THE VAGUS NERVE AND THE NICOTINIC ANTI-INFLAMMATORY PATHWAY

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Abstract | Physiological anti-inflammatory mechanisms are selected by evolution to effectively control the immune system and can be exploited for the treatment of inflammatory disorders. Recent studies indicate that the vagus nerve (which is the longest of the cranial nerves and innervates most of the peripheral organs) can modulate the immune response and control inflammation through a 'nicotinic anti-inflammatory pathway' dependent on the α7-nicotinic acetylcholine receptor (α7nAChR). Nicotine has been used in clinical trials for the treatment of ulcerative colitis, but its clinical applications are limited by its unspecific effects and subsequent toxicity. This article reviews recent advances supporting the therapeutic potential of selective nicotinic agonists in several diseases. Similar to the development of α- and β-agonists for adrenoceptors, selective agonists for α7nAChR could represent a promising pharmacological strategy against infectious and inflammatory diseases.

SEPSIS
The clinical signs of a systemic inflammatory response to infection; 'severe sepsis' refers to organ dysfunction observed during systemic inflammation even in the absence of confirmed infection.

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Inflammation is a fundamental physiological process that is crucial for survival, but at the same time is one of the major causes of human morbidity and mortality. One of the most dramatic examples of the pathological potential of inflammation is SEPSIS, the leading cause of death in intensive care units[1,2]. Severe sepsis is the third leading cause of death in developed societies, equals the number of fatalities from acute myocardial infarction and accounts for 9.3% of overall deaths in the United States annually[3–5]. Sepsis was originally defined by the clinical signs of a systemic inflammatory response to infection, and 'severe sepsis' refers to organ dysfunction observed during systemic inflammation even in the absence of infection[5–7]. Despite the use of antibiotics, severe sepsis remains a major cause of death, in part because antibiotics cannot control inflammation and because severe sepsis is not exclusively caused by infections[7]. Given that inflammation contributes to wound healing and tissue repair, systemic inflammation can be produced in diverse clinical scenarios and not only during infection[7]. SHOCK, trauma, ISCHAEMIA and severe injury can contribute to severe sepsis, which is characterized by an overwhelming production of pro-inflammatory cytokines (such as tumour-necrosis factor (TNF), interleukin-1 (IL-1) and high-mobility group box 1 (HMGB1) that causes organ damage and failure[7–11]. The production of pro-inflammatory cytokines is beneficial and protects the organism against infections and injury. However, an excessive production of these cytokines can cause lethal systemic inflammation that can be more dangerous than the original infection or injury[10–14]. These pro-inflammatory cytokines are validated pharmacological targets for the treatment of a variety of clinical disorders (FIG. 1). Currently, experimental therapies that neutralize pro-inflammatory cytokines (monoclonal anti-TNF antibodies, IL-1 receptor antagonists and TNF-receptor fusion proteins) are successfully used in rheumatoid arthritis, Crohn's disease, ankylosing spondylitis and psoriasis[15–17]. However, these specific therapeutic approaches have produced limited effects on severe sepsis[18–20], which indicates that

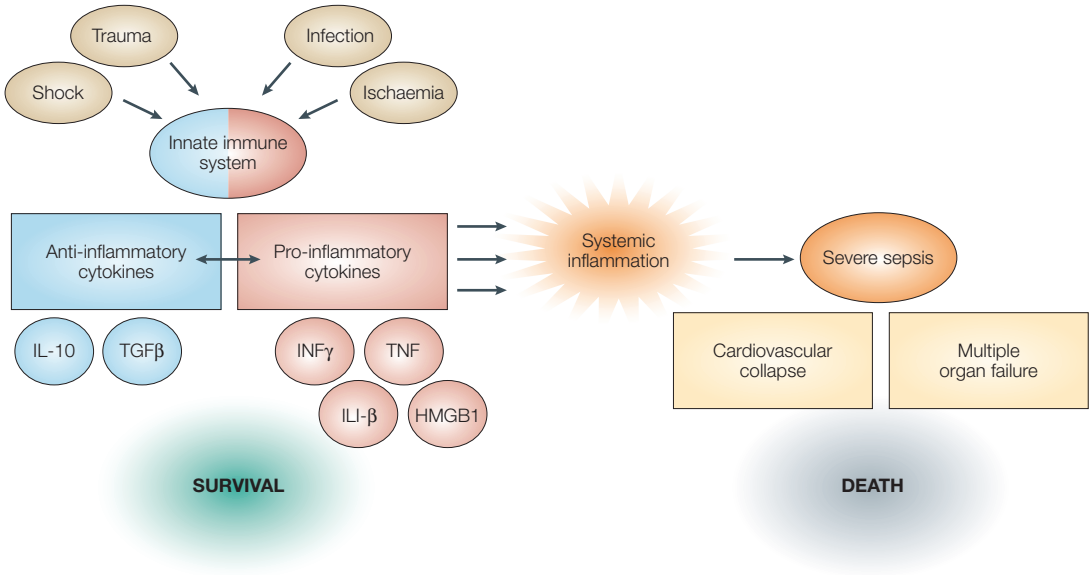

Figure 1 | **Inflammation contributes to the pathological progression of a variety of disorders.** Pro-inflammatory cytokines are one of the major causes of human morbidity and mortality, and they are successful pharmacological targets for the treatment of a variety of human infectious and inflammatory disorders. Shock, trauma, ischaemia and sepsis activate the innate immune system to trigger a defensive inflammatory response that is normally regulated by physiological anti-inflammatory mechanisms. Overwhelming production of pro-inflammatory cytokines (interferon-γ (INFγ), tumour-necrosis factor (TNF), interleukin 1 (IL-1) and high-mobility group box 1 (HMGB1)) can be more dangerous than the original stimuli and cause the characteristic cardiovascular collapse and multiple organ failure associated with severe sepsis. Anti-inflammatory strategies to control the production of these pro-inflammatory cytokines represent a therapeutic approach for infectious and inflammatory disorders.

successful treatment might require the inhibition of several, if not all, inflammatory cytokines. This article reviews the recent advances supporting the therapeutic potential of selective nicotinic agonists to control a variety of pro-inflammatory cytokines during infectious and inflammatory diseases.

Physiological anti-inflammatory mechanisms provide a major advantage to the design of novel pharmacological strategies against inflammatory diseases. The central nervous system is a pivotal regulator of the immune response, and controls inflammation at various levels. Several neuropeptides, such as adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (α-MSH) and glucocorticoids, can modulate cytokine production and subsequent toxicity[21–23]. These molecules have therapeutic potential, and pharmacological analogues to glucocorticoids are currently used for the treatment of diverse inflammatory disorders. Recent studies indicate that stimulation of the vagus nerve can control systemic inflammation in rodents[24–29]. ACETYLCHOLINE, the principal neurotransmitter of the vagus nerve, can limit the production of pro-inflammatory cytokines from human macrophages through a mechanism dependent on the α7 NICOTINIC ACETYLCHOLINE RECEPTOR (α7nAChR)[25,27]. Similar to the design of α- and β-agonists for adrenoceptors, selective NICOTINIC AGONISTS could represent a promising novel pharmacological strategy to control inflammation. There is therefore a great deal of interest in studying this 'nicotinic anti-inflammatory pathway' and its pharmacology to control inflammation.

