The complement cascade in the regulation of neuroinflammation, nociceptive sensitization, and pain

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The complement cascade is a key component of the innate immune system that is rapidly recruited through a cascade of enzymatic reactions to enable the recognition and clearance of pathogens and promote tissue repair. Despite its well-understood role in immunology, recent studies have highlighted new and unexpected roles of the complement cascade in neuroimmune interaction and in the regulation of neuronal processes during development, aging, and in disease states. Complement signaling is particularly important in directing neuronal responses to tissue injury, neurotrauma, and nerve lesions. Under physiological conditions, complement-dependent changes in neuronal excitability, synaptic strength, and neurite remodeling promote nerve regeneration, tissue repair, and healing. However, in a variety of pathologies, dysregulation of the complement cascade leads to chronic inflammation, persistent pain, and neural dysfunction. This review describes recent advances in our understanding of the multifaceted cross-communication that takes place between the complement system and neurons. In particular, we focus on the molecular and cellular mechanisms through which complement signaling regulates neuronal excitability and synaptic plasticity in the nociceptive pathways involved in pain processing in both health and disease. Finally, we discuss the future of this rapidly growing field and what we believe to be the significant knowledge gaps that need to be addressed.

The complement system consists of over 40 soluble and membrane-bound proteins that are rapidly mobilized through a cascade of enzymatic reactions (Fig. 1) in response to infection or tissue injury. Activated components of the complement system participate in canonical host defenses through a range of mechanisms, including the activation and chemotaxis of immune cells, as well as opsonization (*i.e.*, tagging) and killing of pathogens or diseased cells (1–4). The actions of the complement cascade are considered as a functional bridge between the two branches of the immune system, linking the intrinsic activity of the innate immune system to the lymphocytes that drive the adaptive responses (5). It is becoming increasingly appreciated that complement also orchestrates multiple host processes and particularly those related to the function of the nervous system in health and disease. These include complement-dependent regulation of synaptic remodeling, axonal regrowth, neuronal damage, nociceptor sensitization, and pain. Despite recent progress, significant knowledge gaps exist in our understanding of molecular and cellular mechanisms that mediate the effects of complement cascades on neurons in the central and peripheral nervous systems. Furthermore, there are numerous examples when the same component of the complement system may impose opposite effects on neurons under pathological conditions, by either promoting neuronal recovery or exacerbating neuronal damage and aberrant neuronal activity. This suggests that the same complement factors can drive distinct outcomes through state-dependent mechanisms we have yet to fully elucidate.

Here, we systematically discuss what is known about the complex effects of activated complement products on neurons during the normal host response to injury as well as in the development and maintenance of neurological pathologies and propose classification of these effects based on common underlying molecular and cellular mechanisms. We focus particularly on the complement-dependent changes in the function of the somatosensory system that take place in response to injury or illness and discuss how these mechanisms contribute to nociceptive sensitization and the development of acute and chronic pain. Finally, we discuss what we consider as critical open questions for future research as well as challenges and opportunities in this rapidly emerging field of complement neurobiology.

Activation of the complement cascade

Under normal conditions, complement proteins are continuously produced as inactive zymogens by the liver and secreted into the plasma. Other tissues can serve as local sources and provide focal enhancement of complement signaling and inflammation after injury (6). Each inactive zymogen is cleaved into its active products by a factor-specific protease. For example, the C5 protein is inactive until it is cleaved into the small "a" fragment (C5a), which is soluble, and the larger "b" fragment (C5b), which is membrane-bound through a covalent bond. This a/b nomenclature is used

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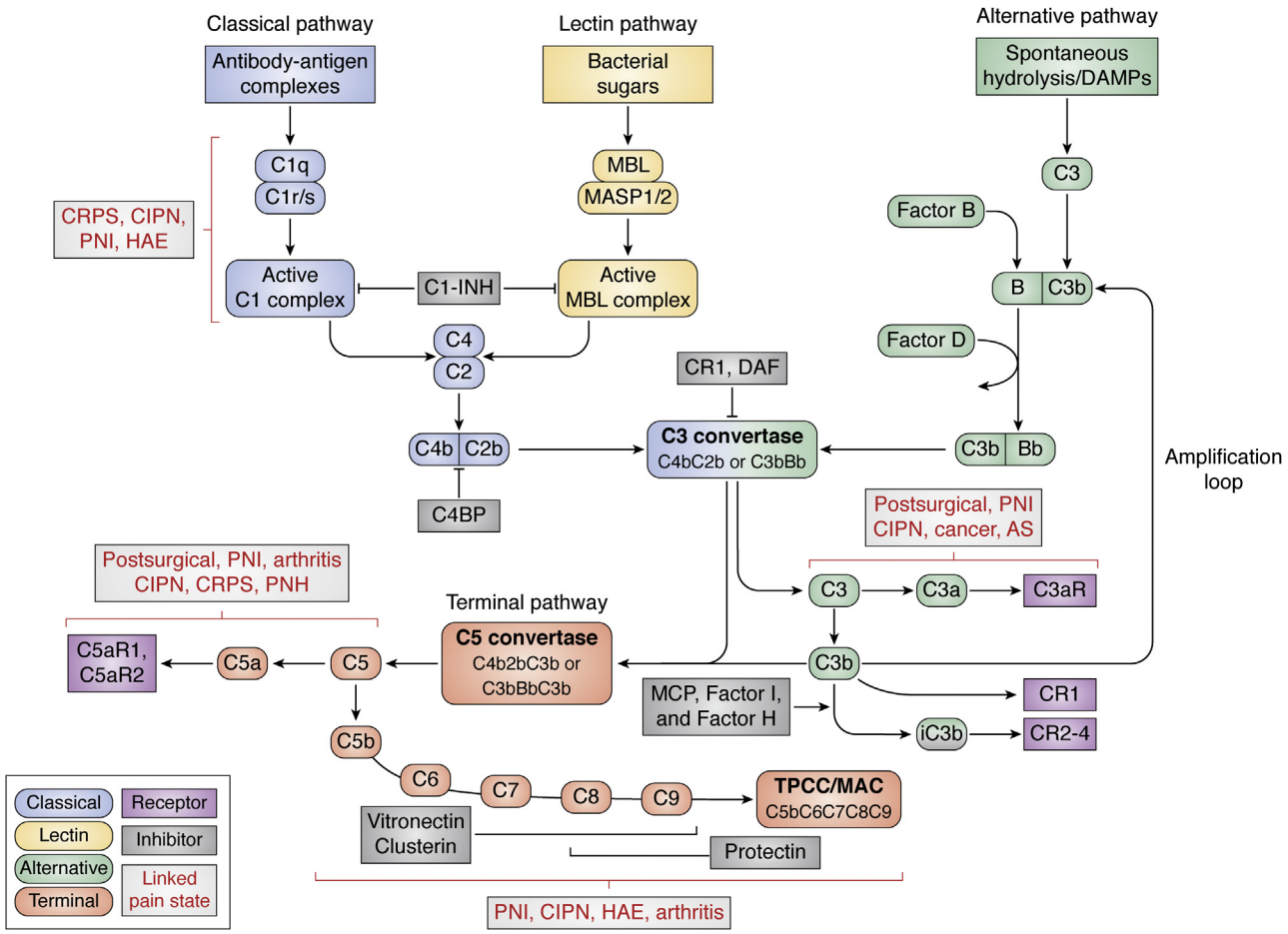

Figure 1. The complement system integrates distinct stimuli into a unified immunological response. The three pathways of complement activation, Classical (*blue*), Lectin (*yellow*), and Alternative (*green*), are activated by distinct perturbations to organism homeostasis such as antibody-based recognition of antigens or danger-associated molecular patterns (DAMPs), to drive a cascade of enzymatic activity merging in the terminal pathway (*red*). The cumulative result is the production of membrane-bound fragments (*e.g.*, C3b and C5b) and soluble factors (*e.g.*, C3a and C5a) that activate signaling through surface receptors (*purple*) that are highly expressed on immune and glial cells, and to a lesser extent, on neurons. Uncontrolled activation of the terminal pathway also promotes assembly of the cell-lysing terminal pathway complete complex (TPCC), also known as the membrane attack complex (MAC), which is regulated by multiple steric inhibitors and proteases (*gray*). This system controls the formation of complement products, their activity once formed and proteolytic degradation. A key component of the system is the amplification loop within the alternative pathway, which provides a positive feedback loop to ensure maximal activation of complement when appropriately triggered. However, this loop can also exacerbate complement-induced pathology under the conditions when complement inhibitors are dysregulated. Linked pain states are highlighted in red square brackets and include postsurgical pain, pain associated with peripheral nerve injury (PNI), complex regional pain syndrome (CRPS), and arthritic pain, as well as pain associated with paroxysmal nocturnal hemoglobinuria (PNH), hereditary angioedema (HAE), ankylosing spondylitis (AS), and chemotherapy-induced peripheral neuropathy (CIPN). In this context, CIPN also includes a painful condition associated with the use of anti-ganglioside-GD2 antibody for treating neuroblastoma cancer (9–11).

across complement components, which are numbered according to the order of their discovery rather than their sequence of activation. When tissue becomes damaged or an autoimmune reaction occurs, the action of the two split products is complementary: the "a" fragment attracts and activates immune cells, whereas the "b" fragment opsonizes the offending cell to promote its recognition and clearance by phagocytes. The activation of the complement cascade can be driven *via* three major pathways: classical, lectin, and alternative.

