REVIEW ARTICLE

Axonal Regeneration and Neuroinflammation: Roles for the Translocator Protein 18 kDa

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After a traumatic injury of the nervous system or in the course of a neurodegenerative disease, the speed of axonal regeneration and the control of the inflammatory response are fundamental parameters of functional recovery. Spontaneous regeneration takes place in the peripheral nervous system, although the process is slow and often incomplete. There is currently no efficient treatment for enhancing axonal regeneration, including elongation speed and functional reinnervation. Ligands of the translocator protein 18 kDa (TSPO) are currently under investigation as therapeutic means for promoting neuroprotection, accelerating axonal regeneration and modulating inflammation. The mechanisms of action of TSPO ligands involve the regulation of mitochondrial activity and the stimulation of steroid biosynthesis. In the peripheral nervous system, TSPO expression is strongly up-regulated after injury, primarily in Schwann cells and macrophages, but also in neurones. Its levels return to low control values when nerve regeneration is completed, strongly supporting an important role in regenerative processes. We have demonstrated a role for the benzoxazine etifoxine in promoting axonal regeneration in the lesioned rat sciatic nerve, either after freeze-injury or complete transection. Etifoxine is already clinically approved for the treatment of anxiety disorders (Stresam®, Biocodex, Gentilly, France). Daily treatment with etifoxine resulted in a two-fold acceleration in axonal regeneration, as well as in a marked improvement of both the speed and quality of functional recovery. The neuroregenerative effects of etifoxine are likely to be mediated by TSPO, and they may involve an increased synthesis of pregnenolone and its metabolites, such as progesterone. After freeze-injury of the sciatic nerve, administration of etifoxine also strongly reduced the number of activated macrophages and decreased the production of the inflammatory cytokines tumour necrosis factor-α and interleukin-1β. Thus, this drug offers promise for the treatment of peripheral nerve injuries and axonal neuropathies. It may also be used as a lead compound in the development of new TSPO-based neuroprotective approaches.

Key words: translocator protein 18 kDa, etifoxine, neurosteroids, axonal regeneration, inflammation.

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Need for efficient treatment of peripheral nerve injuries and diseases

In the case of traumatic nervous system injury, the primary insult leads to neuronal and vascular damage. Afterwards, secondary damage occurs, including hypoxia, ischaemia, the production of pro-inflammatory cytokines and free radicals, and a decrease in energy metabolism. The consequences of these processes can be a slowdown in regeneration, neuronal death, impaired sensitivity and pain. In the peripheral nervous system (PNS), spontaneous axonal regeneration takes place after traumatic injury, as well as in the case of peripheral neuropathies such as chronic inflammatory demyelinating polyneuropathy, chemotherapy or diabetic-induced neuropathies. Nevertheless, such regeneration is slow and often incomplete.

Increasing the speed and efficacy of axonal regeneration is important not only for shortening the period of disability after injury or neuropathies, but also to avoid post-degeneration muscle atrophy and tendinous retraction. Indeed, both the function and viability of skeletal muscles are dependent on intact innervation, and degenerative changes in muscles can be observed within a few weeks after denervation (1). Thus, a delay in muscle reinnervation can result in poor recovery, leading to permanent functional disability (2). In addition, the regenerative capacity of injured axons also decreases with time (3).

A number of drugs with different therapeutic targets have been tested to promote PNS regeneration. They include growth factors that often have serious side-effects, including immunophilins, which regulate the mitochondrial permeability transition pore (MPTP); erythropoietin, which is an anti-inflammatory agent that is able to induce neurogenesis; caspase inhibitors that block apoptosis; antioxidants; and steroids such as progesterone, which enhances myelination (4) and reduces myelin abnormalities during the ageing process, as well as in the case of peripheral neuropathies (5–7). These drugs were shown to improve specific parameters in experimental models. Nevertheless, to date, no successful treatment is available for enhancing the rate of axonal regeneration and assuring its completeness in patients.

As noted above, mitochondria appear to be involved in many deleterious processes occurring during degeneration, both in neurones and in glia. Hence, TSPO, which is part of the mitochondrial membrane and is able to bind an array of various chemical ligands, represents an emerging therapeutic target.

Structure, expression and function of TSPO

TSPO is a well-conserved ubiquitous protein, primarily localised in the outer mitochondrial membrane (8–10). Because it binds the benzodiazepine diazepam, it was formerly known as peripheral benzodiazepine receptor, and also as mitochondrial benzodiazepine receptor. It consists of five transmembrane α helices (11) (Fig. 1, right), forming a channel-like structure that may accommodate the import of cholesterol or other lipophilic molecules inside mitochondria (12,13). The carboxyl-terminus cytosolic end of TSPO contains a high-affinity cholesterol recognition amino-acid consensus domain

(14–17). Consequently, TSPO plays an important role in cholesterol transport from a cytoplasmic donor to the inner mitochondrial membrane. Furthermore, TSPO is a crucial component of the multimeric MPTP complex, whose components are located in the outer and inner mitochondrial membranes. This complex contains: the 32-kDa voltage-dependent anion channel, which is necessary for benzodiazepine and endozepine binding to TSPO (18,19); the 30-kDa adenine nucleotide translocase (18); the TSPO-associated protein 1 (20) and the protein kinase A regulatory subunit RIalpha-associated protein (21). Although the TSPO monomer binds drug ligands and cholesterol with nanomolar affinities, associated proteins may interfere with binding and related functions (22). Furthermore, homopolymerisation of TSPO monomers can occur under particular conditions, such as cell proliferation and steroid synthesis (23,24), as well as in other biological processes where there is increased generation of reactive oxygen species (25).