Vagus nerve controls inflammation

Recent studies indicate that the vagus nerve represents a physiological target for pharmacological anti-inflammatory compounds such as CNI-1493. The mechanisms underlying the anti-inflammatory properties of CNI-1493 are not well understood. However, recent studies suggest that the anti-inflammatory effects of this compound are dependent on the activation of the vagus nerve. Also known as semapimod, CNI-1493 is a synthetic guanylhydrazone (FIG. 2) that attenuates the production of TNF in a wide variety of conditions, ranging from experimental stroke and sepsis in mice to inflammatory bowel diseases in humans[29–32]. A Phase I study in cancer patients demonstrated the safety of the compound and confirmed its activity in inhibiting TNF synthesis in humans[31]. Cytokine Pharmasciences, Inc. conducted successful proof-of-principle studies leading to Phase II clinical trials for the treatment of Crohn's disease[31].

Experimental studies of cerebral ischaemia showed that direct application of CNI-1493 into the cerebral ventricles suppressed TNF production both in the brain and also in peripheral organs, including liver, spleen and heart in rodents[29,30]. CNI-1493 administered via the intracerebroventricular route was 100,000-fold more effective than intravenous dosing at inhibiting ENDOTOXIN-induced peripheral TNF production[29]. These studies suggest that CNI-1493 acts on the brain, and activates a neuronal anti-inflammatory network. The vagus nerve is the longest of the cranial nerves and links the brain to most of the peripheral organs, and so it can potentially act as a neuronal

SHOCK
Bodily collapse or near collapse caused by inadequate oxygen delivery to the cells; characterized by reduced cardiac output, rapid heartbeat, circulatory insufficiency and pallor. Loss of blood is an important cause of shock.

ISCHAEMIA
A decrease in the blood supply to a bodily organ, tissue or body part caused by constriction or obstruction of the blood vessels.

ACETYLCHOLINE
A crystalline derivative of choline that is released at the ends of nerve fibres in the somatic and parasympathetic nervous systems and is involved in the transmission of nerve impulses in the body.

NICOTINIC ACETYLECHOLINE RECEPTORS
One of the two classes of cholinergic receptors. Nicotinic receptors are defined by their preference for binding nicotine over muscarine.

NICOTINIC AGONISTS
Chemical analogues of nicotine that can bind to a specific subset of nicotine acetylcholine receptors.

ENDOTOXIN
A toxin produced by Gram-negative bacteria and released from the bacterial cell.

VAGOTOMY
Surgical sectioning of fibres of the vagus nerve, previously used to diminish acid secretion of the stomach and control a duodenal ulcer.

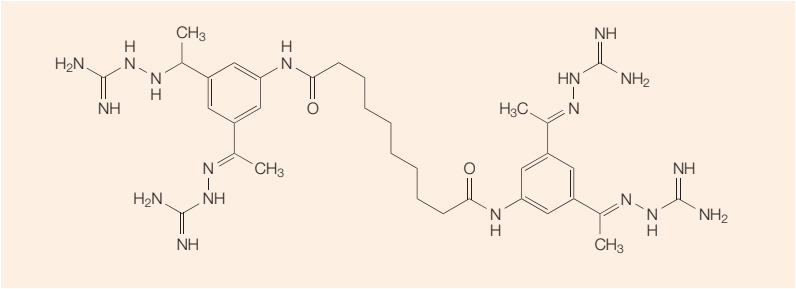

Figure 2 | **Pharmacological activator of the vagus nerve.** Chemical structure of CNI-1493. CNI-1493 (*N*,*N*′-bis[3,5-diacetylphenyl]decanediamide tetrakis[amidinohydrazone]tetrahydrochloride) is an anti-inflammatory compound that is currently in Phase II clinical trials for the treatment of Crohn's disease. CNI-1493 stimulates a neuronal anti-inflammatory response that is mediated by the vagus nerve, and represents a previously unrecognized role of this nerve in mediating the action of a pharmacological anti-inflammatory agent.

network for peripheral surveillance. Accordingly, surgical VAGOTOMY increases the susceptibility of rodents to septic shock[29], suggesting that the vagus nerve can function as a physiological anti-inflammatory system that modulates the immune response. CNI-1493 administered directly into the brain activates vagus nerve electrical activity, and surgical vagotomy abrogates the anti-inflammatory effects of CNI-1493 when administered directly to the brain or peripherally[29,30]. These results suggest that CNI-1493 stimulates a neuronal anti-inflammatory system mediated by the vagus nerve. In agreement with this hypothesis, electrical stimulation of the cervical vagus nerve in rodents attenuates levels of TNF in serum, liver, lung and heart[24–27]. This effect is clinically significant because it confers protection against lethal endotoxaemia by preventing endotoxic shock and the characteristic cardiovascular collapse associated with circulating TNF[29,30]. Similar to CNI-1493, melanocortin peptides can control systemic inflammation through a mechanism dependent on the vagus nerve. Melanocortin peptides — namely, those of the adrenocorticotropin/α-melanocyte-stimulating hormone (ACTH/α-MSH) group — have a life-saving effect in animal and human conditions of haemorrhagic shock[35]. This effect is associated with the reduction of circulating TNF levels through a mechanism that is adrenal-independent and dose-dependent, and which can be achieved with intracerebroventricular injection of doses significantly lower than those needed by the intravascular route[35].

Similar to CNI-1493, ACTH-(1–24) activates efferent vagus nerve and limits circulating TNF levels through a mechanism dependent on the vagus nerve because bilateral cervical vagotomy abolishes its anti-inflammatory effect[35]. These studies provide evidence for a previously unrecognized role of the vagus nerve in mediating the action of pharmacological anti-inflammatory agents. Although this mechanism has been well established for CNI-1493 and melanocortin peptides, further studies are needed to determine whether the vagus nerve contributes to the anti-inflammatory effect of other pharmacological compounds currently used for the treatment of inflammatory disorders.

MUSCARINIC RECEPTORS
One of the two major classes of cholinergic receptors. Muscarinic receptors were originally defined by their preference for muscarine over nicotine.

MUSCARINE
A highly toxic alkaloid related to the cholines, derived from the red form of the mushroom *Amanita muscaria* and found in decaying animal tissue.

The vagus nerve represents a bidirectional connection between the brain and the immune system. The immune system can activate sensory fibres of the vagus nerve that ascend to synapse in the nucleus tractus solitarius[28]. The brain can process the information and, in return, activate efferent fibres of the vagus nerve to control the peripheral immune system. Effector neurons originating in the dorsal motor nucleus of the vagus nerve can inhibit the production of pro-inflammatory cytokines from tissue macrophages. This bidirectional capability of the vagus nerve can provide a therapeutic mechanism for neurological disorders. In 1997, the US FDA approved the use of implantable electrical stimulators of the vagus nerve for the treatment of refractory epilepsy[31,32]. This vagus-nerve stimulator has been implanted in more than 30,000 patients worldwide and shown to be effective and safe[32]. The FDA has also approved the use of these vagus-nerve stimulators for the treatment of chronic and recurrent depression.

Although its mechanism of action remains unknown, a growing number of studies indicate that pro-inflammatory cytokines can contribute to the pathogenesis of these neurological disorders, and vagus nerve stimulation might exert a therapeutic anti-inflammatory effect[33,34]. Vagus nerve stimulation attenuates TNF production, and so vagal stimulators could represent a potential therapeutic strategy for TNF-related inflammatory disorders such as rheumatoid arthritis or Crohn's disease[35–38]. The therapeutic potential of this mechanism and the need for surgical procedures to implant these stimulators have promoted the study of pharmacological strategies to mimic the anti-inflammatory effect of vagus-nerve stimulation. The most common pharmacological approach has been to study the effect of acetylcholine (the principal neurotransmitter of the vagus nerve) on immune cells, the physiological source of inflammatory cytokines.

