More recently, the axonal regeneration field has partially shifted to elucidating the intrinsic growth capacity of CNS neurons. Not surprisingly, many well-defined embryonic developmental pathways have become validated in adult stem cell proliferation, regeneration, and differentiation (Tsokos et al., 1987; Terenghi, 1999; Esposito et al., 2005; Harel and Strittmatter, 2006; Reimer et al., 2009). This supports the theory that regeneration can be accomplished by a revival of developmental signals.

Followings an acute central nervous system (CNS) injury, axonal regeneration and functional recovery are extremely limited. This is due to an extrinsic inhibitory growth environment and the lack of intrinsic growth competence. Retinoic acid (RA) signaling, essential in developmental dorsoventral patterning and specification of spinal motor neurons, has been shown through its receptor, the transcription factor RA receptor β2 (RARβ2), to induce axonal regeneration following spinal cord injury (SCI). Recently, it has been shown that in dorsal root ganglion neurons (DRGs), cAMP levels were greatly increased by lentiviral RARβ2 expression and contributed to neurite outgrowth. Moreover, RARβ agonists, in cerebellar granule neurons (CGN) and in the brain in vivo, induced phosphoinositide 3-kinase dependent phosphorylation of AKT that was involved in RARβ-dependent neurite outgrowth. More recently, RA-RARβ pathways were shown to directly transcriptionally repress a member of the inhibitory Nogo receptor (NgR) complex, Lingo-1, under an axonal growth inhibitory environment in vitro as well as following spinal injury in vivo. This perspective focuses on these newly discovered molecular mechanisms and future directions in the field.

Keywords: RA, RARβ, axonal regeneration

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It is believed that CNS neurons possess the ability, albeit limited, to regenerate. For example, the growth of CNS neurons into a PNS environment is possible (David and Aguayo, 1981; Benfey and Aguayo, 1982). It has been shown that the elongation of axons is linked to de novo transcription, and that blocking transcription immediately after injury inhibits regeneration from occurring (Smith and Skene, 1997). Furthermore, regeneration associated genes (RAGs) have been found following PNS injury that are absent following CNS injury in adults (Makwana and Raivich, 2005). These include GAP-43 and CAP-23 (growth cone proteins) (Chong et al., 1994; Mason et al., 2002), Spr11a and SCG-10 (cytoskeletal proteins) (Mason et al., 2002; Starkey et al., 2009), Galanin (neuropeptide) (Holmes et al., 2000), brain derived neurotrophic factor (BDNF) (Tonra et al., 1998), as well as Ch11 and Lgals1 (cell adhesion molecules) (Zhang et al., 2000), and galectin-1 (McGraw et al., 2004). Furthermore, following a conditioning lesion, upregulation of cAMP activates protein kinase A (PKA), which in turn phosphorylates the transcription factor cAMP response element-binding protein (CREB), inducing transcription thus leading to neurite outgrowth (Neumann et al., 2002; Qiu et al., 2002; Gao et al., 2004). Interestingly, it has been shown that when neurons mature they lose their ability to overcome an inhibitory environment and this transition coincides with a decrease in cAMP levels (Cai et al., 2002). Another transcription factor thought to be involved in the retrograde signal following a conditioning lesion is signal transducer and activator of transcription 3 (STAT3) (Bareyre et al., 2011), which is activated via phosphorylation by tyrosine kinase Janus kinase (JAKs) associated with the receptor subunit, which is the carrier protein involved in the transport of retinol from the liver storage site to peripheral tissue and by cellular retinol binding protein (CRBP), which is the intracellular carrier involved in intracellular movement of retinol (Le Doze et al., 2000). However, in the absence of ligand, RARβ binds DNA in concert with co-repressors (nCo-R, SMRT, HDAC, and mSin3) and inhibits transcription (Glass and Rosenfeld, 2000). There have been several documented cases where RA bound RARβ was found to occupy promoters independently from RXX and to repress transcription (Glass et al., 1989; Lipkin et al., 1992; Schoorlemmer et al., 1994). RA signaling typically involves direct transcriptional regulation, even though there are some less-defined cases involving non-transcriptional dependent RA signaling (Lopez-Carballo et al., 2002; Masia et al., 2007; Ohashi et al., 2009).