TSPO is present in many tissues but is particularly concentrated in those where active steroidogenesis (adrenals, gonads and brain) or bile salt synthesis (liver) take place (24). The fundamental role of TSPO in steroidogenesis has been extensively studied. TSPO gene
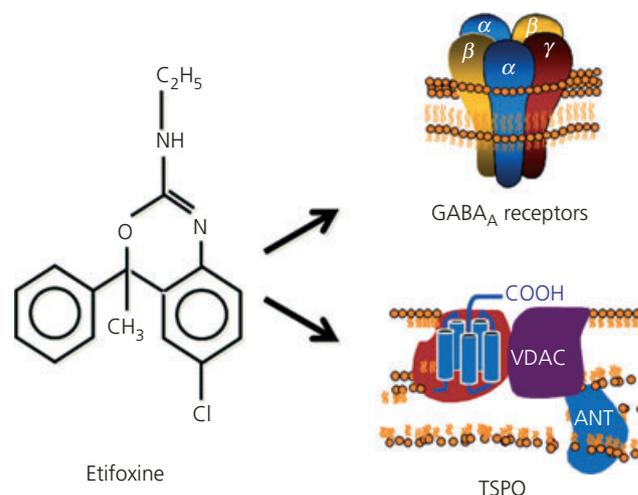
Fig. 1. Chemical structure and binding properties of etifoxine. Left: Two-dimensional structure of etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride; Stresam®). The molecule is structurally unrelated to benzodiazepines and neurosteroids. Right part: Etifoxine binds to both GABA$_A$ receptors and translocator protein 18 kDa (TSPO). GABA$_A$ receptors: Etifoxine competitively inhibits the binding of [^{35}S]t-butylbicyclophosphoro-thionate ([^{35}S]TBPS), a ligand and blocker of the GABA$_A$ receptor chloride channel, in membrane preparations of cerebral cortex with an IC$_{50}$ of 6.7 ± 0.8 μM (74). The direct binding of etifoxine to the β-subunit of GABA$_A$ receptors mediates part of its anxiolytic effect (75). TSPO: primarily localised in the outer mitochondrial membrane, it is arranged as five transmembrane helices (10). Several proteins are associated with TSPO, among them the voltage-dependant anion channel (VDAC) and the adenine nucleotide transporter (ANT). Etifoxine inhibits in a noncompetitive manner the binding of a radiolabelled specific TSPO ligand [^{3}H]PK11195 in membrane preparations of brain, with an IC$_{50}$ of 18.3 ± 1.2 μM. Scatchard analysis showed that etifoxine decreased B$_{max}$ values relative to control, although K$_d$ values were unaltered (62). Thus, etifoxine binds to TSPO at a site probably different from that of PK11195 (62).

disruption in Leydig cells completely suppressed intramitochondrial cholesterol transport and steroid production (26). Conversely, over-expression of exogenous TSPO in cells devoid of the protein induced cholesterol uptake and transport (24). TSPO facilitates the transport of cholesterol from the outer to the inner mitochondrial membrane, which is a rate-limiting step in steroidogenesis. Cholesterol is then metabolised, via the activity of cytochrome P450 cholesterol side chain cleavage enzyme (P450scc; CYP11A1), to pregnenolone, the precursor of all other steroid hormones (24). Other roles have been ascribed to TSPO, and these are essentially related to mitochondrial function, including protein import, porphyrin transport and heme biosynthesis, ion transport, cellular respiration, oxidative processes, immunomodulation, apoptosis and cell proliferation (27–38). By modulating these functions, TSPO ligands may exert a major influence on degenerative and regenerative processes in the nervous system after injury or during neurodegenerative disease.

Up-regulation of TSPO in response to injury and neurodegenerative diseases

In the healthy nervous system, TSPO is expressed at low levels in glia, and also in some populations of neurones (9,39,40). By contrast, TSPO is highly expressed in the injured or diseased central nervous system (CNS) or PNS at the lesion sites. In the CNS, TSPO is up-regulated in microglia, astrocytes and infiltrating macrophages, and probably also in some neurones (9,39,40). Increased TSPO expression is observed in neurological disorders such as multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease and stroke. Levels of TSPO are also elevated in the case of gliomas, herpes, HIV encephalitis and traumatic brain injury, in various models of inflammation and in ischaemia-reperfusion lesions (39,41,42). These observations have led to the development and use of radiolabelled TSPO drug ligands as neuroimaging agents for positron emission tomography, and they may become important diagnostic tools for brain inflammation associated with various neuropathological conditions (10,43–45).

After peripheral nerve injury, TSPO expression is transiently increased in dorsal root ganglia sensory neurones, in Schwann cells and in resident and infiltrating macrophages (46–48). Changes in TSPO expression in response to peripheral neuropathies remain to be explored. Nevertheless, in the case of experimental diabetic neuropathy, it has been shown that a TSPO ligand (Ro5-4864) is neuroprotective (49). By contrast to the CNS, TSPO has so far not been used as a diagnostic tool in the PNS.

TSPO ligands and their use in experimental models of neuroinjury and neurodegeneration

Cholesterol, porphyrins and endozepines are endogenous ligands of TSPO. Endozepines, identified by their capacity to displace benzodiazepines from their GABA$_A$ receptor binding site, are derived from a common precursor, the diazepam-binding inhibitor. The proteolytic cleavage of diazepam-binding inhibitor gives rise to the biologically active peptides octadecaneuropeptide and triakontatetraneuropeptide (50). These peptides probably play an important role in the responses of glial cells to insult and disease because they were shown to stimulate mitochondrial steroid synthesis (51).

A number of synthetic drug ligands have been developed to target TSPO, belonging to different chemical classes. Classical synthetic TSPO ligands include the high affinity isoquinoline carboxamide PK-11195 and the benzodiazepine 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Ro5-4864). Although PK-11195 solely binds to TSPO, the binding of Ro5-4864 requires the presence of other components of the MPTP. Other chemically different drugs also display TSPO binding properties: imidazopyridines such as alpidem, indoleacetamides such as FGIN-1-27 and SSR180575, pyrrolobenzoxazepines, phenoxyphenyl acetamides, sterols such as olesoxime (TRO19622), phenylpurine acetamide such as XBD173, the benzoxazine etifoxine, and others (10).