Acetylcholine receptors on macrophages

As macrophages are a major source of pro-inflammatory cytokines, the anti-inflammatory mechanism of acetylcholine has been studied in human macrophages stimulated with endotoxin (lipopolysaccharide (LPS)) and treated with cholinergic agonists. Cholinergic agonists such as acetylcholine and carbachol inhibit TNF production in both human and murine macrophages in a concentration-dependent manner[24,27]. Acetylcholine signals through either MUSCARINIC (G-protein-coupled receptors) or nicotinic (ligand-gated ion channels) receptors[39–41], and so selective agonists were used to identify the receptors involved in the control of TNF production in macrophages (FIG. 3). MUSCARINE slightly attenuates macrophages at supraphysiological levels, but nicotine is more efficient than acetylcholine at inhibiting TNF production from human macrophages in a dose-dependent manner[24–27]. Nicotine was also more efficient than acetylcholine at inhibiting the production of other pro-inflammatory cytokines such as IL-1, IL-6 and HMGB1. This effect was specific for pro-inflammatory cytokines and neither acetylcholine nor nicotine inhibited the production

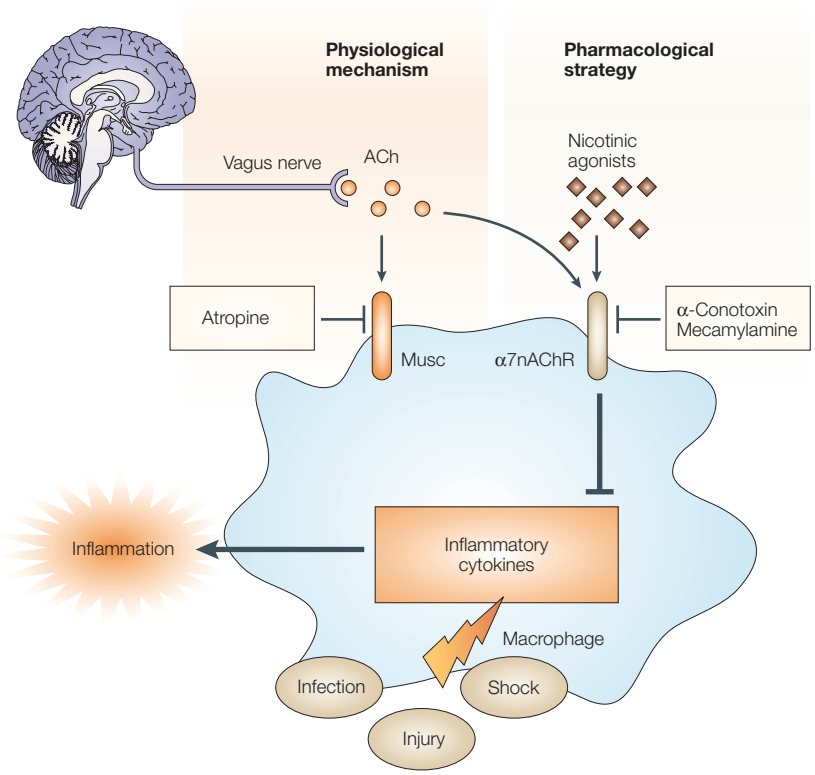

Figure 3 | **The 'nicotinic anti-inflammatory pathway'.** The vagus nerve can modulate the innate immune response and prevent inflammation through a physiological mechanism that can be translated into a pharmacological strategy. Acetylcholine, the principal neurotransmitter of the vagus nerve, signals through either muscarinic or nicotinic receptors, and so selective agonists (atropine, α-conotoxin or mecamylamine) were used to identify the receptors involved in the control of macrophages. This mechanism has been called the 'nicotinic anti-inflammatory pathway' because acetylcholine can inhibit the production of pro-inflammatory cytokines from macrophages through a nicotinic acetylcholine receptor. Nicotine, a more selective cholinergic agonist, is more efficient than acetylcholine at inhibiting the production of pro-inflammatory cytokines from macrophages through a mechanism that is dependent on the α7-nicotinic acetylcholine receptor (α7nAChR).

of anti-inflammatory cytokines such as IL-10[25,27]. To confirm that acetylcholine controls macrophage activation through a nicotinic acetylcholine receptor, selective antagonists against muscarinic (atropine) or nicotinic (α-conotoxin, α-bungarotoxin or mecamylamine) receptors were used on macrophages stimulated with endotoxin and treated with acetylcholine (FIG. 4). Atropine, even at concentrations as high as 1 mM, failed to restore TNF synthesis in macrophages treated with acetylcholine[25,27].

By contrast, the selective nicotinic antagonists α-conotoxin, mecamylamine and α-bungarotoxin significantly and dose-dependently reversed the cholinergic anti-inflammatory effect on macrophages, and re-established the production of pro-inflammatory cytokines in the presence of acetylcholine[25,27]. These results have additional clinical relevance because α-conotoxin Vc1.1 (also known as ACV1) seems to be a potential analgesic for the treatment of painful neuropathic conditions[88]. This effect is apparently mediated by inhibition of neuronal nAChRs, and Vc1.1 suppresses selective stimulation of sensory nerves in rats and antagonizes nicotine-induced axonal excitability[88]. Nicotinic antagonists such as Vc1.1 could antagonize (inflammatory) pain by inhibiting the acetylcholine-induced release of calcitonin gene-related peptides and/or by preventing nociceptor sensitization[89]. Although the specific target of Vc1.1 remains unknown, several data suggest that this analgesic effect could be mediated by α5nAChR. Ectopic lesioning of axons causes abnormal sensitivity to mechanical, thermal and chemical stimuli contributing to the development of neuropathic pain. Nerve injury by spinal ligation does not affect the expression of α4, α7, β2, β3 or β4-nAChR subunits, but it causes bilateral and ipsilateral increased of α3 and α5, respectively[90]. Knockdown of the α5nAChR in spinal nerve-ligated rats alleviates mechanical ALLODYNIA,[91] suggesting that this receptor might mediate the analgesic effect of α-conotoxin.

Although acetylcholine is historically referred to as a neurotransmitter, it can also function as an immune cytokine and might represent a common ancestral mediator in cellular biology. Acetylcholine is synthesized by both nervous and immune cells, and represents a link for neural–immune coordination[26]. From a pharmacological perspective, the anti-inflammatory effect of acetylcholine on macrophages seems to be mediated through nicotinic receptors, and nicotine is a more selective pharmacological agonist to control the production of pro-inflammatory cytokines.

α7-nicotinic acetylcholine receptor

Nicotinic acetylcholine receptors (nAChRs) are a family of ligand-gated ion channels created by a diversity of subunits that form homo- or heteropentameric receptors with distinct pharmacological properties[39–41,46,47]. A significant number of nicotinic subunits have been identified, including ten α (α1–10), four β (β1–4) and γ, δ and ε subunits (FIG. 5). Nicotinic AChRs have been mainly studied in neurons and muscle, and are associated with neuronal synapses and neuromuscular junctions[41–45]. According to their physiological distribution, nAChRs are classified as muscle nAChRs (which are made of up to five different type of subunits) or neuronal nAChRs (which are made of α and β subunits)[41–45]. Neuronal receptors can be functionally differentiated into two principal classes that differ in their affinity for nicotine and α-bungarotoxin[41–45,83]. Subunits that bind nicotine with high affinity contain α2–6 as ligand-binding subunits and require β-subunits for proper activation. A second class of subunits (α7–10) binds nicotine with lower affinity, has high affinity for α-bungarotoxin, and can function as homomeric or α–heteromeric ion channels *in vitro*. α7nAChR is the most abundant α-bungarotoxin receptor identified in mammalian brain — α8-subunits seem to be expressed only in chickens, and α9- and α10-subunit expression is limited to mechanosensory cochlear hair cells and the pituitary[42–45,83].