RA SIGNALING IN NEURITE OUTGROWTH AND AXONAL REGENERATION

Classically involved in development, neuronal differentiation, ventral neural patterning, and motor neuron specification (Maden et al., 1996; Díez del Corral et al., 2003; Novitch et al., 2003; Sockanathan et al., 2003), more recently RA also became a prime candidate to induce neurite outgrowth in neurons. The addition of exogenous RA and/or RARβ has been shown to induce neurite outgrowth in vitro in dorsal root ganglion neurons (DRGs), adult cortical neurons, and postnatal cerebellar granule neurons (CGN) (Corcoran and Maden, 1999; Corcoran et al., 2000; Wong et al., 2006; Yip et al., 2006; Puttagunta et al., 2011). In embryonic and adult DRGs as well as CGN, RA upregulated the expression RARβ2 in correlation with induced neurite outgrowth (Corcoran and Maden, 1999;
Corcoran et al., 2000; Puttagunta et al., 2011). Neurotrophins, such as nerve growth factor (NGF), essential in the development of the PNS (Piirsoo et al., 2010), have been shown to upregulate retinaldehyde dehydrogenase-2 (RALDH-2) and RARβ2 expression to induce neurite outgrowth of adult mouse DRGs (Corcoran and Maden, 1999). Moreover, a RARβ agonist, but not a RARα or RARγ agonist, induced neurite outgrowth from embryonic DRGs (Corcoran et al., 2000). Additionally, both cortex and DRG explants from adult rats treated for 14 days with a RARβ agonist induced neurite outgrowth in contrast to vehicle treated animals (Agudo et al., 2010). As a proof of principle that RA mediates its effect on neurite outgrowth exclusively through RARβ, it was shown in postnatal CGN that RARβ null CGN fail to extend neurites in response to RA (Puttagunta et al., 2011). Whereas, embryonic spinal cord explants respond to RA by inducing neurite outgrowth, adult spinal cord explants do not, and the limited RARβ2 expression in the adult spinal cord in comparison to the embryonic spinal cord is believed to be the reason for this difference (McCaffrey and Drager, 1994; Zetterstrom et al., 1999; Corcoran et al., 2002; So et al., 2006; Yip et al., 2006). Furthermore, when RARβ2, yet not RARγ4, was virally introduced into the adult spinal cord in organotypic preparations, it induced neurite outgrowth (Corcoran et al., 2002). Therefore, it can be concluded that RA acts specifically through RARβ2 in inducing neurite outgrowth in both embryonic and adult neurons.

Having established RARβ2 as the receptor responsible for RA induction of neurite outgrowth, the next logical step was to show that overexpression of RARβ2 in vivo would induce axonal regeneration. RARβ2 lentiviral infection of adult rat DRGs induced axonal growth and functional recovery of injured sensory neurons into the dorsal root entry zone (DREZ) following dorsal root lesion (Wong et al., 2006). In support of these findings, it was also shown that RARβ2 null mice have less axonal regeneration following a peripheral nerve crush versus wildtype mice (So et al., 2006). Additionally, RARβ2 lentiviral infection of the sensorimotor cortex three weeks prior to spinal cord lesion induced axonal and functional regeneration of the cortical spinal tracts (CST) in adult rats (Yip et al., 2006). Finally, RARβ specific agonist, CD2019, CD2019 induced neurite outgrowth in both embryonic and adult neurons.