Some of these drugs have already been tested successfully in preclinical models of lesions or diseases of the nervous system. In the PNS, some TSPO ligands were shown to display neuroprotective properties in models of toxic and diabetic-induced neuropathies, (49,52,53). Furthermore, SSR180575 promoted functional recovery in young rats whose facial nerve had been injured (52) and olesoxime enhanced regeneration of the mouse crushed sciatic nerve (54). Neuroprotective and anti-inflammatory actions of TSPO ligands were also shown in models of excitotoxic or traumatic brain injury (10,55–57).

The use of TSPO ligands to protect nervous tissues and even enhance axonal regeneration is of special interest because TSPO is up-regulated in response to injury. Consequently, these ligands may exert their protective and trophic effects where they are needed. However, the use of some of them is limited, either as a result of their low solubility, their poor penetration in nervous tissues, their inconsistent effects when tested in humans, or even their toxic side-effects. We now discuss the results obtained with the TSPO-binding drug etifoxine.

## Etifoxine, a clinically approved anxiolytic drug, binds to TSPO

Etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride), which is chemically unrelated to benzodiazepines, has anxiolytic-like properties in rodents (58,59). Interestingly, etifoxine is a clinically approved drug, and has been used for years in the treatment of adjustment disorders with anxiety. Randomised, controlled and double-blind studies performed for this indication showed that etifoxine preserved psychomotor, attention and memory performances (60,61). Its anxiolytic effect was attributed in part to the potentiation of GABA$_A$ receptor function by a direct allosteric effect, as well as to an indirect mechanism involving the activation of TSPO (59). Indeed, etifoxine was shown to inhibit (in a noncompetitive manner) the binding of the selective TSPO ligand [^{3}H]PK11195 (IC$_{50}$ = 18 μM) (62) (Fig. 1). Furthermore, i.p. injection of etifoxine increased plasma and brain concentrations of pregnenolone, progesterone, 5α-dihydroprogesterone and allopregnanolone by two- to four-fold (62). These findings suggested that etifoxine, both as a TSPO ligand and as a compound able to enhance neurosteroid levels, represented a good candidate molecule for providing neuroprotection and promoting neuroregeneration.

Etifoxine and axon regeneration

Axonal regeneration was studied after local freeze-injury of the rat sciatic nerve (Fig. 2B), a well standardised model which, similar to nerve crush, results in the rapid destruction of axons. In this model, preservation of the continuity of connective tissues in the injured nerve facilitates spontaneous regeneration (4,63).

The right sciatic nerve was frozen 15 mm above the separation between the tibial and peroneal branches (six successive cycles of freezing–thawing, using a 1-mm diameter copper cryode, precooled
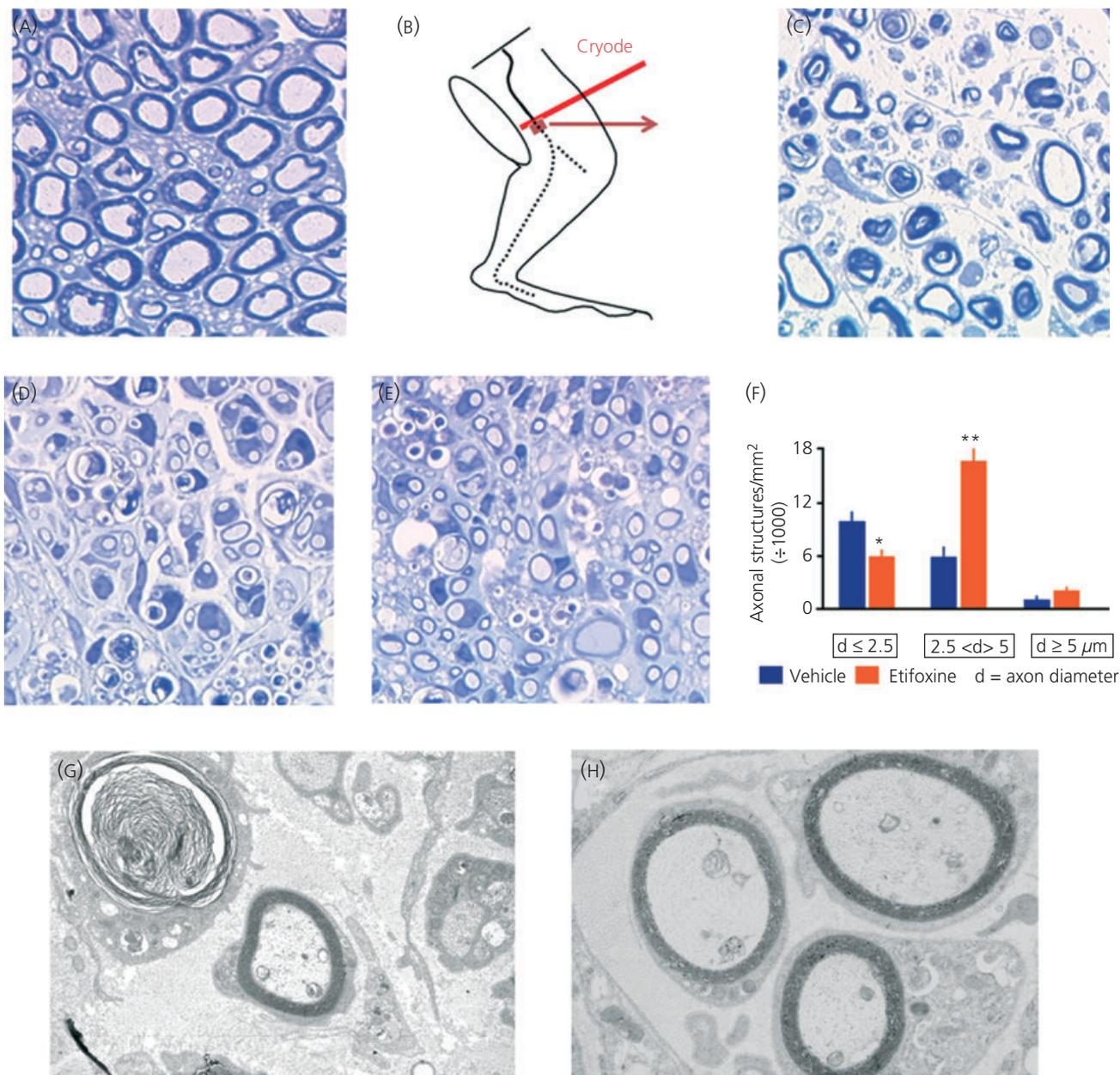