Recent studies indicate that nAChRs expressed on macrophages regulate the immune response and limit systemic inflammation. The presence of acetylcholine receptors in human macrophages was first confirmed

ALLODYNIA
Pain originating from a non-injurious stimulus to the skin.

by using fluorescein isothiocyanate (FITC)-tagged α-bungarotoxin, an antagonist peptide that binds to a specific subset of nicotinic receptors[40,45]. α-Bungarotoxin binds to the surface of human and murine macrophages, and pretreatment with nicotine prevents this interaction, indicating that nicotine and α-bungarotoxin compete for binding to the same receptors. α-Bungarotoxin binds to nicotinic α1, α7 and α9 subunits[27,45]. Although α7 and α9 subunits can form homopentameric nicotinic receptors, the α1 subunit forms heteropentameric nicotinic receptors with β1, δ and either ε (adult) or γ (fetal) subunits to regulate muscle contraction[41,42]. Macrophages express mRNA for the α1 and α7 but not for α9 subunit, and are therefore deficient in α9nAChR[27]. Similar experiments failed to detect mRNA for the δ subunit (a necessary component of the α1 heteropentameric nAChR), suggesting that human macrophages are deficient in functional α1nAChR. The identity of the α7 subunit expressed on macrophages was confirmed by cloning and sequencing, and is identical to the α7 subunit expressed in neurons[43]. Protein synthesis of the α7 subunit was confirmed in both differentiated macrophages and undifferentiated monocytes. Western-blot analysis revealed that the α7 subunit protein expressed in macrophages has a relative molecular mass of 55 kDa, identical to that described in neurons[44,45]. The specific inhibition of the expression of the α7 subunit significantly abolished the surface binding of FITC-labelled α-bungarotoxin to human macrophages, indicating that α-bungarotoxin and nicotine compete for binding to α7nAChR. Together, these results indicate that human macrophages lack functional α1nACh and α9nACh receptors, and that nicotinic modulation of macrophage activation is mediated by α7nACh receptors[25,27].

Both acetylcholine and nicotine inhibit the production of pro-inflammatory cytokines from macrophages through nicotinic signalling that is dependent on α7nAChRs. The specific inhibition of α7nAChRs in macrophages, using phosphorothioate antisense oligonucleotides, re-established TNF production even in the presence of acetylcholine[25,27]. Control sense oligonucleotides to α7nAChR and antisense oligonucleotides against similar regions to α1nAChR did not significantly affect the inhibitory effect of acetylcholine or nicotine on LPS-induced TNF release. Macrophages derived from α7nAChR-knockout mice were also refractory to cholinergic agonists, and produced TNF even in the presence of acetylcholine or nicotine. Macrophages are a major source of TNF[25,46], and so α7nAChR-knockout mice were used to determine whether this receptor could control circulating levels of pro-inflammatory cytokines *in vivo*. Mice lacking α7nAChR develop normally and show no gross anatomical defects, but they are more susceptible to systemic inflammation and lethal endotoxaemia[27,47,48]. Endotoxin significantly causes higher levels of circulating TNF, IL-1 and IL-6 in α7nAChR-deficient mice than in wild-type mice. During endotoxaemia, α7nAChR-deficient mice also produce greater amounts of these cytokines in the liver and the spleen compared with wild-type mice[27], indicating that α7nAChR contributes to the physiological regulation of the systemic response to infection. α7nAChR-deficient mice show a higher susceptibility to endotoxaemia similar to that induced by surgical vagotomy, suggesting that this receptor contributes to the physiological anti-inflammatory mechanism induced by the vagus nerve. Accordingly, vagus-nerve stimulation significantly attenuated endotoxin-induced serum TNF levels in wild-type mice, but not in α7nAChR-deficient mice[27]. Inhibition of α7nAChR therefore rendered the vagus nerve ineffective at regulating circulating TNF levels during endotoxaemia. These findings indicate that α7nAChR is crucial in the regulation of the immune response and has renewed the interest of studying nicotinic AChRs expressed in immune cells.

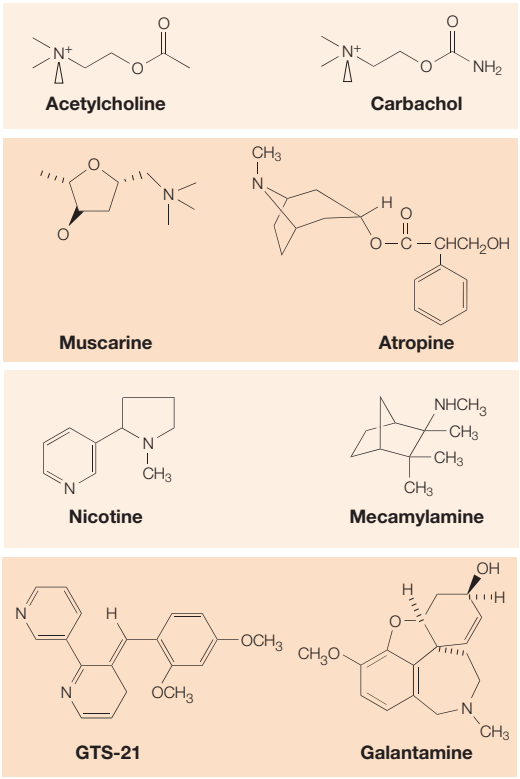

Figure 4 | **Chemical structure of selective cholinergic agonists and antagonists.** Although acetylcholine is historically referred to as a neurotransmitter, it can also function as an immune cytokine and could represent a common ancestral mediator in cellular biology. Nicotine, a more selective cholinergic agonist, is more efficient than acetylcholine at regulating the production of pro-inflammatory cytokines. This figure shows the chemical structure of general cholinergic agonists (acetylcholine and carbachol), and the characteristic agonists and antagonists for muscarinic (muscarine versus atropine) and nicotinic (nicotine versus mecamylamine) receptors. The figure also includes GTS21 and galantamine, which represent selective agonists and allosteric enhancers for α7 nicotinic acetylcholine receptors. Nicotine has been used in clinical trials for ulcerative colitis and it is extensively used in smoking cessation programmes. GTS21 is in clinical trials for the treatment of Alzheimer's disease. Galantamine hydrobromide (Reminyl; Johnson & Johnson) is currently used for the symptomatic treatment of schizophrenia and Alzheimer's disease.

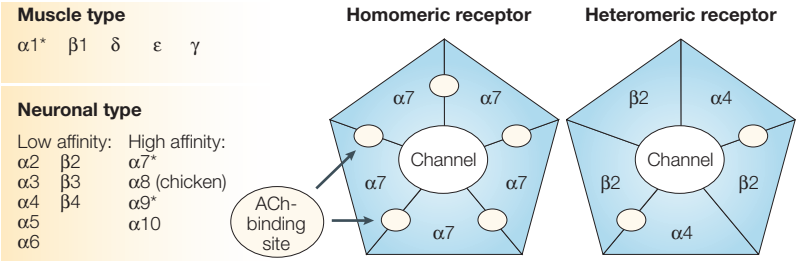

Figure 5 | **Human nicotinic acetylcholine subunits.** Nicotinic acetylcholine receptors (nAChR) are a family of homo- and heteropentameric receptors composed of a diversity of subunits including ten α (α1–10), four β (β1–4) and γ, δ and ε subunits. The α8-containing receptors have been found only in the chick nervous system[83]. The heterogeneity of nAChRs is sub-classified (as low or high affinity) according to the binding to α-bungarotoxin, an antagonist peptide that binds to a specific subset of nicotinic subunits including α1, α7 and α9. Among them, the α7 subunit can form homopentameric α7nAChR and can control the production of pro-inflammatory cytokines from macrophages. *Higher binding to α-bungarotoxin.

