CD40L/CD40 bidirectional signaling is a major regulator of neuronal morphology in the developing nervous system

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Appropriate nervous system function depends on a precise but plastic neural architecture. Neuronal morphology determines how neurons interact with each other and with other cells. Every kind of neuron has its own morphological characteristics, which are determined by both intrinsic and extrinsic factors. In addition to intrinsic genetic programmes and patterns of neural activity, a variety of extrinsic factors regulate the growth and branching of neural processes and synaptogenesis. Work over the past decade has revealed that several members of the tumor necrosis factor superfamily (TNFSF) are potent positive and negative physiological regulators of neural process growth and branching in the developing nervous system without affecting neuronal survival. Extensively characterized in the immune system, where they play key roles in orchestrating and regulating immune responses, TNFSF members bind one or more members of the TNF receptor superfamily (TNFRSF), initiating canonical forward signaling. In addition, several TNFRSF members can act as ligands for the membrane-integrated TNFSF, triggering reverse signaling that has distinctive cellular responses to forward signaling (Eissner et al., 2004). In the developing nervous system, TNFSF/TNFRSF bidirectional signaling plays a major role in modulating neuronal architecture and has been studied most extensively for CD40 ligand (CD40L, TNFSF5) and CD40 (TNFRSF5).

In recent years, several studies have reported that CD40L/CD40 bidirectional signaling is a major physiological regulator of axon and dendrite growth and branching for multiple clinically important populations of neurons in the developing peripheral and central nervous system. Direct morphological comparison of neurons between CD40-deficient animals and wild type animals has revealed the physiological significance of CD40L/CD40 signaling, and a battery of *in vitro* experiments on neurons from *Cd40*$^{-/-}$ or *Cd40*$^{+/+}$ mice has determined whether forward or reverse signaling mediates the physiological effect for each population of neurons. Additional *in vitro* studies have also begun to ascertain the downstream intracellular events that mediate the influence of CD40L/CD40 signaling on axons and dendrites.

Here we review the roles of CD40L/CD40 bidirectional signaling in the development of multiple peripheral and central neuronal populations. This work on development sets the scene for investigation of the potential role of CD40L/CD40 signaling in neuronal plasticity in the adult nervous system, in neural regeneration and in neural degeneration and whether CD40L/CD40 signaling could have potential therapeutic applications.

Experimental approaches: *In vivo* comparison of the morphology of neurons in CD40-deficient and wild-type mice has provided an indication of the physiological significance of CD40L/CD40 signaling in the regulation of neuronal morphology. In the PNS, this has been done by selectively staining axons in whole-mount preparations and in the central nervous system (CNS) by labelling neurons using the Golgi technique.

Because genetic deletion of CD40 disrupts both forward and reverse signaling, *in vitro* studies on neurons isolated from *Cd40*$^{-/-}$ or *Cd40*$^{+/+}$ mice have been carried out to ascertain the direction of signaling in each case. Typically, when neurons from CD40-deficient mice are cultured under appropriate conditions, they replicate the phenotypic change observed *in vivo*. *In vitro* experiments to determine the direction of signaling utilize soluble CD40L and soluble CD40. These are able to activate forward and reverse signaling, respectively, or can inhibit reverse and forward signaling, respectively, by competing with endogenous CD40L and CD40. In addition, in the case of reverse signaling, soluble CD40, but not CD40L, is able to rescue the phenotype of CD40-deficient neurons. Furthermore, studies of the cellular expression of CD40L and CD40 together with other studies can ascertain whether CD40L/CD40 signaling occurs via an autocrine or paracrine mechanism. Additional biochemical experiments and the use of variety of pharmacological reagents that affect different intracellular signaling pathways have begun to elucidate the downstream signaling pathways that mediate the effects of CD40L/CD40 signaling on neural processes.

CD40L and CD40 in developing peripheral neurons (PNS): The accessibility and ease with which PNS neurons are studied both *in vivo* and *in vitro* facilitated the discovery of the neurotrophin family. These secreted proteins, of which nerve growth factor (NGF) is the founding member, regulate neuronal survival and promote the growth and branching of sensory and sympathetic axons in innervated target tissues. Neurons become dependent on and responsive to neurotrophins when their axons first reach their targets (Davies et al., 1987; Vogel and Davies, 1991). A growing body of work has shown that PNS neurons subsequently become responsive to several members of TNFSF, acting by either forward or reverse signaling mechanisms, when their axons are ramifying in their targets later in development. They generally do this by modulating, either positively or negatively, the response of neurons to the axon growth-promoting actions of neurotrophins without affecting neuronal survival.