Fig. 2. Complete degeneration of nerve fibres after cryolesion of the rat sciatic nerve and nerve fibre regeneration promoted by etifoxine treatment. Semi-thin cross-sections (1 μm) were stained with thionine blue for light microscopy (A, C–E) and ultra-thin sections were stained with uranyl acetate and lead citrate for electron microscopy (G–H) (n = 3). (B) Diagram illustrating cryolesion of the rat sciatic nerve 15 mm above the separation between the peroneal and tibial branches; dotted lines: nerve undergoing Wallerian degeneration. (A) Morphology of nerve fibres in the intact rat sciatic nerve. (C) Destruction of myelinated axons 3 days after cryolesion. Remaining myelin sheaths surround empty spaces left by degenerated axons and no spared fibres were observed. (D) Regenerated nerve fibres 15 days after cryolesion in rats treated with vehicle and (E) in rats treated with etifoxine. (F) Regenerated axons were counted 15 days after cryolesion. At this stage, the number of regenerated medium sized axons ($\geq$ 2.5 and $\leq$ 5 μm) surrounded by myelin sheaths was increased by etifoxine. By contrast, the number of small calibre myelinated axons ($\leq$ 2.5 μm) was decreased by etifoxine treatment. (G) Examination of regenerated nerve fibres by electron microscopy in rats treated with vehicle and (H) in rats treated with etifoxine. *P $\leq$ 0.05; **P $\leq$ 0.01.

in liquid nitrogen). The site of lesion was marked with an epineural suture. Rats were injected daily using an i.p. route, either with etifoxine (50 mg/kg) or with an equal volume of vehicle (1% Tween 80 in 0.9% NaCl solution), with the first treatment beginning 24 h after surgery. Rats were killed 1, 2, 3, 7, 10, 15, 21 or 29 days after surgery. Five to eight rats per time and per condition were used for histological, immunological and molecular studies.

At the lesion site, myelinated axons were visualised in semi-thin and ultra-thin sections (Fig. 2). Destruction of myelinated axons was observed 3 days after injury, both in vehicle and etifoxine injected animals (Fig. 2A,C). By contrast, 15 days after injury, regeneration was much more advanced in etifoxine (Fig. 2E) than in
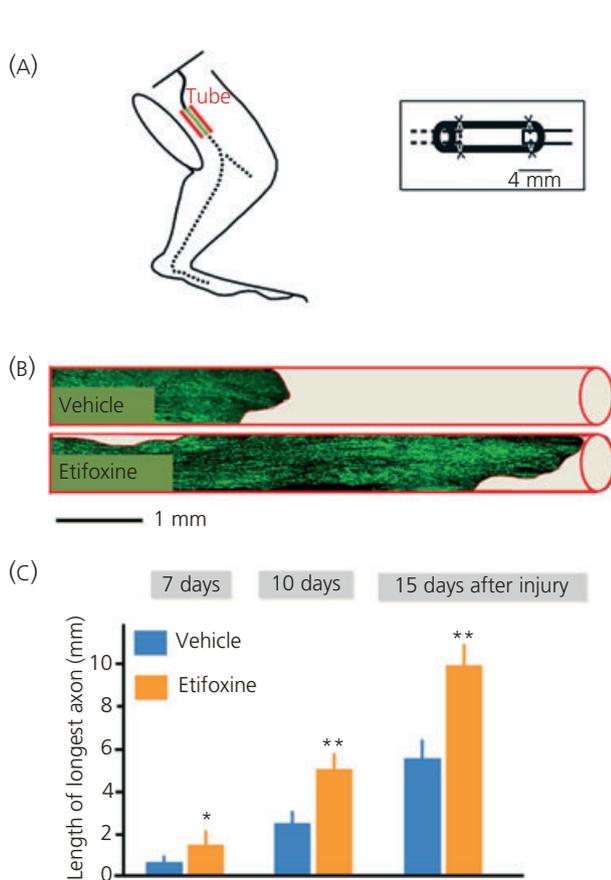
vehicle-treated rats (Fig. 2D). Regenerated nerves treated with etifoxine showed a significant increase in medium-sized axons (diameter in the range 2.5–5 μm) compared to vehicle-injected controls (Fig. 2F). A decrease of small diameter axons (< 2.5 μm) was also observed. Although 15 days after injury degenerated nerve fibres were still visible at the lesion site of vehicle-treated rats (Fig. 2G), the regenerating axons were of more regular shape, and were surrounded by morphologically normal myelin in etifoxine-treated rats (Fig. 2H) (64).