RA signaling in neurite outgrowth in an inhibitory environment. RA signaling increases neurite outgrowth, decreases RhoA activation and inhibits Lingo-1 gene and protein expression in a myelin-inhibitory environment (however, not on a CSPG-inhibitory substrate) in CGN, specifically through RARβ. By in silico analysis, we discovered a RARE in the Lingo-1 promoter, which bound RARβ but not RXR upon RA treatment in a myelin-inhibitory environment. Moreover, we showed this RARE to be functional by a luciferase assay that when mutated did not respond to RA signaling. Furthermore, addition of Lingo-1 in RA-treated CGN in a myelin-inhibitory environment abrogated RA-RARβ induced neurite outgrowth. Finally, in vivo RA treatment decreased Lingo1 protein expression following SCI in wildtype but not in RARβ null mice, providing physiological relevance to the in vitro findings (Puttagunta et al., 2011) (Figure 1A). Importantly, it has previously been shown that Lingo1 antagonists promoted axonal sprouting, improved functional recovery, decreased RhoA activation, and increased oligodendrocyte and neuronal survival following rubrospinal or CST transaction (Ji et al., 2006). It will be important to determine if this RA-dependent decrease in Lingo1 is found exclusively in the neurons or also in the oligodendrocytes that make up the myelin sheathing and if RA signaling, such as seen with Lingo1 inhibition (Mi et al., 2005, 2007, 2009), leads to an increase in remyelination. In fact, for proper functionality following SCI, axons must re-grow, reinnervate their targets, and remyelinate their axons.

THE MOLECULAR MECHANISMS OF RA SIGNALING IN AXONAL REGENERATION

While it is known that RA signaling does induce neurite outgrowth and axonal regeneration only limited research has been done on the precise molecular mechanisms involved. It was shown that lentiviral RARβ2 expression induces neurite outgrowth in DRGs and increases cAMP levels (Wong et al., 2006). The effect of RARβ2 on neurite outgrowth was significantly decreased when an adenylate cyclase inhibitor, 2′,5′-dideoxyadenosine (DDA) or a cell–permanent inhibitor of cAMP-dependent PKA was used (Wong et al., 2006). While this suggests that cAMP is involved in RA signaling, it is not sufficient, as use of dibutyryl cAMP in adult DRGs in the DREZ model did not promote functional recovery as robustly as RARβ2 alone (Wong et al., 2006). In fact, it was shown in postnatal CGN that the positive affect of a RARβ agonist (CD2019) on neurite outgrowth was fully attenuated by a PI3K inhibitor but not a PKA inhibitor. Furthermore, it was shown that RARβ induced phosphorylation of AKT in CGN and in vivo, exclusively in injured neurons (Agudo et al., 2010). Interestingly, it has been previously shown that RA-RARβ2/RXR directly activates AKT during the differentiation of human neuroblastoma cells (Lopez-Carballo et al., 2002; Ohashi et al., 2009).

Recently, we have discovered a direct transcriptional target of RA signaling in neurite outgrowth in an inhibitory environment. RA signaling increases neurite outgrowth, decreases RhoA activation and inhibits Lingo-1 gene and protein expression in a myelin-inhibitory environment (however, not on a CSPG-inhibitory substrate) in CGN, specifically through RARγ. By in silico analysis, we discovered a RARE in the Lingo-1 promoter, which bound RARβ but not RXR upon RA treatment in a myelin-inhibitory environment. Moreover, we showed this RARE to be functional by a luciferase assay that when mutated did not respond to RA signaling. Furthermore, addition of Lingo-1 in RA-treated CGN in a myelin-inhibitory environment abrogated RA-RARβ induced neurite outgrowth. Finally, in vivo RA treatment decreased Lingo1 protein expression following SCI in wildtype but not in RARβ null mice, providing physiological relevance to the in vitro findings (Puttagunta et al., 2011) (Figure 1A). Importantly, it has previously been shown that Lingo1 antagonists promoted axonal sprouting, improved functional recovery, decreased RhoA activation, and increased oligodendrocyte and neuronal survival following rubrospinal or CST transaction (Ji et al., 2006). It will be important to determine if this RA-dependent decrease in Lingo1 is found exclusively in the neurons or also in the oligodendrocytes that make up the myelin sheathing and if RA signaling, such as seen with Lingo1 inhibition (Mi et al., 2005, 2007, 2009), leads to an increase in remyelination. In fact, for proper functionality following SCI, axons must re-grow, reinnervate their targets, and remyelinate their axons.