The *classical* pathway (Fig. 1, blue) is activated by binding of an antibody (IgM or IgG) to an antigen on a microbial or host cell, forming an antibody–antigen complex, also known as an immune complex. In essentially any autoimmune disorder where aberrant self-recognition occurs, the production of immune complexes drives high levels of complement activity. The primary detector of immune complexes and pathogen-associated molecular patterns (PAMPs) is the C1 complex, which consists of the pattern recognition protein, C1q, and two protease complexes formed by C1r and C1s (Fig. 1). The binding of an immune complex to the C1 complex results in autocatalytic cleavage of the latter, producing the active form of C1 complex. This in turn cleaves C4 to produce C4b, which rapidly forms a covalent bond with a membrane. The anchoring of C4b to a membrane limits all subsequent cleavages to the immediate vicinity of the site at which the immune complex was first detected, preventing the spread of activation and providing a focal source of the downstream terminal chemotactic fragments, such as C3a and C5a. Aberrant activation of the classical pathway induced by B cell

overproduction of antibodies can drive immunological pain disorders such as complex regional pain syndrome (CRPS; Fig. 1) (7, 8). Additionally, activation of the classical pathway by cytotoxic agents such as antiganglioside-GD2 antibody for treating neuroblastoma cancer, contributes to severe pain, which is a common side effect of anticancer therapeutics (9–11) (Fig. 1).

Membrane-bound C4b binds C2 that is subsequently cleaved by the C1 complex, resulting in the active serine protease complex, C4b2b, termed the classical C3 convertase (Fig. 1, blue/green). Up to this point, the only "a" fragments produced are C4a and C2a, both of which have essentially no activity (12). Formation of the C3 convertase is a critical transition to terminal pathway signaling and the generation of highly active C3a and C5a components, both of which are powerful drivers of neuroinflammation and pain (Fig. 1) (13–17).

The *lectin* pathway (Fig. 1, yellow) is activated primarily by extracellular sugar residues, most prominently mannose residues on bacterial cells. The pattern recognition complex of the lectin pathway is homologous to the C1 complex of the classical pathway at both the structural and functional levels. It consists of the mannose-binding lectin (MBL) paired with two protease complexes formed by MBL-associated serine proteases, MASP-1 and MASP-2. MASP1/2 are closely related to C1r/s and likely diverged from a common ancestor after gene duplication. Other collagenous lectins such as the ficolins and collectin-11 are also capable of activating the lectin pathway by interacting with MASPs (18, 19). Upon recognition of a substrate, the MBL complex is converted to an active serine protease and cleaves C4 and C2 similarly to the classical pathway, producing the classical C3 convertase, C4b2b.

The *alternative* pathway (Fig. 1, green) is not activated by a specific pathogen, but rather has constitutive low-level activity, which enables to continuously probe cellular surfaces for defects. The spontaneous hydrolysis of C3 allows binding of factor B that is proteolytically activated by factor D to produce the C3 convertase of the alternative pathway, C3bBb. Although this C3 convertase can form on both healthy and pathogenic surfaces, complement inhibitors present in healthy cells rapidly inactivate the convertase and its activation products. In contrast, complement activation proceeds unfettered on foreign cells and can be further amplified through various mechanisms. For example, factor P, also known as properdin, stabilizes the alternative pathway C3 convertase and localizes it to pathogenic substrates, thereby providing amplification of its activity (18). Furthermore, the alternative pathway can significantly amplify signaling from all three pathways through a positive feedback loop. Once a C3 convertase has formed, the cleaved C3b binds factor B to form another C3 convertase, thus amplifying the initial signal. This feedback loop is estimated to be responsible for 80–90% of all complement activity regardless of which initiation pathway was triggered (20).

The *terminal* pathway represents the final stage of complement activation (Fig. 1, red). It begins with the addition of C3b to the C3 convertase complexes, resulting in C4bC2bC3b complex for the classical and leptin pathways and C3bBbC3b complex for the alternative pathway. These complexes function as C5 convertases that cleave C5 into two split products, C5a and C5b. A highly reactive product, C5a functions as a potent inflammatory mediator that was originally described as an anaphylatoxin due to its ability to activate mast cells and induce massive release of histamine. Its profound role in neuroinflammation and pain is discussed below. The other product, C5b, assembles with C6, C7, C8, and the pore-forming subunit C9 on the surface of a cell to put together the terminal pathway complete complex (TPCC) (21), also known as the membrane attack complex (MAC). The complex forms a large channel (up to 100 Å) in the surface membrane, which ultimately causes cell lysis. Notably, overactivation of the TPCC/MAC has been implicated in various pain states, including arthritis, neuropathic pain, and chemotherapy-induced peripheral neuropathy (CIPN; Fig. 1) (11, 22, 23).

Regulators of the complement cascade

Rapid and substantial activation of the complement system in response to foreign invaders and injury also requires equally potent and coordinated mechanisms for limiting its activity in order to prevent uncontrollable inflammation, autoimmunity, and destruction of healthy tissues (18, 24). Multiple molecular and cellular mechanisms have evolved to regulate complement activation through various inhibitory signaling processes (Fig. 1, gray). These mechanisms help to prevent activation of the complement cascade in healthy tissues, accelerate the removal of opsins deposited on host cells, and refine the target of complement signaling by localizing convertases to the site of infection or injury. These inhibitors act through a variety of mechanisms and at all stages of complement signaling, but most target the alternative pathway amplification loop (Fig. 1). Notably, Factor H, Factor I, complement receptor 1 (CR1), decay-accelerating factor (DAF or CD55), and membrane cofactor protein (MCP or CD46) all specifically regulate C3b-containing convertases, further underscoring the importance of controlling the alternative pathway amplification loop under various conditions (18, 19, 25).

The initiation steps of complement activation are also controlled at multiple levels. With respect to the classical and lectin pathways, the C1 complex inhibitor (C1-INH/serpin G1) is a soluble protein that circulates throughout the body and limits the amount of protease activity "leaked" from the site of primary activation. It also limits the spread of spontaneous activation such as seen in chronic pain associated with hereditary angioedema (HAE; Fig. 1), for which the first-line treatment is to provide a plasma-derived preparation of C1-INH, Cinryze (26, 27). Similarly, at the level of inhibiting C3 convertases, the C4b-binding protein (C4BP) displaces C2b in the classical C4b2b/C3 convertase by competitively binding with C4b to inhibit activity of the enzyme (Fig. 1). Acting together with the soluble inhibitors of C3b, Factor I and Factor H, these mechanisms reduce the levels of both alternative and classical C3 convertases within the circulatory system, while permitting their localized actions.

The other inhibitors of complement signaling act primarily at the cell surface. These proteins vary widely in their mechanisms of action, but all prevent damage to the healthy cells

that express them and protect local tissue. For example, the membrane-bound protein MCP (CD46) binds to C3b deposited on the cell surface making it susceptible to cleavage and inactivation by Factor I, which prevents formation of C3 and C5 convertases (Fig. 1). Other membrane-bound inhibitors, such as CR1 and DAF (CD55), competitively bind to C3b to prevent the formation of a convertase. They can also displace Bb from an active convertase to promote decay. These cell surface regulators are especially critical for protecting healthy tissue near an area of injury with active complement signaling, as in the case of nerve damage. DAF and other membrane-localized regulators protect healthy cells from complement deposition throughout the period of inflammation and recovery, while dysregulation of these inhibitory factors leaves healthy cells vulnerable to the actions of immune cells recruited to the site of injury in the context of neuropathic pain (23, 28).

An additional level of complement regulation occurs through various glycosaminoglycans such as heparan sulfate. These sugar molecules expressed on host and pathogen membranes interact with various complement inhibitors and activators. For example, interaction of heparan sulfate with Factor H prevents complement activation on a host cell (29). Mechanistically, heparan sulfate promotes localization of Factor H to host membrane and acts as a cofactor for Factor H to enhance localized cleavage of C3b (30–33). Furthermore, some glycosaminoglycans are involved in complement-mediated regulation of neurological diseases. In particular, heparan sulfate has been found to localize Factor H to Aβ plaques and also to inhibit binding of β-amyloid peptides by C1q, which likely prevents clearance of the plaques and contributes to Alzheimer's disease progression (34, 35).

The last line of defense against complement activity, whether spontaneous or pathogenic, is provided by regulators of the formation and insertion of the TPCC/MAC lytic channel (Fig. 1). Protectin, also known as CD59, prevents assembly of the TPCC by binding to transmembrane proteins C8 and C9. Similarly, vitronectin and clusterin bind to various proteins in the TPCC, preventing its assembly. These proteins are widely expressed on both healthy and apoptotic cells. Thus, formation of the TPCC is normally restricted to pathogenic cells, whereas formation of the TPCC on host cells is commonly associated with pathological conditions. A clear example of importance of TPCC inhibition is found in a rare disease called paroxysmal nocturnal hemoglobinuria (PNH). Patients with PNH experience episodes of severe pain, which is associated with complement-mediated lysis of red blood cells due to the absence of protectin and DAF on host cells (4, 36, 37) (Fig. 1).