Recent studies indicate that human leukocytes selectively express an apparent isoform or duplicate α7-related protein (dup-α7) that could contribute to the regulation of the immune response. However, nicotine and acetylcholine do not elicit a current in leukocytes, and they are consistently negative for α-bungarotoxin–rhodamine staining, suggesting the absence of a typical nicotinic-binding site in this α7-related protein[40]. Previous studies have shown that part of the human α7-subunit gene (exons 5–10) is duplicated in the chromosome 15q13 region[39]. This dup-α7 transcript has several open reading frames, and one encodes a truncated α7-related protein that is compatible with the 45-kDa band observed by Western blot in leukocytes[40]. This duplication is characterized by the presence of four novel exons, named α7D, C, B and A (from 5′ to 3′), located upstream from the original exon 5[39]. Reverse transcriptase PCR (RT-PCR) analyses indicate that exons α7C, B and A are translated together with wild-type exons 5–10 to produce a transcript that contains an alternative amino-terminal sequence of about 110 amino acids[40]. This dup-α7 isoform therefore has different pharmacological properties because it fails to bind to acetylcholine, nicotine or α-bungarotoxin. The putative physiological relevance and stimulus for this isoform remain unknown, but given that nAChRs are present on many types of leukocytes, a neurotransmitter such as acetylcholine would be a relatively nonspecific cell-type agonist. Physiological and structural characterization of this isoform will be crucial in determining its pharmacological and therapeutic relevance. A major obstacle to determining the therapeutic potential of these receptors is the characterization of the intracellular signalling pathway and molecular mechanisms that control the production of inflammatory cytokines.

The nicotinic anti-inflammatory pathway

A growing number of studies indicate that α7nAChRs are crucial to the physiological regulation of systemic inflammation and have renewed interest in the specific 'nicotinic anti-inflammatory pathway' that controls the production of inflammatory cytokines from macrophages. Stimulation of human macrophage with endotoxin induces the transcription of a number of pro-inflammatory cytokines (TNF, IL-1, IL-6 and IL-18), and neither acetylcholine nor nicotine decreases the mRNA levels of these cytokines, suggesting that acetylcholine controls these cytokines at the post-transcriptional level without affecting intracellular mRNA levels[25,50] (FIG. 6a). Acetylcholine inhibits endotoxin-induced HMGB1 secretion from macrophages, and nicotine mimics this regulation in a dose-dependent fashion[25]. This mechanism is especially relevant because, unlike the other cytokines, HMGB1 protein synthesis is required for survival, and acetylcholine should therefore not affect the intracellular levels of this cytokine (FIG. 6b). HMGB1-deficient mice die within hours after birth, in part due to deficiency in gene expression induced by transcriptional factors, such as glucocorticoids receptors[53]. HMGB1 was originally described as a nuclear protein that binds DNA, and functions as a co-factor that is required for proper transcriptional regulation and gene expression[49–52]. Endotoxin and other inflammatory stimuli activate macrophages to secrete HMGB1 into the extracellular milieu[49,50].

Extracellular HMGB1 functions as a pro-inflammatory cytokine that contributes to the pathogenesis of different inflammatory disorders, including rheumatoid arthritis and severe sepsis[7,25,38]. Treatment with endotoxin and/or nicotine did not affect HMGB1 mRNA or total HMGB1 protein levels in macrophages. However, nicotine can halt HMGB1 secretion from macrophages by inhibiting its translocation from the nucleus to the cytoplasm, a crucial step for its secretion[25]. HMGB1 lacks a typical secretory signal sequence, and little is known about its mechanism of secretion[54–56]. General inhibitors of protein synthesis (such as a cycloheximide) do not block HMGB1 secretion, suggesting that nicotine might regulate HMGB1 secretion at a post-translational level. As HMGB1 secretion and binding to DNA can be modulated by post-translational regulation[57–58], nicotine could impinge directly on macrophage activation by modulating HMGB1 acetylation or phosphorylation[25,50].

Endotoxin activates toll-like receptor 4 (TLR4) and induces the activation of a number of intracellular pathways. Among them, the nuclear factor-κB (NF-κB) pathway is crucial for macrophage activation and the production of pro-inflammatory cytokines[59–62]. Nicotine prevented the endotoxin-induced activation of the NF-κB pathway in a concentration-dependent manner[25]. This effect is dependent on α7nAChR: specific inhibition of this receptor in macrophages restored the endotoxin-induced activity of the NF-κB pathway even in the presence of nicotine[25]. These results suggest that nicotine controls the production of pro-inflammatory cytokines from macrophages by inhibiting the NF-κB pathway through an α7nAChR-dependent anti-inflammatory pathway. These results have been confirmed in other cell types and nicotine inhibits the NF-κB pathway in human U937 monocytic[104] and