RA AND EPIGENETICS

Epigenetics changes can affect gene regulation by modifying the surrounding chromatin environment. They include DNA methylation and a number of histone post-translational modifications such as phosphorylation, acetylation, and methylation. For example, methylation of promoters or histones often inhibits gene expression, yet acetylation of histones or transcription factors induces gene expression by promoting transcription factor occupancy within a more accessible chromatin environment (reviewed in (Kouzarides, 2007; Mueller and von Deimling, 2009). These epigenetic changes can be transient or more long lasting thereby allowing extrinsic and intrinsic cellular signals to immediately and strongly influence gene expression (Giusconi and Puri, 2009). The acetyltransferases, CBP/p300, are essential for mammalian cell proliferation and development (Yao et al., 1998). Importantly, p300 appears to be a critical cofactor for RA transcriptional activity, even leading to differentiation and somitic
FIGURE 1 | RA-RARβ dependent direct transcriptional repression of inhibitory myelin signaling and a hypothetical schematic of RA involvement in retrograde signaling, RAGs expression, and axonal regeneration. (A) Following RA treatment HDAC3 binding to the RARE of the Lingo-1 promoter decreases acetylation of H3 lysine 9 and possibly displaces PCAF binding. Meanwhile, an increase in methylation of H3 lysine 27 is observed, coupled with strong binding of a RARβ homodimer to the RARE of the Lingo-1 promoter repressing Lingo-1 expression. This is believed to displace the equilibrium of the NogoR complex and block the activation of RhoA, thus leading to neurite outgrowth. Other possible members of this transcriptional repressor complex may include NCoR and SMRT (indicated with dashed lines). (B) Following a conditioning peripheral lesion and RA treatment an increase in RARβ, pCREB, and pSTAT3 levels are observed. All three have been shown to be vital to PNS axonal regeneration. CREB and STAT3 have been shown to play a role in retrograde signaling involved in the induction of RAGs expression prior to axonal regeneration. It will be intriguing to examine if RA signaling activates these three transcription factors to work in concert regulating RAGs expression to induce both axonal CNS and PNS regeneration in vivo.

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symmetry (Kawasaki et al., 1998; Yao et al., 1998; Vilhais-Neto et al., 2010). Furthermore, in spinal motor neuron development, CBP is recruited by RA activated RAR to promoters for acetylation of histones, increasing accessibility of the promoter to transcription factors and inducing the expression of spinal motor neuron genes (Lee et al., 2009). RA signaling initiates PCAF binding to RAR/RXR heterodimers displacing co-repressor complexes and inducing gene expression (Blanco et al., 1998). Preliminary findings show (personal observations) that acetylation of histone 3 at lysine 9 (AcH3K9) at the RARE of the Lingo-1 promoter is significantly decreased upon RA treatment in a myelin-inhibitory environment. In accordance, we saw an increase in histone deacetylase 3 (HDAC3, known to form a repressor complex with NCoR/SMRT at RA regulated genes) (Hartman et al., 2005) on the RARE of the Lingo-1 promoter. In addition, we observed an increase in methylation on H3 at lysine 27 (H3K27Me3, also known to be previously linked to RA mediated differentiation) (Gillespie and Gudas, 2007). These observations further extend upon recent published work showing a decrease in Lingo-1 expression upon RA treatment in a myelin-inhibitory environment (Puttagunta et al., 2011) (Figure 1A), however, further examination is required to determine complete

**FIGURE 2 | RA-dependent non-transcriptional involvement in neurite outgrowth.** Inhibition of PTEN leads to increased levels of PIP3 via PI3K. This in turn activates AKT by phosphorylation. Active pAKT has many functions such as the inhibition of GSK-3, Bad, or Bax, conversely it can activate Raf, Rac1, and NF-κB. Recently in axonal regeneration it has been shown that active pAKT leads to the activation of the mTOR pathway (which can be inhibited via rapamycin). This leads to increased protein synthesis and ribosome biogenesis as well as cell growth through the phosphorylation of the ribosomal protein S6 and the release of the eukaryotic initiation factor 4E (eIF-4E) from inhibition. RA-RARα signaling has shown an inhibition of PTEN and a direct activation of pAKT in two separate studies. It will be of interest to examine if RA signaling induces downstream mTOR signaling in axonal regeneration.
activator and repressor complexes involved in RA-dependent Lingo-1 regulation.