Complement receptors

Although many of the actions of the complement system are directed at its targets through generic PAMPs or ubiquitous tagging of lipid membranes, specific receptors present on the host cells can facilitate recruitment to the site of injury, recognition and phagocytosis of opsonized pathogens, or even stimulation of cellular replication to aid in tissue recovery (4). The early components of the complement cascade (C1, C2, C4, MBL, and MASPs) have few well-characterized receptors as they are involved primarily in detection of threats and initiation of the terminal pathway. The only well-characterized receptors are those for C1q, such as C1qR, cC1qR, and gC1qR. These receptors are expressed on phagocytes and thought to be important for recognizing C1q bound to the immune complexes and promoting phagocytosis. The remaining complement receptors recognize primarily fragments of C3 and C5 (Fig. 1, purple).

The first class of complement receptors in this group consists of those that recognize membrane-bound fragments of C3 and include CR1 (CD35), CR2 (CD21), CR3 (CD11b+CD18), and CR4 (CD11c+CD18) (Fig. 1). CR1 binds C3b with high affinity and the inactivated form of C3b (iC3b) with low affinity, whereas CR2-4 receptors universally recognize iC3b. When these receptors are expressed on phagocytes, activation promotes phagocytosis of opsonized material. Monocytes, macrophages, and neutrophils all express CR1, CR3, and CR4, whereas dendritic cells express CR4 (6). As mentioned earlier, CR1 also has decay-accelerating characteristics, by competitively binding C3b and displacing Bb from the C3 convertase. B cells also express CR1 and CR2, and iC3b binding to these receptors enhances B cell activation and antibody secretion (18). The latter exemplifies how the complement system augments adaptive immune responses.

The remaining receptors bind the soluble complement fragments C3a and C5a. Both C3a and C5a act through classical seven-transmembrane G-protein-coupled receptors (GPCRs) called C3aR and C5aR1 (CD88), respectively (Fig. 1). These receptors have similar expression patterns on endothelium (38), smooth muscle (39), glia (40, 41), and throughout all myeloid cells including neutrophils, monocytes, macrophages, and restricted populations of dendritic cells (42, 43). Activation of these receptors produces a variety of effects, including increase in vascular permeability, chemotaxis, production and release of inflammatory factors, and stimulation of phagocytosis. C3aR- and C5aR1-mediated effects are particularly prominent in various pain states, including postsurgical pain, neuropathic pain, CRPS, and arthritis (Fig. 1) (8, 14, 36, 44–47). To limit systemic activation, both C3a and C5a are rapidly "des-arginated" by serum carboxypeptidases. However, while this entirely removes the activity of C3a at C3aR, C5a-desArg retains some, albeit reduced, activity at C5aR1, thus making C5a highly potent in biological tissues (1, 48).

The other C5a receptor, C5aR2 (C5L2; Fig. 1), is less fully characterized and has more nuanced function. Although C5aR2 has structural homology to C5aR1, it is uncoupled from intracellular heterotrimeric G-proteins due to mutations in the G-protein recognition sequence (49). As such, C5a-C5aR2 engagement does not induce classical GPCR signaling events, initially promoting the concept of C5aR2 as a decoy receptor (49, 50). Indeed, deletion of C5aR2 appears to enhance the C5aR1-mediated proinflammatory activity of C5a (51, 52). However, further studies demonstrated that C5aR2 can signal through β-arrestins independent of G-proteins, which may

mediate some functional activities including pro- and anti-inflammatory responses (48, 51, 53, 54).

The interactions of the complement receptors and their targets on various cellular populations promote significant cross-talk among immune cells, allowing complement to integrate and enhance a wide variety of responses throughout the peripheral and central nervous systems (PNS and CNS, respectively; Fig. 2), as well as in other tissues (36, 48, 55). Although much is known about intrinsic mechanisms that drive complement activation, we know comparatively less about the myriad of effects that complement effectors have on downstream targets. In the next sections, we discuss the current literature regarding the roles of complement in the nervous system and outline the key knowledge gaps that need to be filled by additional studies.

Complement cascade in the nervous system: mechanisms and functions

In addition to protecting the nervous system from pathogens, the complement system plays major roles in controlling many fundamental processes within the PNS and CNS during development and aging, as well as in response to injury, disease, ischemic damage, or toxic stress. Figure 2 describes four prominent generalizable mechanisms through which complement acts in the nervous system in both development and physiological homeostasis as well as in neuropathology. Circulating complement components are not traditionally thought to cross the blood–brain or blood–spinal cord barriers in any meaningful concentrations in the healthy state. Rather, the complement proteins present in the CNS are thought to be produced locally within the CNS, predominantly by glia (56, 57). CNS-derived complement components can act on neurons, glia, and immune cells to mediate both physiological and pathological effects (56). During development of the nervous system, complement proteins regulate the proliferation of neural progenitor cells and neuronal migration, as well sculpting developing synaptic networks (58, 59). In addition, deregulation of the complement cascade has been implicated in synaptic loss and neuronal damage in aging and various neurodegenerative diseases including Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) (55, 60). Following stroke, the complement system is rapidly activated, promoting inflammation and exacerbating oxidative tissue damage in the first few days after stroke, while facilitating synaptogenesis, neuronal plasticity, and poststroke recovery at later stages (61–64). Similarly, the complement system plays a

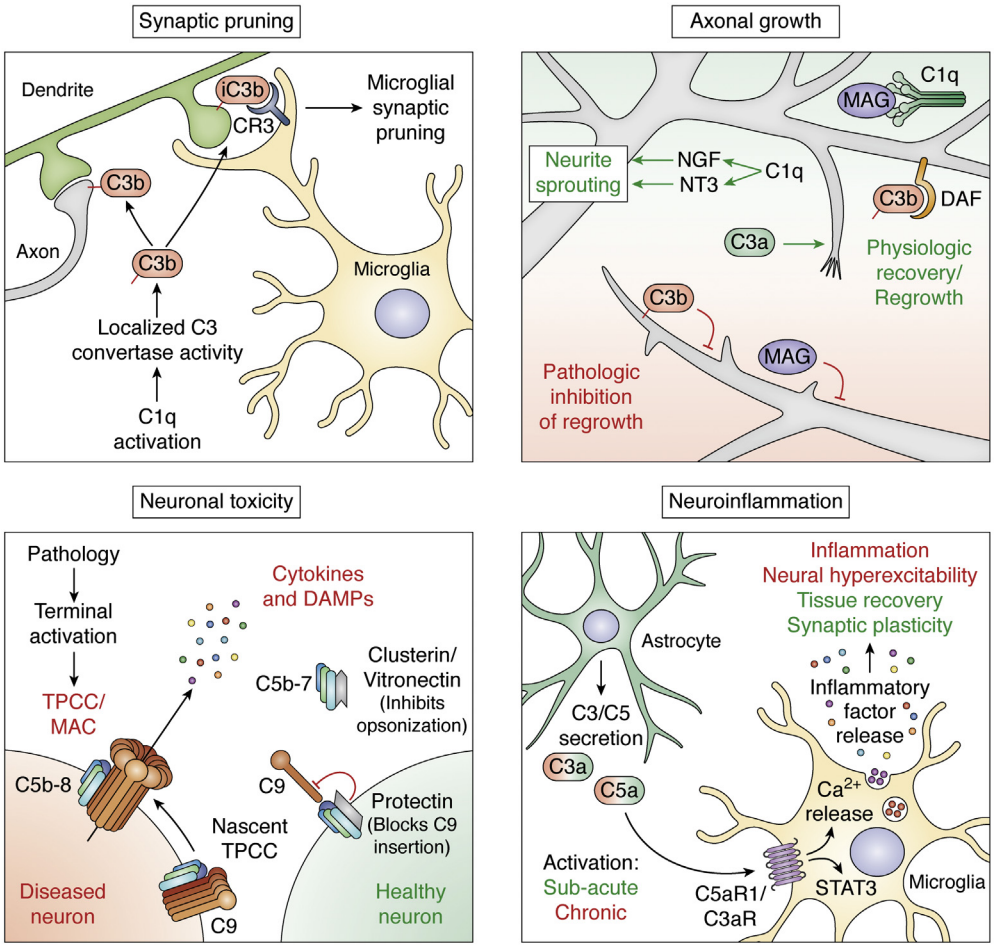

Figure 2. Complement-mediated mechanisms in the regulation of neuronal functions in health and disease. Where labeled, physiological mechanisms are shown in *green* and pathological mechanisms in *red*. Some mechanisms, such as synaptic pruning (*gray*), can contribute to both physiological and pathological processes, depending on developmental state and disease conditions.

dual role following spinal cord injury (SCI), contributing to the inflammatory response and clearance of cell debris and damaged tissues during an acute phase, while facilitating tissue repair during a later recovery phase (65, 66). Another important function of the complement system following tissue or nerve injury is its contribution to sensitization of nociceptive neurons and amplification of pain signaling, which serves to guard and protect injured tissues and support normal healing. However, aberrant activation of the complement cascade can promote the development of conditions characterized by chronic pain, such as complex regional pain syndrome, arthritic pain, and neuropathic pain (7, 22, 45).