The promoting effect of etifoxine on axonal regeneration was also demonstrated by the immunolabelling of stathmin-like 2 protein (STMN-2), peripherin or 200-kDa neurofilament (NF-200)
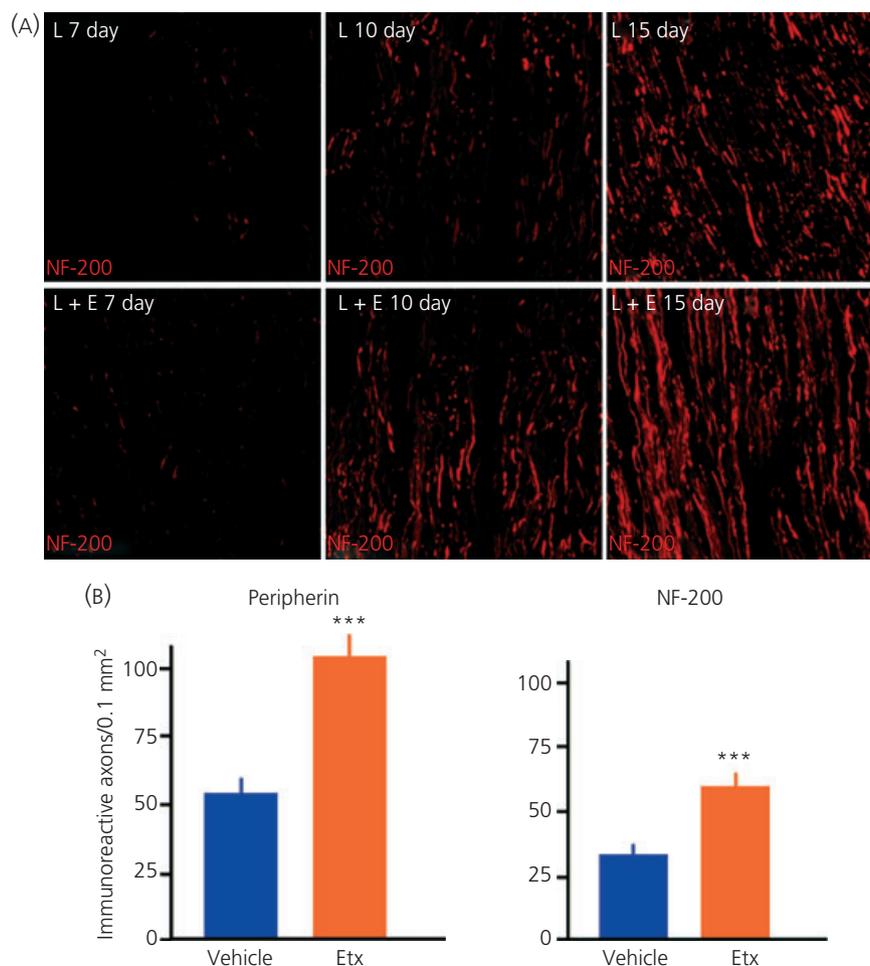
Fig. 3. Etifoxine enhances the outgrowth of axons in the injured site within the distal stump. For immunostaining, nerves were fixed with 4% paraformaldehyde solution, cryopreserved and embedded for subsequent sectioning using a cryostat apparatus. Longitudinal 12 μm-sections were stained with anti-200-kDa neurofilament (NF-200) (Abcam, Cambridge, MA, USA) or anti-peripherin (Chemicon Europe, Chandelers Ford, UK) antibody and observed with an AxioImager A1 microscope (Carl Zeiss, Oberkochen, Germany). An analysis of longitudinal 30 μm sections with a confocal microscope (Carl Zeiss) allowed the counting of all fluorescent fibres within the depth of the sections. (A) Immunostaining of the neurofilament NF-200 at 7, 10 and 15 days after injury in the lesioned area (L) of the sciatic nerve. Injured rats were treated daily either with vehicle (upper panel) or with etifoxine (+E) (lower panel). Note that, after cryolesion, NF-200^{+} linear structures were increased by etifoxine at 10 and 15 days. (B) Number of axonal structures per area in the distal stump of freeze-injured sciatic nerve of rats, treated either with daily injection of etifoxine or of vehicle, 10 days post-injury. According to the type and status of the neurone, the expression of intermediate neurofilaments differs, with peripherin being mainly expressed in small and medium-sized neurones, and the neurofilament triplet in large diameter, myelinated axons. Etifoxine increased the number of axonal structures in the distal stump of the sciatic nerve (mean ± SEM) (Student's t-test: ***P < 0.001).
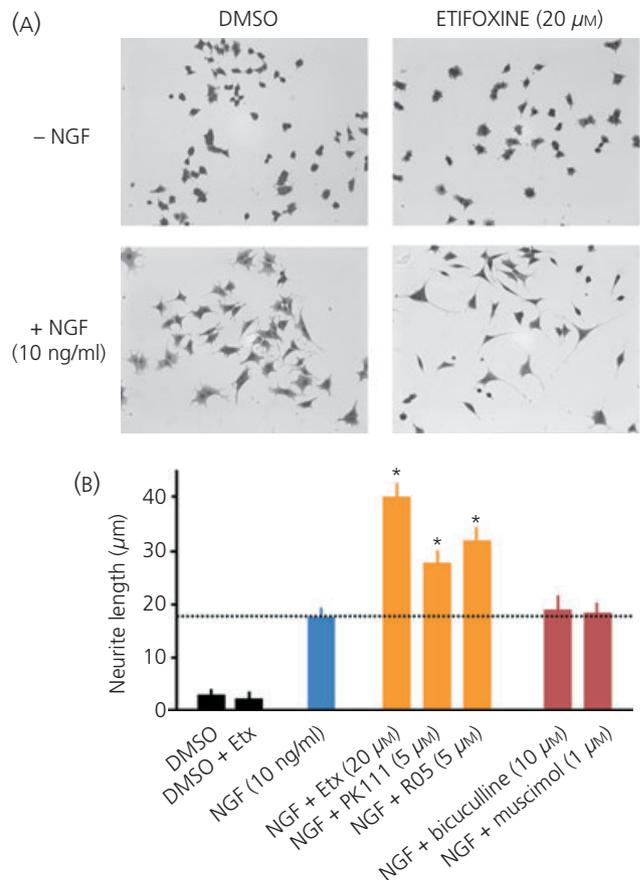
expressing nerve fibres after cryolesion. STMN-2 is selectively expressed in regrowing axons and concentrated in growth cones, thus being a typical marker of axonal regeneration (65). Peripherin and NF-200 are intermediary neurofilaments, with the former being expressed in small-diameter fibres and in regrowing axons (66,67) and the latter being a marker of more mature large diameter axons (68). For all three markers, the number of immunolabelled axons was markedly increased in etifoxine-treated rats, compared to their vehicle-injected counterparts, until 10 days post-injury (Fig. 3) (64). Western blotting and immunolabelling showed that, after 10 days post-injury, peripherin expression began to decrease, and this decrease was more important in etifoxine-treated rats. Conversely,

NF-200 neurofilament expression continued to increase after 10 days post-injury, and this increase was enhanced by etifoxine treatment (Fig. 3A) (64). Thus, the decrease of small diameter axons together with the increase of medium and large-sized axons observed after etifoxine treatment (Fig. 2F) could be the result of an accelerated maturation of axons by etifoxine.