The first evidence that CD40L/CD40 signaling affects developing neurons came from studies of paravertebral sympathetic neurons (McWilliams et al., 2015). Here autocrine CD40-activated CD40L-mediated reverse signaling enhances NGF-promoted axon growth and branching. However, the effect of CD40L reverse signaling on axon growth is only observed at low concentrations of NGF because high levels of NGF suppress CD40 and CD40L expression, effectively turning off the CD40/CD40L autocrine loop. Consequently, a hypoinnervation phenotype in CD40-deficient mice *in vivo* is restricted to paravertebral target tissues that express low levels of NGF. This work not only demonstrated the physiological relevance of CD40/CD40L signaling in the nervous system but also provided the first rationale for autocrine signaling in neurons, a counterintuitive yet widespread phenomenon. This work showed that differential regulation of autocrine signaling within a population of neurons can have region-specific effects on axon growth and tissue innervation.

A later study showed that autocrine CD40-activated CD40L-mediated reverse signaling operates in prevertebral sympathetic neurons over the same late development window as in paravertebral sympathetic neurons, but has opposite effects on axon growth *in vitro* and tissue innervation *in vivo* (Calhan et al., 2019). Here CD40/CD40L impairs NGF-promoted axon growth, resulting in a hyperinnervation phenotype in certain paravertebral sympathetic target tissues in CD40-deficient mice.

In contrast to sympathetic neurons, CD40L signaling in developing sensory neurons operates by a forward signaling mechanism (Howard et al., 2019). Here it enhances sensory axon growth and promotes the innervation of peripheral sensory target tissues *in vivo*. However, in marked contrast

to all other TNFSF members, which act on PNS neurons late in development after they become dependent on neurotrophins, CD40L-activated CD40 mediated forward signaling acts in sensory neurons during early stage of development when axons are growing to their targets. Furthermore, in addition to a CD40L/CD40 autocrine signaling loop that enhances early neurotrophin-promoted axon growth, target-derived CD40 acts directly on sensory neurons to enhance axon growth independently of neurotrophins.

In summary, bidirectional CD40L/CD40 signaling is a physiological regulator of axon growth and target innervation in the developing PNS that acts in a variety of ways in different populations of neurons. Either by forward or reverse signaling, either by enhancing or inhibiting axon growth, either by autocrine or paracrine mechanisms and either by modulation of the axon growth-promoting actions of neurotrophins or by acting independently of neurotrophins.

CD40L and CD40 in developing central neurons: CD40-activated CD40L reverse signaling is a major *in vivo* regulator of dendrite growth and elaboration in at least two populations of clinically important CNS neurons during development. In CD40-deficient mice, the dendrite arbors of excitatory hippocampal pyramidal neurons are greatly reduced in size and complexity compared with wild-type mice, whereas those of inhibitory striatal medium spiny neurons (MSNs) are substantially larger and more branched (Carriba and Davies, 2017). These striking and opposite dendrite phenotypes are replicated in cultured neurons. However, because axons can be easily distinguished from dendrites in cultured hippocampal pyramidal neurons, the finding that a small axon phenotype is observed in CD40-deficient neurons and its rescue to wild type phenotype after the addition of soluble CD40 raises the possibility that CD40L reverse signaling may also regulate axon growth from hippocampal pyramidal neurons *in vivo*.

In MSNs, the effects of CD40L-mediated reverse signaling are not only restricted to regulating dendrite growth and branching but also influence dendrite spine number and morphology (Carriba et al., 2020). Dendrite spines are key structures in the synaptic transmission and neuronal plasticity, and abnormalities in spine morphology are associated with several neuropathological conditions. Although dendrite spines have been widely studied, little is known about the extrinsic factors that control their formation and maturation. While spine density in MSNs is unaffected in *Cd40*$^{-/-}$ mice, because the dendrite arbors of MSNs in these mice are larger and more exuberant than those of wild-type mice, MSNs possess a correspondingly larger total number of spines in *Cd40*$^{-/-}$ mice. In addition, the morphology of the MSN spines in *Cd40*$^{-/-}$ mice resembles that of mature spines. Biochemical analyses showed significant changes in the expression of some members of Rho GTPases, a family of proteins that regulate actin cytoskeleton dynamics, and a reduction in the expression of postsynaptic density protein 95 (PSD-95), a key protein involved in the spine morphology, maturation, and function. Furthermore, the distribution of PSD-95 along MSN dendrites was more clustered in *Cd40*$^{-/-}$ mice and more diffuse in *Cd40*$^{+/+}$ mice. The changes observed in MSN cultured from *Cd40*$^{-/-}$ mice were prevented by activation of CD40L reverse signalling, suggesting that reverse signalling also influences dendrite spine morphology by regulating the expression and distribution of proteins that control spine shape and maturation (Carriba et al., 2020).