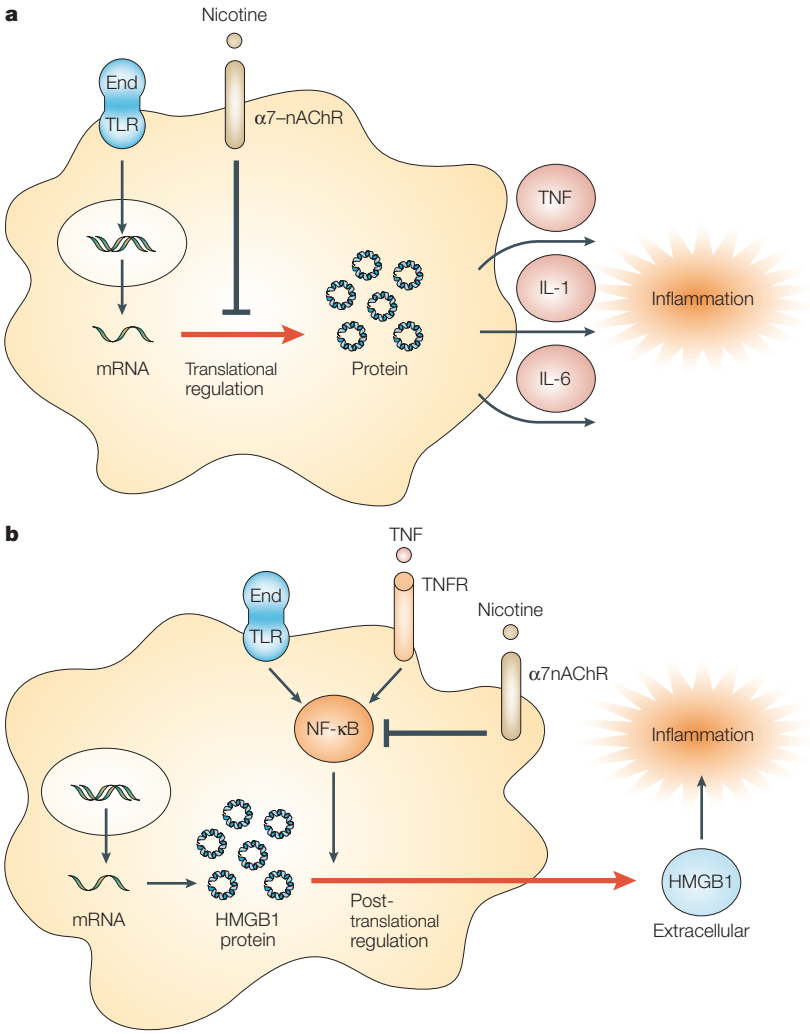

Figure 6 | **Nicotinic regulation of pro-inflammatory cytokines. a** | Endotoxin (End) induces the transcription of a number of inflammatory cytokines (tumour-necrosis factor (TNF), interleukin 1 (IL-1) and IL-6). Acetylcholine and nicotine can inhibit the production of these cytokines through a post-transcriptional mechanism that is dependent on the α7 nicotinic acetylcholine receptor (α7nAChR). Neither acetylcholine nor nicotine decreases the mRNA levels of these cytokines but they both significantly inhibit their protein synthesis. This mechanism is specific for pro-inflammatory cytokines and neither acetylcholine nor nicotine inhibits the production of anti-inflammatory cytokines such as IL-10. **b** | Nicotinic post-transcriptional regulation of pro-inflammatory cytokines. Acetylcholine inhibits endotoxin-induced HMGB1 secretion from human macrophages. This mechanism is especially significant because, unlike other pro-inflammatory cytokines, the continuous protein synthesis of HMGB1 is required for survival and the normal transcriptional regulation of macrophages. Acetylcholine inhibits HMGB1 secretion from human macrophages by signalling through a nicotinic acetylcholine receptor (nAChR). Nicotine, a more selective cholinergic agonist, is more efficient than acetylcholine, and inhibits HMGB1 release induced by either endotoxin or tumour-necrosis factor (TNF). Nicotine prevents endotoxin-induced activation of the nuclear factor-κB (NF-κB) pathway and inhibits HMGB1 secretion through a specific 'nicotinic anti-inflammatory pathway' dependent on the α7nAChR. HMGB1, high-mobility group box 1; TLR, toll-like receptor; NF-κB, nuclear factor-κB.

human microvascular endothelial cells[105]. These studies indicate that nicotine prevents LPS-induced nuclear translocation of the NF-κB complex by preserving cytoplasmic levels of inhibitor of NF-κB (IκB)[25,64,105]. However, it is unknown whether nicotine modulates the transcription of specific IκB inhibitors or inhibits an upstream mediator that prevents IκB ubiquitination and subsequent degradation. Many stimuli and toxins converge in the activation of the NF-κB pathway, and so nicotine can prevent macrophage activation and cytokine production in a variety of inflammatory scenarios. Nicotine also inhibited the activation of the NF-κB pathway induced by peptidoglycan from *Staphylococcus aureus*, suggesting that nicotine can interfere with the activation of macrophages induced by Gram-positive bacteria[25]. These results are clinically relevant because both Gram-positive and -negative bacteria cause sepsis.

Nicotinic agonists inhibit the production of pro-inflammatory cytokines in macrophages through a mechanism that resembles vagus-nerve stimulation[7,25]. Vagus nerve stimulation acts on α7nAChR to inhibit the NF-κB pathway and attenuate TNF production during endotoxaemia[27,31,64]. In a similar manner, nicotinic stimulation acts on the α7nAChR to inhibit the NF-κB pathway and attenuate TNF production in macrophages[25,27]. Moreover, efferent vagus nerve blunts activation of NF-κB signalling by preserving the IκBα inhibitor in the liver[31,64]. Likewise, nicotine blunts NF-κB signalling by preserving the IκBα inhibitor in human monocytic and endothelial cells[104,105]. Although these studies refer to different experimental models (vagus-nerve stimulation was analysed in haemorrhagic shock in rats, whereas the human monocytic and endothelial cells were activated with endotoxin), they both illustrate the crucial contribution of NF-κB and TNF to diverse clinical scenarios and inflammatory disorders. These studies show that it is possible to intervene with drugs that act at different points in this pathway. As an example, ethyl pyruvate is a stable lipophilic pyruvate derivative that inhibits TNF and HMGB1 release from macrophages and rescues animals from established polymicrobial peritonitis[50]. Ethyl pyruvate has no effect on IκB inhibitors but prevents binding of NF-κB to DNA by directly modifying p65(Rel A) at Cys38[107]. Critical Therapeutics, Inc. has recently initiated a Phase II clinical trial using ethyl pyruvate (also known as CTI-01) to prevent systemic inflammation and organ damage in patients undergoing cardiopulmonary bypass[108]. Because α7nAChR can control the NF-κB pathway, a pharmacological target for several inflammatory disorders, nicotine and selective nicotinic agonists could therefore provide a therapeutic potential target for the treatment of a variety of inflammatory disorders.

Clinical use of nicotinic agonists

The therapeutic use of nicotine has been suggested for the treatment of a large number of human diseases, including weight control, depression, Tourette's syndrome, Parkinson's disease, and inflammatory disorders such as Crohn's disease and ulcerative colitis[75,81,82]. To date, ulcerative colitis is the only condition for which controlled trials have provided evidence of the therapeutic potential of nicotine. Ulcerative colitis is an inflammatory bowel disorder epidemiologically related to smoking[65,66]. Most patients with ulcerative colitis are non-smokers, and

patients with a history of smoking usually acquire their disease after they have stopped smoking[66–69]. Patients who smoke intermittently often experience improvement in their colitis symptoms during periods when they are smoking[70,71].

Available treatments for ulcerative colitis are far from satisfactory and new strategies are needed; there are only two major drug options, mesalamine and glucocorticoids, both of which are poorly tolerated or ineffective in regular use. The addition of transdermal nicotine to conventional therapy significantly improves symptoms in patients with ulcerative colitis[70–72]. In one trial, 77 patients with diagnosed active ulcerative colitis were treated with either transdermal nicotine or placebo patches for 6 weeks in a randomized, double-blind study[72]. The patients in the nicotine group achieved a statistically significant greater improvement in the histological and global clinical score of colitis, including lower abdominal pain, stool frequency and fecal urgency. Of the 30 patients that finished the nicotine treatment, 17 had complete remissions, as compared with 9 of the 37 patients in the placebo group[72]. Although several epidemiological studies indicate that the response to nicotine is stronger in men than in women, the small numbers of patients of this clinical trial limited the power of these analyses, and the response to nicotine showed no relationship to age, sex or smoking history[72–76].

This trial included patients who had never smoked, and therefore the tolerance and pharmacokinetics of transdermal nicotine was first studied in normal subjects who were lifelong non-smokers. The nicotine doses were increased in a step-wise manner over a period of 5 days to minimize side effects. Three out of twelve non-smoker subjects were intolerant to nicotine, but the remaining nine tolerated doses of 15 and 25 mg per day[72]. Most patients with ulcerative colitis also tolerated similar doses of nicotine given in step-wise increments, although many had side effects. Five out of 35 patients in the nicotine group withdrew from treatment because of intolerable side effects[72]. The most common side effects were similar in both groups and included nausea, lightheadedness, headache, sleep disturbance and dizziness. The occurrence of side effects in the nicotine group was related to the patients' smoking history; they occurred in 10 of the 11 lifelong non-smokers, compared with 13 of the 24 former smokers[72]. There were no significant correlations between side effects and plasma nicotine or cotinine concentrations. The total mean dose in the nicotine group was 17±6 mg, a concentration that was approximately 35% of the average for smokers[70]. The cutoff point for distinguishing smokers from non-smokers is 14 ng per ml[73]. On average, each increase of 11 ng per ml in the plasma cotinine concentration reflects a nicotine intake of 1 mg (about one cigarette) in 24 hours[71]. During and after the trial none reported a craving for smoking, suggesting that addiction might depend on the sharp increases in plasma nicotine concentrations that follow smoking, which are unlike the steady release achieved with transdermal patches[72,73].