The efficacy of etifoxine for accelerating axonal regrowth was also tested in a model of complete section of the rat sciatic nerve, after which the two ends of the nerve were secured by a silicone tube (Fig. 4A). Etifoxine (50 mg/kg) or its vehicle was injected daily (i.p.), with the first treatment beginning 24 h after surgery. The

Fig. 4. Etifoxine treatment promoted axonal extension after sciatic nerve transection. (A) Diagram illustrating transection of the rat sciatic nerve 15 mm above the separation between the peroneal and tibial branches. The two nerve endings were inserted and secured into the ends of a 10-mm long silicone tube filled with phosphate-buffered saline, leaving a gap of 6 mm between the nerve stumps (right part of the diagram). The dotted line indicates the distal part of the nerve undergoing Wallerian degeneration. (B) Etifoxine (50 mg/kg, i.p.) versus vehicle was injected daily, the first treatment beginning 24 h after surgery. Etifoxine increased the growth of stathmin-like 2 protein (STMN-2)-immunoreactive axons (green; anti-STMN-2 antibody from Chemicon Europe) from the proximal stump into the silicone tube by more than two-fold, 10 days after surgery. (C) The maximal extension of nerve fibres into the tube was quantified at 7, 10 and 15 days after sciatic nerve transection (mean ± SEM). Two-way ANOVA with time and treatment after cryolesion as factors revealed both factors and their interaction to be significant at *$P < 0.05$ or **$P < 0.01$; Bonferroni post-test.


Fig. 5. Etifoxine stimulated PC12 cells neurite extension in the presence of nerve growth factor (NGF). (A) PC12 cells acquire a neuronal phenotype when grown in the presence of NGF. Cells were treated with or without NGF (10 ng/ml) and etifoxine (20 μM). Dimethylsulphoxide (DMSO) was used as vehicle. After 72 h of treatment, cells were fixed and coloured with toluidine blue. Etifoxine stimulated neurite extension solely in PC12 cells grown in the presence of NGF. (B) In PC12 cell culture, the efficacy of different treatments on neurite length was compared with that of etifoxine (Etx, 20 μM): the translocator protein 18 kDa (TSPO) ligands PK11195 (PK111, 5 μM), Ro5-4864 (Ro5, 5 μM) dissolved in ethanol, and the GABA$_A$ receptor ligands bicuculline (10 μM) and muscimol (1 μM) dissolved in water. After 72 h of treatment, cells were fixed and coloured. The length of the longest neurite was measured on 600 cells per culture condition and the mean (± SEM) computed. The TSPO ligands induced neurite extension, but to a lesser extent than etifoxine, although GABA$_A$ receptor ligands did not. *$P < 0.05$ vs. NGF treatment.

immunolabelling of the axons growing from the proximal end inside the guide tube with an STMN-2 antibody showed that, at 7, 10 and 15 days after injury, the longest axons were two-fold longer in

etifoxine-treated rats than in their vehicle-treated counterparts (Fig. 4B,C) (64). The effect of etifoxine on neurite outgrowth was also tested in rat pheochromocytoma PC12 cells, displaying a neuronal phenotype when cultured in the presence of nerve growth factor. We showed that etifoxine stimulated neurite extension by more than two-fold (Fig. 5A). Because etifoxine binds to both GABA$_A$ receptors and TSPO, specific ligands of these receptors were also tested. The selective TSPO ligands PK11195 and Ro5-4864 mimicked the neurotrophic effect of etifoxine, whereas the selective GABA$_A$ receptor ligands muscimol and bicuculline were inefficient in stimulating neuritic extension (Fig. 5B) (64). These results suggested that the neurotrophic effect of etifoxine was related on binding to TSPO.