While hippocampal pyramidal neurons and striatal MSNs co-express CD40 and CD40L, because these neurons cannot be cultured at very low density, it has not been possible to formally demonstrate that CD40L/CD40 signaling operates by an autocrine mechanism in these CNS neurons. Furthermore, because of this and the poor understanding of the roles of neurotrophic factors in regulating the development of CNS neurons, it has not been possible to test whether CD40L/CD40 signaling is regulated by neurotrophic factors in the CNS. However, because MSNs and pyramidal neurons can be harvested in much larger numbers than PNS neurons, there has been much better progress in identifying the intracellular signaling pathways that mediate the effects of CD40L/CD40 signaling in influencing the growth of CNS processes. Using a combination of western blot analysis, pharmacological drugs and siRNA knockdown, we established that the morphological effects mediated by CD40L reverse signaling in pyramidal and MSNs are dependent on activation of protein kinase C (PKC), with the PKCβ isoform required in pyramidal neurons and the PKCγ isoform required in MSNs (Carriba and Davies, 2017).

Furthermore, in pyramidal neurons, we started to identify the downstream molecular components after CD40L engagement. Initial *in silico* analysis using the STRING database (https://string-db.org/), which determines possible protein-protein interactions, identified Syk, a nonreceptor tyrosine kinase highly expressed in cells of the immune system, as a common partner for CD40L and PKCβ. Activation of receptors of the adaptive immune response results in Syk recruitment to the cell membrane in which it plays a key role in signal transduction. Immunoprecipitation studies using pyramidal neurons confirmed the interaction of Syk with PKCβ, but not with PKCγ, suggesting that activation of CD40L reverse signaling in pyramidal neurons results in the formation of a molecular complex comprising CD40L, PKCβ, and Syk (Carriba and Davies, 2020).

This *in silico* analysis also identified extracellular regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) and several regulatory proteins of these signaling pathways. Indeed, western blotting experiments using pharmacological reagents suggest that PKC, ERK1/2 and JNK participate in a molecular network that is responsible for the regulation of axon and dendrite growth in developing pyramidal neurons following the activation of CD40L reverse signaling (Carriba and Davies, 2020). These three signaling proteins have been each separately implicated in the regulation of growth and/or branching in several neuronal models by numerous extrinsic factors, including CD40L/CD40 (Carriba and Davies, 2017; Howard et al., 2019). Engagement of CD40L reverse signaling causes activation of all three signaling pathways in hippocampal pyramidal neurons. However, analysis of the effects of pharmacological activators and inhibitors of these pathways on axon and dendrite growth elicited by reverse signalling revealed that activation of PKC and ERK1/2 enhanced the growth while activation of JNK inhibited growth. Combinations of these pharmacological reagents in morphological and phosphorylation experiments showed that these signaling pathways regulate axon and dendrite growth by functioning as an interconnected and interdependent network. The morphological studies provided the hierarchy in which these proteins act, and the phosphorylation studies showed that when CD40L reverse signaling is engaged the phosphorylation level of each particular protein is differentially and distinctively regulated by the other two signaling pathways. Briefly, our findings showed that JNK plays a dominant role, its inhibition being necessary for the growth-promoting actions of PKC and ERK1/2. Moreover, JNK phosphorylation is fine-tuned by PKC and ERK1/2. These features along with the fact that CD40L engagement phosphorylates JNK suggest that JNK activation may be a checkpoint loop that controls whether or not growth continues depending on the phosphorylation status of PKC and ERK1/2. Remarkably, in all combinations in which PKC was active, the levels of pJNK increased, suggesting that the role of JNK activation is related to growth termination (Carriba and Davies, 2020).