The complement system can regulate various aspects of neuronal life and death both directly, through specific complement receptors expressed on the neuronal plasma membrane, as well as indirectly, by recruiting glial and immune cells that convey complement signaling to neurons through various mechanisms (55, 58, 60). The complex effects of the complement system on neuronal excitability, plasticity, survival, and demise involve intricate molecular, cellular, and intercellular mechanisms that can be broadly divided into four groups: (1) synaptic pruning; (2) regulation of axonal growth; (3) regulation of neuroinflammation; and (4) TPCC/MAC-mediated neuronal toxicity (Fig. 2).

Synaptic pruning

Activity-dependent elimination of synapses, also known as synaptic pruning, is a fundamental mechanism that shapes neuronal wiring during development (58, 67). Recent studies have showed that synaptic pruning in the developing visual system is guided by components of the classical complement cascade (68, 69). This process involves opsonization of synapses by the complement fragment C3b and its interaction with complement receptor CR3 expressed on microglia, to direct phagocytosis of C3b-expressing synapses (Fig. 2) (68–70). The presence of C1q at synapses suggests that C3b is produced locally at these sites as a result of activation of the classical cascade. Similar mechanisms are involved during the development in other brain regions, and they also contribute to synaptic plasticity and learning in the adult brain (71–73). However, excessive synaptic pruning during development and aging has been implicated in psychiatric and neurodegenerative diseases (58, 74). For example, increased expression of the complement protein C4 has been linked to schizophrenia (75). Mechanistically, it has been proposed that elevations in levels of C4 lead to excessive downstream activation of C3 and complement/microglia-mediated elimination of synapses, ultimately leading to impaired synaptic wiring in the prefrontal cortex and other brain regions implicated in schizophrenia (75, 76). Another example of the pathological role of complement in excessive synaptic pruning relates to its involvement in synaptic loss in AD. Indeed, increased expression of the components of the classical complement cascade (*e.g.*, C1q, C3 and C4) has been reported in the hippocampus and frontal cortex of AD patients, as well as in AD mouse models (55, 77). Elevated levels of C1q were specifically associated with synapses in two different Aβ mouse models of AD, whereas inhibition of C1q or deletion of C3 or CR3 in these mice led to reductions in the number of phagocytic microglia and synaptic loss, as well improvements in learning and memory tasks (78, 79). Similarly, in Tau-P301S mice, C1q was found at the synapses in association with phospho-Tau and phagocyting microglia, and a C1q-scavenging antibody rescued synaptic loss in these mice (77).

Thus, complement-mediated synaptic pruning likely represents a general mechanism that plays critical roles in both physiological processes (*e.g.*, synaptic refinement during development and structural remodeling during synaptic plasticity and memory formation in mature brain) and the pathogenesis of various psychiatric and neurological diseases (*e.g.*, schizophrenia, AD, glaucoma).

Regulation of axonal growth

The complement system can both promote and suppress axonal growth, depending on the specific complement factors involved (Fig. 2). The initiating factor of the classical cascade, C1q, was shown to stimulate axonal growth both *in vitro* and *in vivo* through noncanonical mechanisms, independent of classical pathway activation (65, 80). For example, C1q was shown to promote neurite outgrowth in rat primary cortical neurons by upregulating expression of nerve growth factor (NGF) and neurotrophin 3 (NT3) (80) (Fig. 2). In addition, C1q can promote axonal growth through its direct interaction with myelin-associated glycoprotein (MAG), which belongs to a class of proteins known as myelin-associated inhibitors of axonal growth (65, 81). This interaction blocks the growth inhibitory signaling of MAG, thereby promoting cytoskeletal rearrangement and neurite outgrowth (Fig. 2). The described C1q-dependent mechanism is especially important for supporting axonal regeneration following spinal cord injury (65) (Fig. 3).

In contrast, C3 was shown to inhibit axonal growth, as revealed by examining the effects of C3 deletion on axonal regeneration (82). In this study, the regeneration of sensory axons after spinal cord injury was 2-fold higher in mice lacking C3 than in wild-type counterparts. Furthermore, *in vitro* examination suggested that this effect was mediated by the C3 split product, C3b, by either causing neuronal toxicity or inhibiting neural adhesion (82). Notably, the other split product of C3, C3a, mildly promoted, rather than inhibited, neurite outgrowth in cultured cortical neurons (82) (Fig. 2). As discussed below, preferential activation of a growth-promoting *versus* growth-inhibiting mechanism can profoundly affect healing processes and normal pain resolution following injury (Fig. 3).

Regulation of neuroinflammation: the roles of C3a and C5a

Within the CNS, complement activation is commonly found alongside neuropathology and inflammation (55), and is accompanied by robust generation of the inflammatory peptides C3a and C5a (Fig. 2). Through their conjugate receptors, C3aR and C5aR1/2, these peptides can drive both glial and

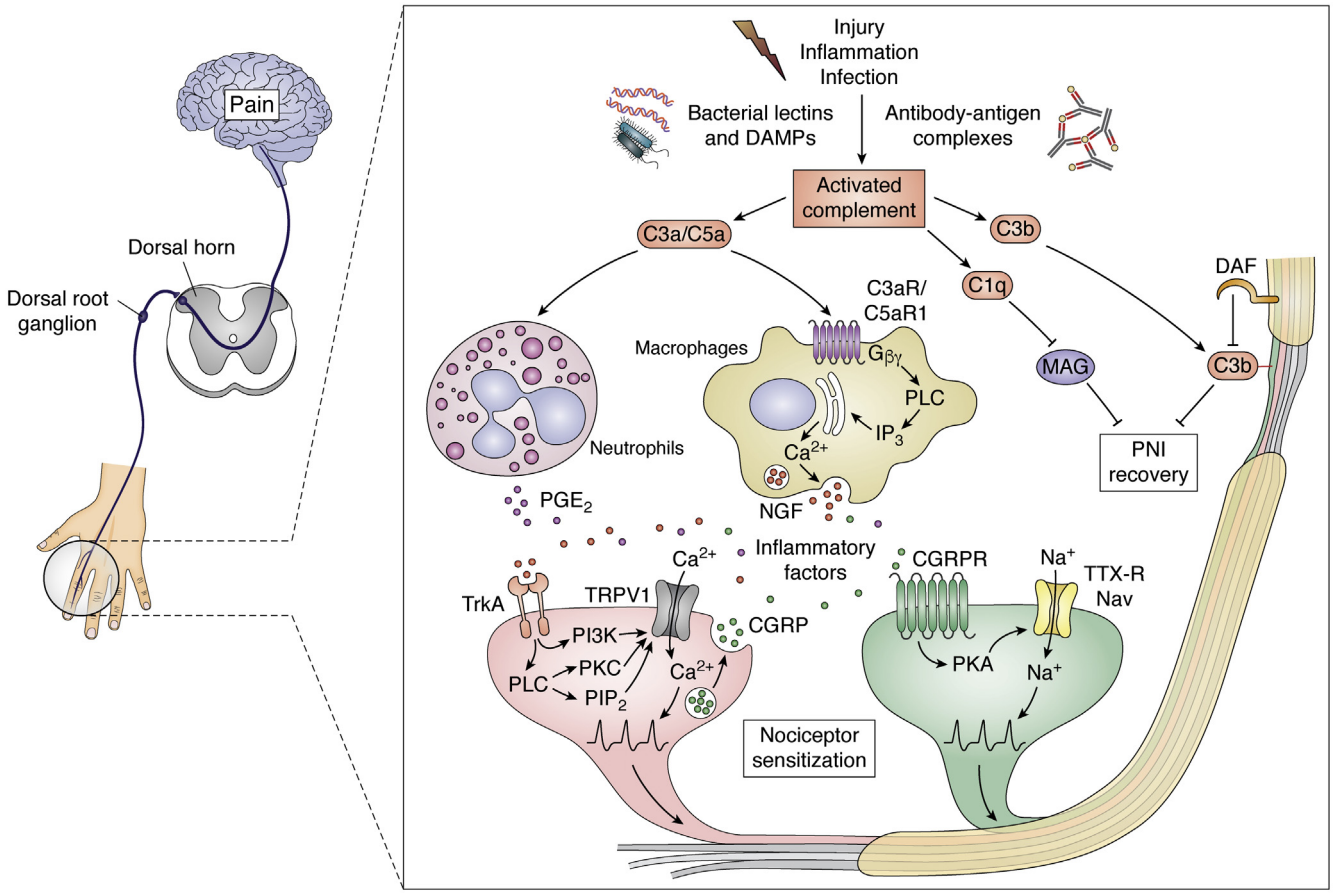

Figure 3. The complement system orchestrates cellular responses to injury in the peripheral nervous system. After injury, inflammation, or infection, activation of the complement system contributes to nociceptor sensitization and wound healing. In the case of peripheral nerve damage, activated components of the complement system (*red*) stimulate immune cells and promote nerve regrowth. Damaged nerves also release myelin-associated glycoprotein (MAG), which can inhibit regrowth of sensory axons. C5a/C3a-dependent recruitment of macrophages helps to clear MAG and to inhibit its action through binding of C1q, which promotes axonal regrowth. C3b, when deposited on nerves, can inhibit regrowth, but is typically inactivated by DAF (CD55) under physiological conditions. The soluble complement factors C3a and C5a act through their receptors, C3aR and C5aR1, respectively, to recruit and activate immune cells. This causes Ca^{2+}-dependent release of numerous inflammatory mediators (*e.g.*, NGF, PGE_2, CGRP), which can sensitize nociceptive neurons by modulating TRPV1, tetrodotoxin-resistant (TTX-R) voltage-gated Na^+ channels, and other ion channels and receptors through various mechanisms (124–126, 134).