Etifoxine and neuroinflammation

It was previously shown that freeze-injury of the rat sciatic nerve is accompanied by a rapid and important increase in TSPO ligand binding capacity (69). This was the result, at least in part, of an increase of TSPO immunoreactivity in activated macrophages and Schwann cells. However, it was unknown whether TSPO ligands have an influence on peripheral nerve inflammation. The number of activated macrophages, as assessed by OX42 immunoreactivity, first rapidly increased in both ends of the cryolesioned nerve and, second, was especially high in the distal end, where Wallerian degeneration takes place. Daily etifoxine treatment exerted a marked immunoprotective action, which was visible as early as 3 days post-injury in the proximal end and, later, at 7 days post-injury, also in the distal end (Fig. 6A) (64). The expression of the pro-inflammatory cytokines tumour necrosis factor (TNF)-α and interleukin (IL)-1β was also profoundly affected by etifoxine treatment. Their expression greatly increased after injury, but daily etifoxine treatment (50 mg/kg, i.p.) caused an important reduction of both cytokines as soon as 2 days post-injury (Fig. 6B). This anti-inflammatory effect of etifoxine may contribute in a significant manner to successful peripheral nerve regeneration because an excess of inflammatory products can become deleterious to neurones. In addition, this excess of inflammatory response could also be involved in the origin of chronic neuropathic pain.
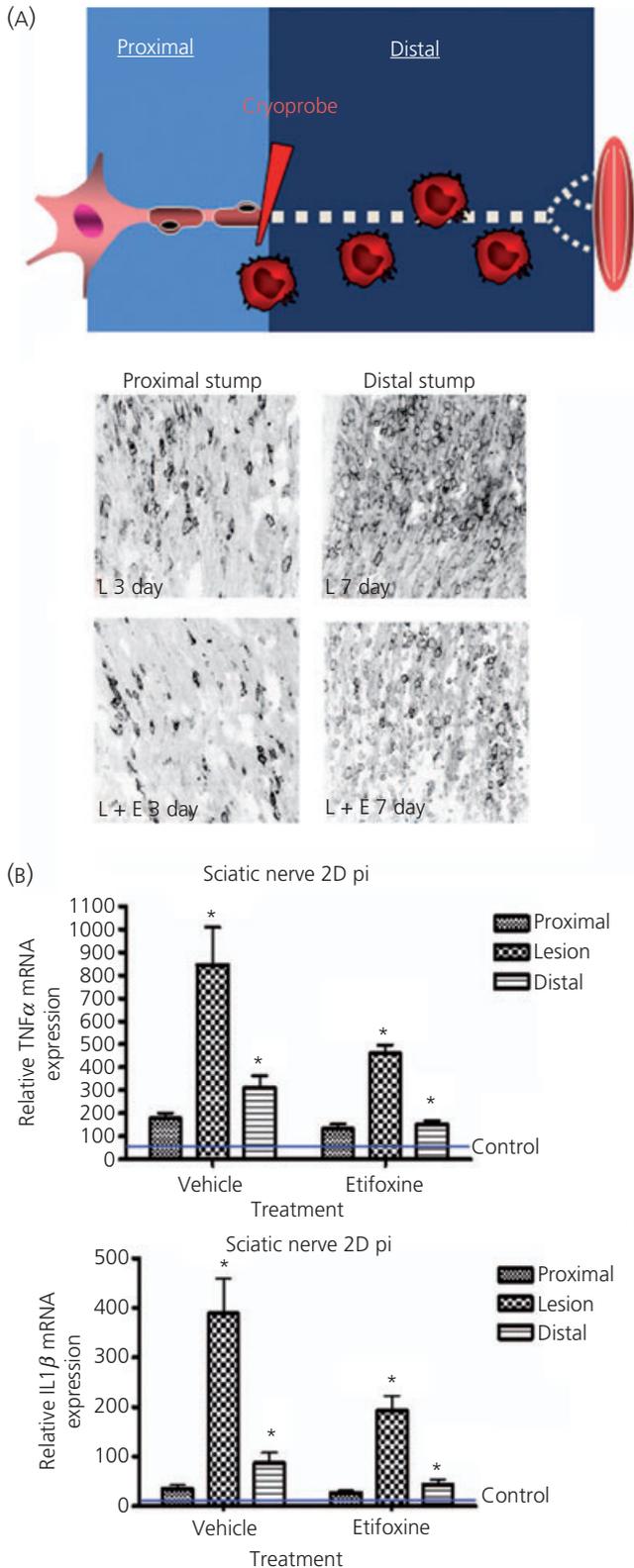
Fig. 6. Etifoxine modulated inflammatory responses in the sciatic nerve after cryolesion. (A) Immunostaining of the activated macrophages with OX42 antibody was performed on the proximal and distal stumps of the freeze-injured sciatic nerve (i.e. an injured axon surrounded by macrophages). Some macrophages are already resident in the nerve, proliferating and differentiating in response to specific stimuli; others are haematogenous monocytes, which are attracted into the injured nerve, where they differentiate. Chronic etifoxine treatment decreased the number of activated macrophages, becoming apparent at 3 days in the proximal end and at 7 days in the distal end. (B) The relative expression of the mRNAs of the inflammatory cytokines tumour necrosis factor-α and interleukin-1β was analysed by reverse transcriptase-polymerase chain reaction. The expression of these cytokines was increased in the whole nerve, as soon as 2 days post-injury, especially within the lesion site and in the distal stump. Etifoxine treatment clearly down-regulated their expression (mean ± SEM, Krustall–Wallis test, Dunn's post-test. *P < 0.05).

Etifoxine and functional recovery

The regeneration of sensory axons, evaluated by nerve pinch test, showed that sensitivity had progressed farther along the injured nerve in etifoxine- than in vehicle-treated rats, as soon as 3 days post-injury (at that time, STMN2 positive fibres can be seen in the distal end of the nerve of etifoxine-treated animals). At 15 days post-injury, etifoxine-treated rats had almost completely recovered their sensitivity, which was not the case for vehicle-treated animals (64).
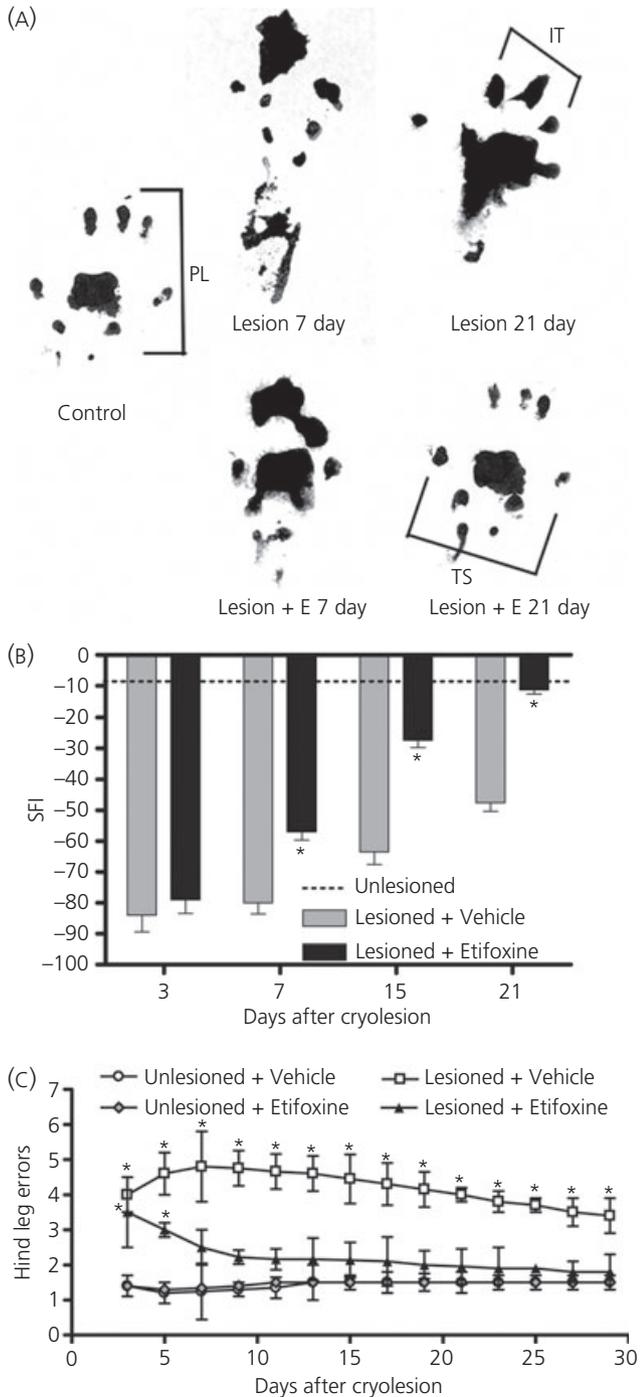
Functional testing was performed on seven rats per time and condition. The same rats were used all along the length of the testing, the rats being injected daily (i.p.), either with etifoxine (50 mg/kg) or with its vehicle. The recovery of motor function was assessed by the walking track test. Rats were trained before injury and treatment. After their injured hind leg had been dipped in ink, they had to walk along a corridor covered with paper on the floor, leaving on it their footprints. Measurements of several parameters on the footprints allow calculation of the 'Sciatic Nerve Index' (SFI) (70). This test revealed faster and better recovery in etifoxine-treated rats compared to vehicle-treated controls (Fig. 7A,B) (64). Importantly, 21 days post-injury, the SFI of etifoxine-treated rats, but not of vehicle-treated animals, was comparable to that of uninjured controls, suggesting that regeneration was complete.