These findings might provide clues about the molecular mechanism by which CD40L reverse signaling regulates the growth in other neuronal cells. In this regard, we have preliminary data indicating that in MSNs the restriction of the neurite growth and elaboration mediated by CD40L reverse signaling is also regulated by PKC, ERK1/2 and JNK, although the function, hierarchy and phosphorylation regulation differ from

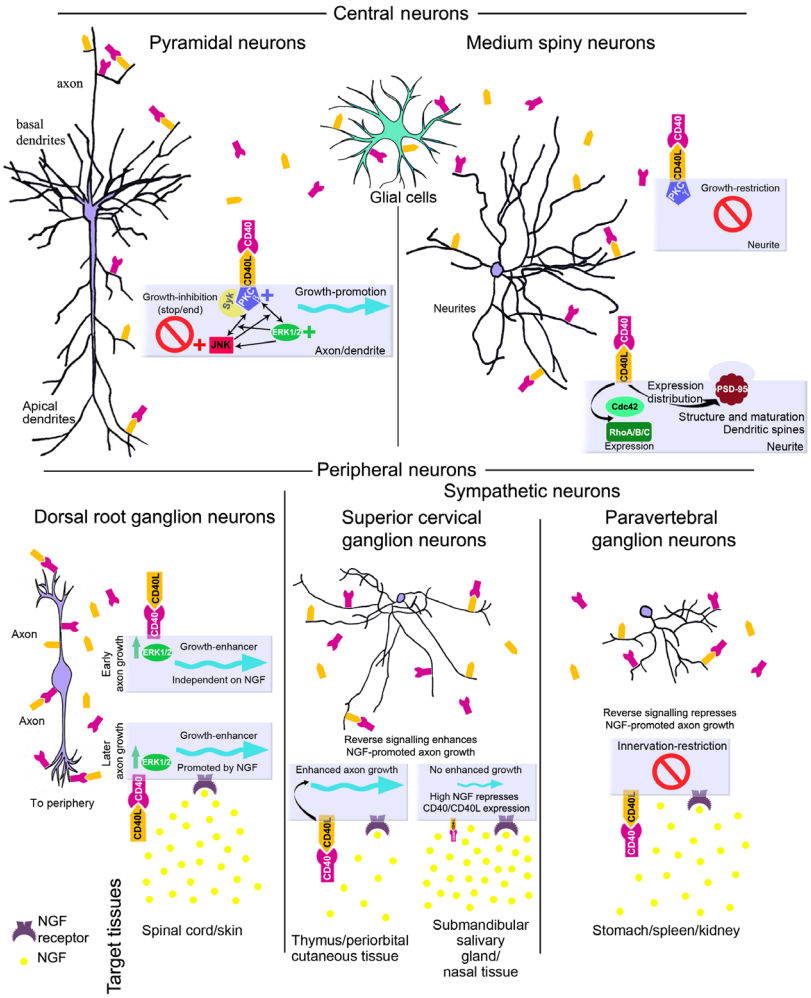

Figure 1 | **Summary of the roles of CD40/CD40L bidirectional signaling in neural development in central and peripheral neurons.**
CD40 and CD40L are co-expressed in pyramidal neurons, medium spiny neurons, dorsal root ganglion neurons, superior cervical ganglion neurons, and paravertebral ganglion neurons. In peripheral neurons, CD40L/CD40 signaling operates by an autocrine mechanism, and in central neurons, CD40L and CD40 can also be obtained from glial cells. See the text for the functions of CD40/CD40L signaling in regulating the growth of neural processes in each kind of neurons. NGF: Nerve growth factor; PSD-95: postsynaptic density protein 95.

pyramidal neurons.

Final remarks: Abnormalities in the size and elaboration of neural processes and in spine number and structure are observed in many neurodevelopmental disorders and neurodegenerative diseases (Koleske, 2013). While several studies have shown that CD40L and CD40 play important and diverse roles in neural development in the PNS and CNS (**Figure 1**), there is some evidence implicating CD40L/CD40 signaling in neuropathology. Mutations in CD40L produce a syndrome characterized by severe developmental delay, ataxia, and seizure disorder with some physical abnormalities (Rauch et al., 1999) and there is some evidence implicating CD40L/CD40 signaling in Alzheimer's disease (Tan et al., 2002). In future work, it will be important to investigate the potential participation of CD40L/CD40 signaling in the etiology and/or progression of neuropathological disorders. Given the important role of CD40L/CD40 signaling in the immune system, the inflammatory response that accompanies many neurological disorders may affect the nervous system by changes in CD40L/CD40 signaling. Moreover, given the marked influence of CD40L/CD40 signaling on the growth of neural processes, it will be important to investigate its potential involvement in neural injury and regeneration. Such work may have important therapeutic implications.

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