neuronal dysregulation in a number of diseases including stroke (55, 61–64), spinal cord injury (66, 83), AD (60, 84, 85), and ALS (86–89). In most cases, elevated levels of the inactive zymogens C3 and C5 are largely attributed to their production by glia, which subsequently leads to the formation of the small soluble fragments, C3a and C5a by the respective convertases (56, 57). Once formed, C3a and C5a activate their cognate receptors, which are expressed on both glia and neurons, though generally at higher levels on glia. Activation of C3aR and C5aR1 initiates a variety of effects, including chemotaxis of immune and glial cells, production and release of inflammatory factors in a Ca^{2+}-dependent manner, and stimulation of phagocytosis (18, 36, 48, 60). Levels of C3a and C5a are significantly elevated in the plasma after stroke (90), and inhibition or genetic ablation of C5a/C5aR1 signaling significantly reduced the infarct volume and functional deficits after stroke (61, 62). Similarly in ALS patients, plasma and leukocyte levels of C5a are increased relative to healthy individuals (88). In addition, levels of complement inhibitors DAF (CD55) and protectin (CD59) were altered at the endplates of motor neurons in ALS patients (91). The role of complement in ALS was also supported by the findings that in mouse models of ALS, genetic or pharmacologic inhibition of C5aR1 delayed disease progression (86, 87, 89) and that complement inhibitors DAF (CD55) and protectin (CD59) were downregulated in ALS mouse models (92, 93). C3 and C3aR are also increased in the brain of AD patients as well as in the mouse brain of an AD model (94). Mechanistically, C3a/C3aR signaling was shown to induce microglia activation *via* STAT3-dependent transcriptional program, which in turn drives tau pathogenesis in AD mice (94). Intriguingly, it was also noted by the same group that C3aR expression and activation on neurons were also capable of driving Aβ production (95), underscoring the functional importance of C3aR on both neurons and microglia to the development of AD.

However, inhibition of C3aR or C5aR1 signaling is not always disease modifying or palliative, as both C3a and C5a can play roles in tissue repair. For example, intranasal administration of C3a drives neurogenesis during recovery from stroke (63, 64), and a higher C3a:C3 ratio in the serum

(indicating enhanced convertase activity) correlates with more positive outcomes following cardioembolic strokes (96). Similarly, in murine models of AD, inhibition or genetic knockout of C3 worsens AD pathology, which is characterized by higher levels of Aβ deposition and neuronal death that in complement sufficient animals (84, 85). Finally, in models of spinal cord injury, genetic or pharmacological blockage of C5aR1 signaling enhanced recovery during the acute phase (<7 days) by reducing microglia-mediated inflammation and cytokine release. However, chronic or later blockade of C5aR1, or C5aR1 genetic deletion, appears to be detrimental to full recovery after injury (66, 97). These apparently contrasting effects for C3a and C5a signaling illustrate the overall dual roles of the complement system in neurological disorders and CNS/PNS injury, where the same complement factor can either exacerbate neuronal damage or stimulate the recovery process, depending on both the type and stage of a particular neuropathological condition.

TPCC/MAC-mediated neuronal toxicity

Unlike many typical signaling mechanisms including those of C3a and C5a, the activity of the TPCC/MAC (21) can occur in the absence of a conjugate receptor on the affected cell (Fig. 2). This unusual property enables the TPCC to form a large lytic pore on any lipid structure of a host cell or pathogen, where it can cause cytolytic destruction (98, 99). In addition, the TPCC can play a signaling role when conditions are sublytic (*i.e.*, a pore is formed but the cell has not lysed), causing an increase in the release of proinflammatory cytokines (100, 101) through a variety of mechanisms, both Ca^{2+}-dependent and independent (99). However, in the context of neuronal toxicity, the lytic function of the TPCC is suspected to be the primary cause of neuronal cell death. This is the case in a variety of neurological disorders including stroke (102–105), traumatic brain injury (TBI) (106–108), spinal cord injury (109–111), ALS (87, 89, 91–93, 112), and neuropathic pain (11).

Many pathologies are characterized by elevated deposition of the TPCC in affected tissues. For example, in ALS, TPCC levels are increased at the end plate of motor neurons (113, 114). In spinal cord injury, TPCCs surround the primary site of injury (109, 111). In TBI, TPCC insertion is positively correlated with the disruption of blood–brain barrier after the injury (106–108). This increased deposition of TPCC in various neuropathologies is likely driven by poor regulation of the formation and insertion of the complex. Indeed, the ubiquitously expressed inhibitor of TPCC formation, protectin (CD59), is downregulated in ALS patients (92, 93), and its genetic knockout impairs recovery in rodent models of stroke (105) and spinal cord injury (110). Although these various pathologies are defined by different types of neurological trauma or neurodegeneration, a common hallmark of these diseases is the contribution of excessive TPCC activity to neuronal death, due to either excessive activation of the TPCC or dysregulation of the inhibitory mechanisms (Fig. 2).

Mechanisms underlying complement action in tissue injury and pain

Tissue injury is commonly associated with an amplified pain sensation in response to a noxious stimulation (hyperalgesia) and/or an innocuous stimulation (allodynia) at the affected site. This sensitized reaction represents a fundamental adaptive mechanism that helps to protect injured tissue and assists in wound healing. However, chronic sensitization is deleterious and serves no physiological function. Peripherally, after an injury there is a complex interplay between the immune cells and nociceptive neurons (Fig. 3) to promote healing and facilitate behavioral guarding of the wound. Complement assists in both these aspects by recruiting and activating immune cells (*e.g.*, macrophages) to fight infection and clear debris, while at the same time stimulating these cells to release inflammatory factors that sensitize neurons to promote behavioral guarding until the wound has healed. However, the persistent or unbalanced signaling of complement factors during chronic pain points to a potential role for complement in the maladaptive mechanisms of peripherally driven chronic pain. Numerous studies have highlighted the critical roles of the complement cascade in various chronic pain conditions. Indeed, elevated levels of many key complement factors (*e.g.*, C3a, C5, and C5a) have been reported in patients with various pathological conditions associated with pain, including osteoarthritis, rheumatoid arthritis, pancreatitis, burns, and surgical trauma (22, 115–120). Similarly, increased production of complement factors C3, C5, and C5a was reported in several rodent models of acute and chronic pain, including models of postsurgical pain, cancer pain, and neuropathic pain (15, 23, 45, 121), while genetic deletion of C3 and C5 as well as pharmacological inhibition of C3aR and C5aR1 produced significant analgesic effects in these models (11, 16, 44–46, 122). Furthermore, a meta-analysis of 20 microarray studies that examined changes in gene expression in various chronic pain models in rodents found that complement was one of the most commonly and highest regulated categories of genes upregulated after induction of both neuropathic (*i.e.*, induced by peripheral nerve injury) and inflammatory (*i.e.*, induced by tissue injury) pain (123). Below, we discuss the major mechanisms that underlie the contribution of the complement system to tissue injury and pain.

Sensitization of nociceptive neurons

Activation of the complement system in peripheral tissues triggers the production and release of numerous inflammatory factors that can significantly modify neuronal excitability. The main soluble effectors of the complement cascade that drive inflammatory responses are C3a and C5a. These peptides have profound effects on a wide array of physiological systems that promote inflammatory responses to infection or injury. Indeed, direct application of C5a to an *ex vivo* skin–nerve preparation sensitized primary afferent fibers to thermal stimulation and also caused spontaneous firing of action potentials (14, 15). Similar effects were induced by C3a, indicating that complement activity is capable of heightening sensitivity of peripheral neurons to cutaneous stimulation.