**FUTURE PERSPECTIVES**

In order to achieve functional axonal regeneration and recovery following SCI several obstacles need to be overcome: (a) an inhibitory glial environment, (b) excessive inflammation, (c) lack of neuronal intrinsic capacity for axonal outgrowth, and (d) axonal demyelination. In fact, RA signaling seems to have the potential to tackle several of these limitations. RA has long been known for its anti-inflammatory benefits (Orfanos and Bauer, 1983; Nozaki et al., 2006). Interestingly, RA has been shown to inhibit the interferon-gamma induced inflammatory response in primary rat brain cultured astrocytes by inducing suppressors of cytokine signaling (SOCS3) and inhibiting JAK and STAT3 activation (Choi et al., 2005). Thus, it will be of interest to elucidate weather RA treatment in vivo following SCI reduces the formation of a glial scar by inhibiting the inflammatory response.

As the RA-RARβ pathway seems to overcome the myelin inhibitory environment, but not necessarily proteoglycan inhibitory signaling, combining ChABC with enhancement of RA-RARβ should strongly reduce the inhibitory signaling following SCI and further promote axonal regeneration than either does alone.

Given that neuronal overexpression of RARβ promotes axonal sprouting and regeneration of the CST and dorsal columns after SCI, it will be interesting to explore whether RA-RARβ is able to enhance the intrinsic ability of CNS neurons to regenerate by driving the expression of specific RAGs. Besides a possible direct transcriptional role for RAGs, RA-RARβ signaling may be involved in the cAMP-dependent retrograde signal following a PNS lesion that induces RAGs expression (Qiu et al., 2002). In fact, RARβ null mice have significantly reduced neurite outgrowth following peripheral nerve crush compared to wildtype mice (So et al., 2006), and, as mentioned previously, cAMP is induced by RARβ overexpression and involved in its ability to induce neurite outgrowth (Wong et al., 2006). Moreover, STAT3 is thought to be a retrograde signal following a PNS lesion and its inhibition limits axonal regeneration (Qiu et al., 2002, 2005; Schweizer et al., 2002; Bareyre et al., 2011). While STAT3 is inhibited by RA signaling in astrocytes, conversely it is induced in a CNFT-dependent manner in differentiated neuroblatoma cells, retinal, and ciliary chick ganglion neurons (Malek and Halvorsen, 1997; Wang and Halvorsen, 1998). It will be of interest to inspect if RA-RARβ, CREB, and STAT3 transcriptional signaling work in concert to induce expression of RAGs and axonal regeneration (**Figure 1B**).

RA may also intersect with PTEN-mTOR signaling after SCI. The inhibition of PTEN results in the activation of AKT (pAKT) inducing axonal regeneration through mTOR by new protein synthesis and growth (Park et al., 2008). Accordingly, pAKT, which decreases after axonal injury (Liu et al., 2010), was shown to be only upregulated in injured CST axons following treatment with a RARβ agonist (Agudo et al., 2010). Therefore, it will be interesting to determine if AKT activation by RA signaling is through the inhibition of PTEN or the direct phosphorylation of AKT following axonal lesions and if this results in mTOR activation (**Figure 2**). In fact, dissection of this pro-regenerative molecular pathway may offer novel molecular targets for the effective enhancement of axonal regeneration.

Moreover, as RA signaling may take place via either paracrine or autocrine pathways and as Schwann cells, astrocytes, meningeal fibroblasts, and macrophages differentially express RA,RAR, or RALDH2 after PNS and CNS injury (Mey, 2006), further investigation of the relative weight of the two modes of action in axonal regeneration is warranted.

While RA pathways seem to positively affect several pathogenic aspects of SCI, further investigation is needed to clarify more direct transcriptional targets of RA-RARβ signaling following SCI and the interaction of RA pathways with other pro-regenerative signals. Only then, may we have the possibility to design RA signaling-dependent molecular therapies that may specifically enhance spinal axonal regeneration and recovery, with limited off target side effects.

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