A second randomized, double-blind clinical trial involving 80 patients with ulcerative colitis in remission indicated that transdermal nicotine alone was no better than placebo in maintaining the remission of ulcerative colitis[77]. The clinical and histological scores worsened over the 6 months of the study, and the number and the time course of the relapses were almost identical in the two groups. Although more patients in the nicotine group had side effects, withdrawals due to ineffective therapy were more common in the placebo group and there were no significant differences in blood pressure, heart rate or any haematological or biochemical measurements between the two groups during the trial[77].

As compared with previous studies of acute colitis, this study presents several changes that could account for the clinical outcome. Transdermal nicotine was administered alone rather than in addition to a dose of mesalamine. Treatment began with a dose of only 2.5 mg followed by a slow build-up to a maintenance dose of 15 mg, instead of 25 mg. The patch was worn only during the day and was removed at bedtime rather than being left on for 24 hours. Although it is not possible to establish how any of these changes influenced the efficacy of treatment, the serum nicotine and cotinine concentrations of these patients were lower than expected. In other studies, the average daily intake of 15 mg of nicotine should produce cotinine concentrations of approximately 120 ng per ml; however, this study recorded concentrations of only 70 ng per ml[70]. If future studies confirm nicotine to be ineffective as a maintenance therapy for ulcerative colitis, but effective for the active disease, then the situation would be analogous to the use of corticosteroids. Although corticosteroids have been repeatedly shown to be effective for acute colitis[72], they are of little value for the maintenance of clinical remission[71,77]. Nicotine might act by inhibiting inflammatory mediators such as TNF and IL-8[74] or by changing adherent surface mucus in the colon[76–78]. Nicotine might also be more effective in reducing the acute neuromotor symptoms of active colitis rather than in suppressing the underlying abnormality responsible for the relapsing nature of the condition[77]. A better understanding of its mode of action should allow the design of efficient therapeutic approaches that overcome clinical limitations of nicotine itself.

Selective nicotinic agonists have been designed to target α7nAChR in pre-synaptic neurons and promote acetylcholine release in patients with Alzheimer's disease. Among other compounds, GTS-21 (3-[(2,4-dimethoxy)benzylidene]-anabaseine dihydrochloride) is a selective agonist that binds to α7nAChR in the cerebral cortex and hippocampus, which are the most affected areas in Alzheimer's patients (FIG. 4). GTS-21 can protect neurons against damage induced by amyloid peptides, at least in cell culture, suggesting that α7nAChRs might have a neuroprotective role. However, further studies are needed to determine whether GTS-21 affects other nicotinic receptors, including α4β2-nAChRs[84,85]. This compound is active

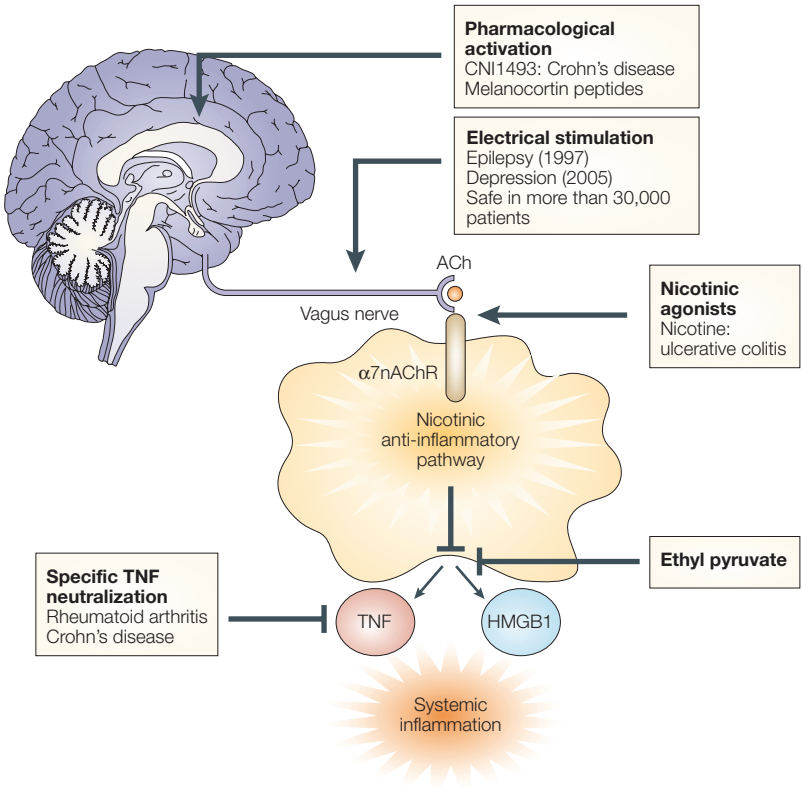

Figure 7 | **Clinical implications of the vagus nerve and the 'nicotinic anti-inflammatory pathway'.** The vagus nerve and the 'nicotinic anti-inflammatory pathway' represent a physiological mechanism that might be useful for the treatment of different infectious and inflammatory diseases. The vagus nerve represents a physiological target for pharmacological anti-inflammatory compounds. CNI-1493 and melanocortin peptides control systemic inflammation through a mechanism that is dependent on the vagus nerve. Electrical stimulation of the vagus nerve is currently used for the treatment of refractory epilepsy and depression. Acetylcholine and nicotine activates the α7nAChR in macrophages and controls the production of inflammatory cytokines. Nicotine has already been used in clinical trials and provided a therapeutic effect against ulcerative colitis. Similar to nicotine, ethyl pyruvate limits systemic inflammation by inhibiting the nuclear factor-κB (NF-κB), and controlling the production of a variety of inflammatory cytokines including tumour-necrosis factor (TNF) and high-mobility group box 1 (HMGB1). These inflammatory cytokines are therapeutic targets for the treatment of diverse inflammatory diseases, including rheumatoid arthritis and Crohn's disease.

in several experimental models and produced beneficial effects in psychological and cognitive tests when given to healthy volunteers in clinical trials conducted by Taiho Pharmaceutical Co.[84].

In one such trial 87 healthy human subjects were enrolled in four Phase I studies to evaluate GTS-21. The first three randomized, placebo-controlled studies indicate that patients tolerated well single doses up to 250 mg GTS-21. The fourth study analysed the pharmacokinetics and cognitive effects of GTS-21 in a multiple-dose study in healthy volunteers[84]. Eighteen subjects had GTS-21 (25, 75 and 150 mg) or placebo administered three times daily for three 5-day sessions. Patients tolerated well doses of up 450 mg per day, and there were no clinically significant differences in adverse events between the treatment groups[84]. GTS-21 induced statistically significant enhancement of three measures of cognitive function (attention, working memory and episodic secondary memory) compared with placebo. A relationship between treatment with GTS-21 and the magnitude of the cognitive response was apparent, with a maximal effect approached for doses between 75 and 150 mg three times a day[84]. GTS-21 has no effect on locomotor activity in mice or dopamine turnover in rats, indicating that it is less toxic than nicotine[85,87]. Nefiracetam (*N*-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl)acetamide) is another potential therapeutic designed by Daiichi Pharmaceutical Co. By September 1999, nefiracetam was in Phase II trials in the US for the treatment of mental symptoms associated with Alzheimer's dementia. However, in February 2002 Daiichi withdrew its Japanese New Drug Application due to insufficient efficacy in the revised trial[86].