Specific factors responsible for complement-induced sensitization have only recently begun to be unraveled and include a variety of local inflammatory mediators, which are released primarily from immune cells but also from peripheral neuronal terminals (Fig. 3). One particularly well-characterized inflammatory mediator is nerve growth factor (NGF). The levels of NGF are prominently elevated after peripheral injury (7, 13, 16), concomitant with the expression of complement factors (15). Subcutaneous NGF can also be increased by injecting activated complement fragments such as C5a (13). NGF promotes sensitization of nociceptive fibers primarily through signaling of the tropomyosin-related kinase A (TrkA) receptor expressed on the peripheral terminals of the afferents. In particular, NGF/TrkA-mediated signaling stimulates plasma membrane insertion of the polymodal nociceptive ion channel TRPV1 and also sensitizes the channel to noxious heat, acidic pH, and some endogenous lipid-derived ligands (124–126). In cases when acute injury is not readily resolved, NGF can promote a transition to chronic pain through various mechanisms, including changes in the expression of nociceptive ion channels, the production of reactive oxygen species, and stimulation of peripheral axonal sprouting (127, 128).

Although the primary cellular source of the NGF that is released in the periphery in response to complement activation is likely macrophages (13, 129) (Fig. 3), another distinct source of complement-induced nociceptive sensitization lies within the afferent fibers themselves. For example, the pronociceptive mediator calcitonin gene-related peptide (CGRP), which is synthesized and released by peptidergic C-fibers in the setting of neurogenic inflammation (130–133), is responsible for the mechanical sensitization caused by the actions of C5a in the dermis (17), and this effect may involve facilitation of tetrodotoxin-resistant voltage-gated Na^+ currents (134) (Fig. 3). In addition, levels of a pronociceptive cytokine, interleukin 1β (IL-1β), increase after injury or downstream of C5aR1 activation (7, 16, 135). IL-1β likely acts through p38 protein kinase-dependent enhancement of voltage-gated Na^+ currents, to increase excitability of nociceptive neurons in various pain states (136, 137). Notably, a blockade of IL-1β receptor produced analgesic effects in a pain model that relies on activation of the alternative pathway (138). Similarly, inhibition of complement signaling reduces pronociceptive signaling and inflammatory factor release in various rodent pain models, including the models of inflammatory and postsurgical pain (13, 15, 44, 139). Overall, these findings suggest that the mechanisms responsible for complement-induced pronociceptive effects usually involve the release of potent inflammatory factors (*e.g.*, NGF, CGRP, or IL-1β) that act on their respective receptors on nociceptive neurons to increase their excitability and sensitize them to noxious and innocuous stimuli (Fig. 3).

Recruitment and activation of immune cells

B cells, neutrophils, and macrophages are rapidly recruited to the site of injury to aid in fighting infections, killing pathogens, and clearing myelin and debris. These are critical steps for promoting tissue recovery because the detritus from injury inhibits the regrowth of sensory afferents, while necrotic death of pathogens and infected cells causes massive release of inflammatory mediators that can increase pain and prevent healing (140–142). When immune cells are stimulated by complement acutely after injury, they produce and release numerous inflammatory factors such as CCL2, NGF, ATP, TNF-α, PGE2, or IL-1β. These factors recruit additional immune cells and promote sensitization of the sensory neurons to guard and protect the injury site during recovery (Fig. 3). Chronically, however, the activation of these immune cells can prove deleterious for both the local tissue and the neurons that innervate it. For example, after surgery or infection, activation of the complement system and infiltration of immune cells are transient events that start with the injury to support the healing process and gradually disappear along with the recovery process. However, during injuries such as a peripheral nerve trauma (143) or autoimmune disorders (7), where pain sensitization persists well past the local tissue recovery, prolonged activity of the complement cascade and the continuous presence of macrophages, neutrophils, T cells, and B cells are key factors contributing to pain chronification.

Numerous studies support a key detrimental role of overactivated complement cascade in various chronic pain conditions. For example, in a model of rheumatoid arthritis (RA), knockout of C5aR1 (but not of C3aR) protects against histological pathology, inflammatory factors, and infiltration of immune cells such as neutrophils, T cells, and macrophages (144). In contrast to RA and osteoarthritis (OA), which are associated with joint pain, ankylosing spondylitis (AS) is a form of arthritis whose primary symptom is lower back pain. In a mouse model of AS, there is substantial activation of the complement along with macrophage and neutrophil activation. Notably, administration of the C3-binding complement inhibitor, Efb-C (C-terminal of extracellular fibrinogen-binding protein), to AS mice significantly attenuates these pathological processes and reduces the disease progression (145). Another example of a posttraumatic chronic pain disorder associated with autoimmunity and complement action is complex regional pain syndrome (CRPS). Indeed, in a mouse model of CRPS, the sensitization of afferents and complement activation/deposition in the skin and on nearby afferent fibers is dependent on antibody production by B cells (7). Similarly, rodent models of peripheral nerve injury (PNI) are characterized by prominent tissue infiltration by immune cells (146) and complement deposition on damaged nerves (147, 148). Generalized pharmacologic inhibition of immune cells (149) and more specific chemogenetic ablation of macrophages are each sufficient to reverse the PNI-induced sensitization of peripheral afferents (146, 150). Thus, complement-dependent recruitment of immune cells is an essential component of tissue responses to injury, which can affect the function of nociceptive neurons through release of inflammatory factors, deposition of complement on nerves that promote cell death/clearance through the TPCC, or clearance of myelin to promote axonal regeneration and tissue repair (Figs. 2 and 3).

Axonal regeneration and recovery after injury

An important property of the dorsal root ganglion (DRG) sensory neurons is that after peripheral nerve injury, these neurons are able to repair and regrow the damaged axons and reinnervate target tissues. Complement signaling appears to contribute to the regulation of this process directly at the site of injury (Fig. 3). After nerve injury there is a significant amount of myelin from the damage, which strongly inhibits DRG axonal outgrowth mainly through myelin-associated glycoprotein, known as MAG (65). This glycoprotein dose-dependently reduced the growth of damaged DRG axons, and the addition of C1q blocked the effects of MAG, leading to unencumbered regeneration of DRG axons (65). Although the presence of C3a similarly led to an increase in average neurite length in DRG cultures, the addition of C3b decreased the viability of DRG neurons and the number of neurons with neurite outgrowth (82). One potential explanation for the cytotoxic effects of C3b deposits on DRG neurons would be its promotion of formation of the TPCC resulting in cell lysis. However, C3b impaired neurite outgrowth even in cultures free of immune cells and serum, suggesting that its inhibitory effects on axonal regeneration were primarily due to the inhibition of cell adhesion (82). While this might initially appear as a net-zero push–pull action of the C3 split products, the described observations are consistent with the proposed role of complement as a "dual-edged sword" (56), where depending on the precise levels of complement factors, receptors, and inhibitors, the complement system can drive seemingly opposing processes. Nevertheless, the observed effects suggest that an appropriate amount of complement activity can promote the recovery of normal neuronal function after injury and that the levels of specific complement factors must be closely regulated both spatially and temporally.

A recent study (28) examining single-nucleus RNA sequencing data from DRG after different types of nerve injury, including some that recover normal sensitivity (sciatic nerve crush) and others that do not (spinal nerve transection), provides an illuminating example of the temporal role and activity of complement inhibitors in recovery after nerve injury. Specifically, in the crush model, the cell surface convertase inhibitor DAF (CD55) was significantly downregulated immediately after injury and then recovered concomitantly with healing of the injury and amelioration of the behavioral deficits. This is consistent with the role of acute complement activity driving tissue remodeling, beneficial inflammation, and overall recovery. In contrast to a transient crush injury, the chronic transection model led to reduced levels of DAF (CD55), which never returned to basal levels indicating that the injury still had not resolved and that the complement cascade was chronically disinhibited. Consistent with this single-cell sequencing study (28), other groups have confirmed similar decreases in DAF/CD55 after spinal nerve transection and that complement depletion prevents the development of pain behaviors (23, 151) further supporting the idea that chronic complement activity can be detrimental to the recovery from nerve injury. Additionally, in transection models, the levels of cell death are generally higher than in crush models due to the axotomy (152–154). Consistent with this, the TPCC inhibitor protectin (CD59) remains highly expressed on all surviving cells after injury, unlike the transient elevation observed in the crush model (28). These findings suggest that the survival of primary afferent neurons after injury is highly dependent on the expression of terminal complement inhibitors as there is a lack of convertase inhibition due to the loss of DAF expression. Finally, global genetic knockout of C6, which inhibits formation of the TPCC, prevented the development of persistent pain in a model of anti-GD2 immunotherapy (9), and global knockout of C5 alleviated neuropathic pain (45).

Thus, various components of the complement system play distinct roles in regulating axonal damage and recovery after nerve injury. The complement factors, such as C1q and C3a, promote axonal regeneration and recovery by facilitating the recruitment and activation of immune cells to clear debris and by interacting with myelin-associated glycoproteins that inhibit axonal growth. In contrast, the components of the TPCC/MAC pathway, C5b-C9, promote axonal degeneration and contribute to the development of pathological pain state. Accordingly, selective therapeutic targeting of specific elements of the complement cascade has the potential of promoting axonal regeneration and effective recovery after injury, as well accelerating resolution of injury-induced pain.