To further monitor fine motor coordination, tests were performed using the Locotronic® device (IntelliBio, Seichamps, France). In this automated test, rats have to walk along a horizontal scale, and the number of hind-leg errors is registered. As early as 5 days after injury, a clear improvement of performances was observed in etifoxine-treated rats compared to the vehicle-treated animals. After 11 days, the response of etifoxine-treated rats had further improved, and became indistinguishable from that of control rats. Conversely, motor coordination remained poor in vehicle-treated animals as late as 29 days post-injury (Fig. 7C) (64).

Putative mechanisms of action of etifoxine involving TSPO

TSPO mediates multiple mitochondrial functions, some of which could play a role in the modulation of inflammatory responses and in axon regeneration. These include mitochondrial respiration, MPTP opening and mitochondria proliferation, which may be fundamental factors in energy metabolism of regenerating axons. The effect of TSPO on cholesterol redistribution could also impact on the synthesis of new cell membranes. Furthermore, its effects on the generation of reactive oxygen species could control both steroidogenesis and apoptosis.

Fig. 7. Etifoxine treatment accelerated and improved functional recovery after cryolesion. (A) Locomotion was assessed by the walking track test, and footprints were monitored at 3, 7, 15 and 21 days after cryolesion and in unlesioned control rats (PL, print length; IT, intermediary toe spread; TS, toe spread). (B) Calculation of the Sciatic Nerve Index (SFI) revealed a more rapid and qualitatively improved recovery of locomotion in etifoxine-treated animals. (C) The recovery of fine motor coordination was assessed in the Locotronic® device (IntelliBio). In response to etifoxine treatment, motor coordination rapidly improved and became indistinguishable from unlesioned animals after 11 days. Motor coordination remained poor in lesioned animals treated with vehicle. For the results of both the walking track test and Locotronic® device, two-way ANOVA with treatment and time after cryolesion as factors revealed both factors and their interaction to be significant at least at $P < 0.01$. *$P < 0.01$ compared to unlesioned rats and #$P < 0.01$ compared to vehicle-treated lesioned rats by Tukey's tests after the two-way ANOVA (n = 35; mean ± SEM).

Taken together, our results show that etifoxine, which is already clinically approved for the treatment of anxiety disorders, displays remarkable efficiency in promoting peripheral nerve regeneration and functional recovery. Furthermore, Aouad *et al.* (2009) have demonstrated that etifoxine reduces mechanical and thermal pain symptoms induced by the chemotherapeutic agent vincristine in rats. In animal models of anxiety (62) and pain (71), it was shown that the effect of etifoxine was, at least in part, a result of the stimulation of allopregnanolone synthesis, which modulates GABAergic synaptic transmission (59). Indeed, it was shown that a single i.p. injection of etifoxine strongly enhanced (two- to four-fold) the plasma and brain levels of steroids, especially of progesterone and its metabolites (62). This was also the case in the sciatic nerves of control animals, although pregnenolone and progesterone levels in the sciatic nerves of freeze-injured, etifoxine-injected rats were still higher (C. Girard, personal communication). Progesterone has been shown to reduce myelin abnormalities in aged rats and in experimental models of peripheral neuropathies (5–7). Moreover, pregnenolone and progesterone were shown to enhance neuritic growth in PC12 cells (72) and the myelination of axons (4). Hence, the stimulation of steroid synthesis by etifoxine, via TSPO, could be part of the events resulting in accelerated regeneration and improved functional recovery after injury. Interestingly, another ligand of TSPO, XBD173, also displays anxiolytic properties in rat and human (73). Its efficacy on neurosteroid biosynthesis and on nerve regeneration will be tested in preclinical models of peripheral nerve injury, and compared with that of etifoxine therapy.

Conclusions and perspectives

We showed that a ligand of TSPO, etifoxine, acting at least in part via the stimulation of progestagen biosynthesis, displays marked regenerative and anti-inflammatory properties in experimental models of traumatic peripheral nerve injury (64). Moreover, the use of etifoxine revealed anti-hyperalgesic properties in a preclinical model of toxic neuropathy (71). This molecule is of particular interest because it is already successfully used in clinical practice for another indication: the treatment of patients suffering from adjustment disorders with anxiety, with a limited administration period of 6 months (60,61). Because etifoxine appears as a molecule with regenerative, anti-inflammatory and likely anti-hyperalgesic properties, vasculitis and polyradiculoneuropathies could be good candidate neuropathies for etifoxine treatment. Because macrophage invasion and axonal injury are observed in these pathologies, etifoxine can be expected to have a beneficial influence on axon regeneration, inflammation and possibly pain.

Acknowledgements

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