Designing selective agonists

A crucial limitation to designing specific nicotinic agonists is the structural characterization of the ligand-binding domain of nAChRs. Although biochemical and electrophysiological information on prototypic nAChRs is abundant, structural data at atomic resolution is limited. Structural information about nicotinic receptors is based on X-ray structure analysis and electron microscopy (EM) studies using Torpedo receptors[92–95]. Most recently, structural analysis of a soluble acetylcholine-binding protein (AChBP) from the freshwater snail *Lymnaea stagnalis* has revealed the prototypic ligand-binding domain of nicotinic receptors[93,94]. The comparison of the EM data with the AChBP crystal structure shows that the α-nicotinic subunits themselves resemble AChBP once ligand is bound[92]. AChBP is a structural homologue of the extracellular ligand-binding domain of nAChRs, and shows ~24% sequence identity with the neuronal α7 subnit. Like the α7 subnit, AChBP assembles into a homopentamer with ligand-binding characteristics that are typical of a nicotinic receptor; unlike nAChR, AChBP lacks the domains to form a transmembrane ion channel[95].

Several structural nAChR models have been created on the basis of the AChBP structure[92,95,96]. Although these predictive models are inaccurate because the homology is weak (<25% identity), they might be relatively good in the domains with higher identity, such as the ligand-binding site. From a physiological perspective, AChBP represents an acetylcholine-binding protein that is released into the synaptic cleft by perisynaptic glial cells to regulate cholinergic transmission in the central nervous system. High concentrations of free acetylcholine will probably activate both postsynaptic neurons and perisynaptic glial cells (EC_{50} in the micromolar range), which will release AChBP into the synaptic cleft[93,94]. Soluble AChBP will bind free acetylcholine, thereby diminishing or terminating cholinergic transmission[94]. Similar regulatory mechanisms involving perisynaptic glial cells have been suggested for glutamate and GABA (γ-aminobutyric acid)-mediated synapses. In this example, presynaptically released glutamate not only binds receptors on the postsynaptic neuron, but can also activate AMPA

(α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors on perisynaptic astrocytes, causing a release of glutamate from glia[97,98]. Glutamate released by the perisynaptic astrocytes in turn activates *N*-methyl-D-aspartate (NMDA)-type receptors on the presynaptic neuron, thereby increasing the release of glutamate and enhancing synaptic transmission[97,98].

In addition to typical nicotinic agonists, nAChR can be activated by a novel class of drugs called allosteric enhancers, so named because they can activate these receptors without binding to the acetylcholine-binding site[99,100]. The most characteristic examples are physostigmine and galantamine (Reminyl; Janssen Pharmaceutica); both belong to a class of acetylcholinesterase inhibitors approved for the symptomatic treatment of schizophrenia and Alzheimer's disease (FIG. 4). These drugs presumably act by raising and prolonging the profile of acetylcholine via an inhibitory effect on the esterase. However, these drugs seem to bind directly to nAChR and modulate its activation[99–102]. Galantamine produces a brief, voltage-dependent channel block, which is consistent with a simple, linear open-channel-blocking mechanism[101]. Galantamine does not interfere with the binding of nicotinic agonists such as acetylcholine, carbachol, choline or ^{125}I-α-bungarotoxin[99]. These acetylcholinesterase inhibitors have no significant effect on either the amplitude or kinetics of α7nAChRs activated by acetylcholine, but they slow the rate of recovery from desensitization through an indirect mechanism[102]. The existence of multiple classes of binding sites is well established for other ligand-gated ion channels. For example, the $GABA_A$ receptor can be activated by several classes of drugs that bind to non-overlapping regions of the receptor: whereas GABA and muscimol interact with the characteristic ligand-binding site, barbiturates and steroids bind different domains[103]. Further studies are needed to identify the precise binding site of these potential allosteric enhancers to nAChRs, and to determine its potential pharmacological interest in inflammatory disorders.

Future strategies

The vagus nerve and the 'nicotinic anti-inflammatory pathway' might represent a mechanism for controlling the production of several inflammatory cytokines, and could therefore potentially be a target for therapeutic approaches in a diverse range of clinical disorders (FIG. 7). Severe sepsis is a major clinical and scientific challenge due to its high mortality rate, and more than 30 unsuccessful clinical trials have assessed sepsis therapeutics[7,8]. Severe sepsis is one of the most dramatic representations of the pathological effects of pro-inflammatory cytokines, and is characterized by elevated serum levels of diverse pro-inflammatory cytokines, including TNF and HMGB1[7]. Nicotine treatment *in vivo* reduces circulating TNF and HMGB1 levels, conferres protection against lethal endotoxaemia and improves survival in experimental sepsis in rodents[25–27,106].

The protective effect of nicotine has also been assessed in severe sepsis caused by polymicrobial peritonitis[25,106]. Nicotine inhibits the secretion of HMGB1, a 'late' mediator of lethal sepsis, and so nicotine treatment was delayed until after the onset of sepsis in order to resemble clinical standards. 'Delayed' nicotine treatment was started 24 hours after the induction of experimental sepsis, the time at which mice show clear signs of sepsis, including lethargy, diarrhea, piloerection, huddling, fever and malaise[25,27]. Delayed administration of nicotine diminished circulating HMGB1 levels, and significantly improved survival in experimental polymicrobial sepsis[25]. Mice were monitored for 3 weeks, and no late deaths were observed, indicating that nicotinic treatment confers lasting protection and reverses the clinical signs of sepsis.

Although further experimental studies are needed to evaluate the therapeutic potential of nicotinic agonists, a major advantage of this approach would be their capacity to inhibit the production of both TNF and HMGB1 in a clinically relevant timeframe, and to improve survival even when the treatment is started after the onset of sepsis.

A growing number of studies indicate that different pro-inflammatory cytokines participate in a variety of clinical scenarios, and anti-inflammatory strategies against these cytokines might therefore provide a potential therapeutic approach to diverse inflammatory diseases. The vagus nerve and the 'nicotinic anti-inflammatory pathway' represent a physiological 'neuro–immune' connection between some neurological and immune disorders. Electrical stimulation of the vagus nerve is currently approved by the FDA for the treatment of refractory epilepsy and recurrent depression (FIG. 7). Similarly, nicotine-like treatment has been proposed for the treatment of neurological and immune disorders, including schizophrenia, Alzheimer's and Parkinson's diseases and ulcerative colitis[75,81,82]. The development of nicotinic agonists for the treatment of these disorders has been limited by two factors: the characterization of the specific receptors for drug targeting, and the potential long-term effect on physiological variables[26,28,81,82]. The vagus nerve and the 'nicotinic anti-inflammatory pathway' represent two alternative targets that might provide a way to surmount these limitations. Vagus-nerve stimulators seem to be safe in long-term regular use and might provide an advantage for the treatment of chronic inflammatory disorders such as a Crohn's disease or rheumatoid arthritis[38]. On the other hand, the recent characterization of α7nAChR in macrophages supports the design of selective nicotinic agonists that might overcome the unspecific side effects of nicotine mediated by other receptors[74].

The toxicity of nicotine has emerged as a major issue in the use of nicotine-substitution preparations to aid in the cessation of smoking. These products are effective as aids to smoking cessation, but questions have been raised about the potential increased risk of myocardial infarction, and the effects of nicotine on the cardiovascular system, conception and fetal development. Future studies are needed to evaluate the potential use of specific α7nAChR agonists and to determine their effects on physiological variables including blood pressure, respiratory status and renal function[26,82].

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Competing interests statement

The author declares no competing financial interests.

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