Spinal mechanisms of pain processing

In addition to the peripheral roles of the complement system in regulating tissue repair, nociceptor sensitization, and pain, recent work has highlighted multifaceted roles of complement in controlling central mechanisms of pain processing in the spinal cord (Fig. 4). Indeed, gene microarray studies comparing changes in gene expression in several different models of neuropathic pain showed a marked enrichment of genes related to the complement system within the spinal cord, including C1q, C3, and C4 (45, 155). These complement transcripts were also the most strongly upregulated of all genes examined and were expressed primarily in microglia (45), clearly indicating a common role for the complement system after most types of injury. An increased expression of C1q and C3 in the spinal cord could potentially contribute to synaptic remodeling *via* localized production of C3b and C3b-dependent synaptic pruning, as described in Figure 2. In support of this idea, treatment with recombinant C1q was shown to reduce dendritic spine density in spinal cord neurons both *in vitro* and *in vivo* (156). The same study reported that C1q expression in the spinal cord was upregulated following peripheral nerve injury, but was downregulated after peripheral inflammation. In the latter case, intrathecal administration of C1q partially reversed an increase in the dendritic spine density associated with peripheral inflammation and also reduced inflammatory pain (156). Although the significance of spinal C1q upregulation in the context of neuropathic pain has not been specifically tested in this report, the described analgesic effects of intrathecal C1q administration suggest that an increase in C1q expression is an

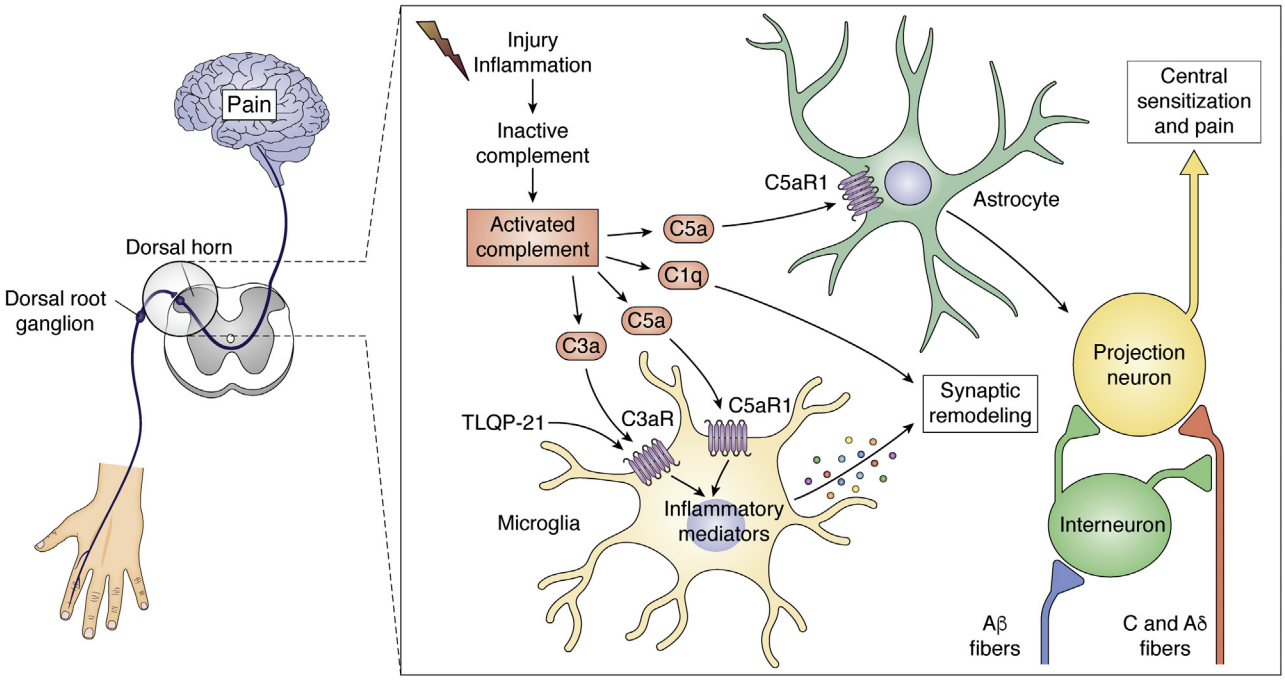

Figure 4. Model describing the regulation of nociceptive signaling *via* complement in the spinal dorsal horn. The activity of complement in the spinal cord has distinct roles following nerve injury. C1q has been shown to contribute to the clearance of damaged tissue, synaptic remodeling, and neurite outgrowth. In contrast, C3a and C5a are thought to amplify nociceptive signaling through microglia and possibly astrocytic activation. This activation releases inflammatory mediators and growth factors that regulate neuronal excitability and synaptic plasticity in the spinal dorsal horn circuit, which includes local interneurons, projection neurons, and the central terminals of primary afferent fibers (C-fibers: slow-conducting unmyelinated primary afferents that transduce pain and thermal stimuli; Aδ-fibers: intermediate-conduction velocity thinly myelinated fibers that transduce pain and thermal stimuli; Aβ-fibers: fast-conducting myelinated fibers that transduce innocuous mechanical stimuli). These primary afferent inputs are processed by the interneurons and are integrated by projection neurons that transmit sensory information to the brain. The specific mechanisms through which activated complement factors modulate neuronal excitability and synaptic transmission in the spinal cord remain to be determined.

adaptive response serving to promote tissue healing and recovery following nerve injury. This is also consistent with the described noncanonical roles of C1q in regulating axonal growth and neuronal survival (65, 80, 157).

The components of the terminal complement cascade, C5 and C5aR1, are also highly upregulated in spinal cord microglia after peripheral nerve injury, and activation of C5aR1 through intrathecal administration of C5a produces cold allodynia, whereas intrathecal administration of a potent and selective antagonist of C5aR1, PMX53, alleviated neuropathic pain (45). These latter findings suggest that C5a-mediated activation of microglia is a key event in the spinal cord in the context of neuropathic pain (Fig. 4). This notion is further supported by numerous studies showing that after peripheral nerve injury, microglia are strongly activated in the dorsal horn of the spinal cord (45, 148, 149, 158, 159) and that pharmacological inactivation of microglia by intrathecal administration of minocycline, a tetracycline antibiotic known to also inhibit microglia activation (160, 161), prevents the development of allodynia and elevation of proinflammatory markers (149, 158).

Besides the products of the complement cascade, other factors, such as VGF-derived neuropeptide TLQP-21, have been reported to activate the complement receptors C3aR and gC1qR (also known as C1qBP) in rodents (162) (Fig. 4). Notably, TLQP-21 has been implicated in the spinal mechanisms that underlie both neuropathic and inflammatory pain (163). Inhibition of TLQP-21 signaling in the spinal cord attenuated mechanical hypersensitivity in rodent models of inflammatory and neuropathic pain, whereas its activation promoted mechanical sensitization (46, 163). The pronociceptive action of TLQP-21 involves microglial activation *via* C3aR, with the receptor being significantly upregulated after nerve injury (46).

How activation of complement receptors on microglia translates to the amplification of nociceptive signaling in the spinal cord following peripheral nerve injury remains unclear. Mechanistically, central sensitization caused by enhanced synaptic activity in the dorsal horn of spinal cord is a hallmark of neuropathic pain, and many aspects of central sensitization are controlled by microglia, including profound structural and functional changes within the nociceptive synaptic network (164–167). Thus, it is plausible that complement-dependent activation of microglia (and possibly other glial cells, *e.g.*, astrocytes) and the release of inflammatory factors contribute to synaptic remodeling within the spinal cord through molecular and structural modifications, as well as through changes in neuronal excitability and synaptic transmission, ultimately leading to central sensitization and the amplification of pain processing (Fig. 4). Future studies will help to identify specific molecular and cellular mechanisms that link complement activation with amplified nociceptive signaling within the spinal cord.

Concluding remarks and perspectives

Accumulating evidence suggests that the complement cascade is strongly activated and upregulated in a variety of

neuropathological states, although the mechanisms underlying the complement-dependent modulation of neuronal activity are still being unraveled. Preclinical data show that inhibition of particular aspects of complement signaling can ameliorate or even prevent conditions such as inflammatory or neuropathic chronic pain, identifying exciting new alternatives to opioids for the treatment of these conditions (122). However, the homeostatic role of the complement system also critically depends on the time after injury, as modulating a single specific component could have either deleterious or healing effects, as in the case of C5aR1 inhibition in a model of spinal cord injury (66). This highlights one of the major questions in the field that is important to address: How does the role of specific components of the complement system change during the progression of injury or disease, and what are the underlying mechanisms and consequences of these changes?

Indeed, we now have evidence that complement plays multifaceted roles in both disease progression and recovery. The specific roles and timing can be substantially different for each pathology. For example, in the context of acute neurological trauma and stroke, activation of the complement system during early stages exacerbates inflammation and tissue damage, whereas at later stages, the activity of complement factors (*e.g.*, C3a and C5a) is key to axonal regeneration and the restoration of synaptic connectivity (149, 158). On the other hand, in chronic neurodegenerative diseases such as AD and ALS, complement activation during early stages may help to clear toxic protein aggregates and damaged cells. As these chronic diseases progress, the complement cascade becomes persistently activated, overwhelming complement-regulating inhibitory mechanisms and leading to uncontrolled inflammation, synaptic loss, and neurotoxicity (55, 60). Major tasks for future research in this area will be to establish the mechanisms that drive specific programs of time-dependent complement activation in the context of neurological trauma, chronic pain, and neurodegeneration and to define the complement factors and neuroimmune cellular interactions that contribute to these neuropathological conditions.

With the relatively recent discovery of complement involvement in neuronal remodeling, there are also many unanswered questions as to how complement, neurons, glia, and immune cells act to regulate axonal regrowth, neurogenesis, and synaptic pruning. Recent evidence suggests that complement can act directly on peripheral nerves to both inhibit and promote axonal recovery after injury, but the molecular mechanisms are largely unknown (65, 82). Also remaining to be identified are the factors and mechanisms that regulate complement activity at the synapse and recruit glia to prune synapses or drive sprouting.

Progress in our understanding of complement-mediated mechanisms in the nervous system will require overcoming some of the limitations of current models commonly used to study the complement cascade. One of the limitations is a sparsity of transgenic conditional mouse models that permit temporally controlled deletion or modification of complement proteins in a tissue-specific manner. This is a significant problem because the net effect of activated complement cascade on neuronal functions commonly depends on many cell types, including glia, immune cells, and vasculature cells (13, 17, 45, 46, 66, 78, 94). Another important factor defining the complement action is timing in relation to a disease progression or developmental stage (55, 59, 60, 66). Recent generation of three new floxed reporter knock-in mouse lines for C3aR, C5aR1, and C5aR2, respectively (42, 168, 169), represents important progress in addressing these limitations. These mice allow both monitoring expression of these receptors in various tissues and temporally controlled tissue/cell-specific deletion of the receptors when crossed with an inducible Cre-expressing mouse line (170). Future studies using these new mouse lines as well as development of additional conditional-ready lines for other components of the complement system will be essential for dissecting the logic of cross-cellular interaction in executing many complement-mediated effects on the nervous system in health and disease.

Another important limitation concerns the use of animal models for studying and developing treatments for complement-dependent human diseases, including chronic pain conditions. Despite many similarities, there are substantial differences in complement signaling between rodents and humans. For example, in humans, the degradation product of C5a, C5a-desArg, has lower overall activity for C5aR1 than C5a. However, in the mouse, C5a-desArg appears to exert equal potency with that of C5a for C5aR1 (171). In a similar vein, human CR1 and CR2 show substantially different responses to C3 cleavage products relative to CR1 and CR2 in the mouse (26). Additionally, while there are numerous articles positing a role of C4 in various neurological and psychiatric disorders, including AD and schizophrenia (75, 172–174), elucidation of C4-driven mechanisms is complicated by the fact that mouse C4 has little catalytic activity compared with human C4 (175), suggesting that C4 might have significant variability in function between species. These examples highlight some of the challenges associated with the use of rodent models in studying complement-mediated human disease mechanisms.

Several strategies could be used to address these limitations in the future. *First*, combining human pluripotent stem cells (hPSC)-derived neurons, astrocytes, microglia, and other cells in culture has the potential to provide an *in vitro* platform for modeling molecular processes that take place in human neurological diseases. A recent study demonstrated the utility of this strategy for studying neuroinflammatory processes and complement activation in AD (176). Combining the hPSC-based approaches with recently developed methods for isolating and culturing human DRG neurons (177, 178) will be instrumental for modeling molecular and cellular aspects of human pain conditions *in vitro*, including investigation of complement signaling. *Second*, development of humanized mouse models expressing specific human complement genes will enable studying their roles *in vivo*. A recent report used this approach to provide a mechanistic insight into the link between schizophrenia and the C4A and C4B isoforms of C4 (174). Mice only express C4, making them a poor model for investigating the roles of C4A and C4B isoforms, which

prompted the development of humanized C4A- and C4B-expressing mice to study the roles of these isoforms *in vivo* (174). *Third*, continued efforts toward generating a data bank of essential biomarkers of complement signaling associated with specific diseases, including chronic pain, will be of great utility for the field. There are ongoing efforts within the pain research community to generate a comprehensive database of biomarkers associated with chronic pain and to better understand the underlying mechanisms in humans (179). This program will involve creation of clinical centers for pain phenotyping and surgical specimen acquisition, centers for tissue recovery from organ donors, and finally, centers for sample analysis using whole-genome sequencing, epigenomics, transcriptomics, proteomics, and other cutting-edge molecular approaches (179). The data will be widely available for research community and are expected to provide important insight for many areas of pain research, including those focusing on complement signaling and neuroinflammation.

Overcoming the described limitations will be essential for success in therapeutic targeting of the complement system for treating chronic pain and other neuropathological conditions. Despite the many remaining unanswered questions about the complement system and the complexities inherent to targeting its components for therapeutic purposes, there are already clinically utilized tools targeting the complement system such as cinryze, eculizumab, ravulizumab, and pegcetacoplan (26, 27, 47, 180). Cinryze is a human plasma-derived C1-INH inhibitor that is indicated for treating hereditary angioedema (HAE), a genetic disease characterized by episodic attacks of swelling of extremities, face, and gastrointestinal tract, with the latter causing severe abdominal pain (181). Another drug, eculizumab, is a monoclonal antibody against C5 that inhibits C5a production and TPCC/MAC formation; it is approved by the FDA for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) (27, 36). Patients with PNH experience bouts of severe pain associated with complement mediated lysis of red blood cells, and eculizumab helps to control these symptoms. A longer-acting analog of eculizumab, ravulizumab, was also recently approved for treating PNH patients. Eculizumab is also approved for treating patients with the neurogenerative disease neuromyelitis optica, which affects the spinal cord and optic nerve, and in antiacetylcholine receptor antibody-positive generalized Myasthenia Gravis patients. However, eculizumab is a very expensive humanized monoclonal antibody (~$500,000/year) with severe side effects, including an increased risk of meningococcal infections and other serious infections (27, 182). Thus, there is immense interest in developing more financially accessible small-molecule inhibitors with better safety profiles. Indeed, the recent approval of the C3-inhibitor pegcetacoplan for PNH (180) heralds on advance in development of new nonbiologic approaches to target complement and is expected to dramatically shift the landscape for therapeutic targeting of complement in disease.

Several recently developed small-molecule inhibitors of C5aR1 have also shown promise in preclinical and clinical studies for various conditions (26, 27, 47). Selective targeting of C5aR1 without concomitant inhibition of the TPCC/MAC complex is expected to provide a better safety profile for these drugs than that for eculizumab or ravulizumab, particularly where chronic dosing of elderly individuals is required. Indeed, clinical trials using the C5aR1 antagonists, CCX168 and PMX53, have demonstrated safety of both drugs (183, 184). CCX168 (also known as avacopan) is currently pending FDA approval for treating patients with ANCA-associated vasculitis and is in clinical trials for other chronic conditions (27, 47, 185). PMX53 has demonstrated beneficial effects in animal models for a number of neurological conditions, including chronic pain (8, 44, 45, 55). Despite promising preclinical data, PMX53 did not provide benefits to patients with rheumatoid arthritis in clinical trials (183), likely due to its poor oral bioavailability and tissue penetration (26, 47). PMX205, a newer more lipophilic analog of PMX53, has shown an improved oral bioavailability and significantly better penetration through the blood–brain barrier (186). These improved pharmacokinetic characteristics combined with promising preclinical data in animal models of AD, ALS, and spinal cord injury, make PMX205 a promising candidate for future therapeutic development. Many additional drug candidates that target C5a/C5aR1 signaling and other components of the complement system are currently at various stages of clinical trials for a variety of chronic pathological conditions (26, 27, 47). A more nuanced understanding of specific complement-mediated mechanisms that drive neurological symptoms and of disease-specific temporal aspects of complement system activity is expected to identify windows of opportunity as well as novel targets for the treatment of a wide range of neuropathological conditions for which efficacious clinical approaches have been lacking.

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Abbreviations—The abbreviations used are: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ANKA, antineutrophil cytoplasmic antibody; AS, ankylosing spondylitis; C3aR, C3a receptor; C5aR1/2, C5a receptor 1/2; C1-INH, C1-complex inhibitor;

C4BP, C4b-binding protein; CGRP, calcitonin-gene related peptide; CNS, central nervous system; CRPS, complex regional pain syndrome; DAF, decay-accelerating factor; DAMPS, danger-associated molecular patterns; DRG, dorsal root ganglion; GPCR, G-protein-coupled receptor; HAE, hereditary angioedema; IL-1β, interleukin 1β; MAC, membrane attack complex; MASP, MBL-associated serine protease; MAG, myelin-associated glycoprotein; MBL, mannose-binding lectin; MCP, membrane cofactor protein; NGF, nerve growth factor; OA, osteoarthritis; PAMPs, pathogen-associated molecular patterns; PNH, paroxysmal nocturnal hemoglobinuria; PNI, peripheral nerve injury; PNS, peripheral nervous system; SCI, spinal cord injury; TBI, traumatic brain injury; TPCC, terminal pathway complete complex; TrkA, tropomyosin-related kinase A; TRPV1, transient receptor potential vanilloid 1.

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