Published in final edited form as:

Exp Neurol. 2016 September ; 283(Pt A): 224–234. doi:10.1016/j.expneurol.2016.06.019.

Peripheral and central neuronal ATF3 precedes CD4+ T-cell infiltration in EAE

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Abstract

Experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis produced by immunization with myelin oligodendrocyte glycoprotein (MOG) and adjuvants, results from profound T-cell mediated CNS demyelination. EAE is characterized by progressive, ascending motor dysfunction and symptoms of ongoing pain and hypersensitivity, in some cases preceding or concomitant with the motor deficits. In this regard, the EAE model mimics major features of multiple sclerosis, where a central neuropathic pain state is common. Although the latter condition is presumed to arise from a CNS loss of inhibitory controls secondary to the demyelination, dysfunction of sensory neurons may also contribute. Based on our previous studies that demonstrated the utility of monitoring expression of activating transcription factor 3 (ATF3), a sensitive marker of injured sensory neurons, here we followed both ATF3 and CD4+ T cells invasion of sensory ganglia (as well as the CNS) at different stages of the EAE model. We found that ATF3 is induced in peripheral sensory ganglia and brainstem well before the appearance of motor deficits. Unexpectedly, the ATF3 induction always preceded T cell infiltration, typically in adjacent, but non-overlapping regions. Surprisingly, control administration of the pertussis toxin and/or Complete Freund's adjuvants, without MOG, induced ATF3 in sensory neurons. In contrast, T cell infiltration only occurred with MOG. Taken together, our results suggest that the clinical manifestations in the EAE result not only from central demyelination but also from neuronal stress and subsequent pathophysiology of sensory neurons.

Keywords

Experimental allergic encephalomyelitis; Demyelination; ATF3; Dorsal root ganglion; Spinal cord; Myelin oligodendrocyte glycoprotein; Pertussis toxin

1. Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system that manifests with significant neurological deficits, including profound motor dysfunction and a central neuropathic pain state that is very difficult to manage (Noseworthy et al., 2000).

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Although the pathophysiological basis of the neurological deficits undoubtedly arises from patchy demyelination in the CNS, patients with MS also have significant inflammation and oligodendrocyte degeneration (Ellwardt and Zipp, 2014; Trapp et al., 1998). Indeed, frank neuronal dysfunction secondary to persistent inflammation may be the primary cause of non-relapsing, irreversible neurological disability (O'Connor et al., 2008).

Experimental autoimmune encephalomyelitis (EAE), the most widely used model to study pathological events occurring in MS, is a primarily T-cell-mediated disorder induced in rodents by immunization with myelin oligodendrocyte glycoprotein peptide (35–55; MOG). The major features of the MOG-EAE model are an ascending paralysis that begins in the lower limbs and a relatively predictable disease progression. Disease severity is scored on a 0 to 5 scale, with the first symptoms appearing around days 10 to 12 post MOG inoculation. The neurological manifestations occur in concert with characteristic neuropathological features, notably infiltration of CD4+ T-cells. Of particular interest to the present study is that pain hypersensitivity is common in several EAE mouse models and depending on dose of MOG and adjuvant can occur before or concomitant with motor dysfunction (Khan and Smith, 2014; Lu et al., 2012; O'Connor et al., 2008; Olechowski et al., 2009; Thibault et al., 2011; Tian et al., 2013).

Unquestionably, the profound degeneration of spinal cord white matter is the major contributor to the motor abnormalities. On the other hand, there is much more limited understanding of the mechanisms responsible for the sensory abnormalities, ongoing pain and reduced pain thresholds observed in both MS patients and in the EAE mouse models, particularly those that occur at very early stages (Khan and Smith, 2014; O'Connor et al., 2008; Olechowski et al., 2009). Loss of CNS inhibitory controls is generally considered critical to producing this central neuropathic pain condition. However, molecular changes in primary afferents, including induction of activating transcription factor 3 (ATF3), a marker of cellular stress (Hai et al., 1999) are also associated with neuropathic pain (Ogawa et al., 2014). Interestingly, previous studies reported upregulation of TNF α in the DRG of EAE rats (Begum et al., 2013) and Thorburn and colleagues reported that there is T-cell invasion of sensory ganglia as well as increased expression of glial fibrillary acidic protein in surrounding satellite cells (Thorburn et al., 2016). The authors of these studies suggested a possible contribution of these changes to the pain phenotype.

In the present report, we induced MOG-EAE in mice and monitored CNS and sensory (dorsal root and trigeminal) ganglia expression of ATF3 and of the CD4 receptor, a marker of a subset of immune T-cells (Romagnani, 1991). We report that there is significant induction of ATF3 and infiltration of CD4+ T-cells in peripheral sensory ganglia and throughout the brainstem, well before the appearance of motor deficits. Interestingly, the ATF3 induction always occurred prior to T-cell infiltration. Unexpectedly, we found that the traditional adjuvant vehicle used to produce EAE induced extensive ATF3 expression in sensory ganglia, even in the absence of MOG.

2. Materials and methods

2.1. Mice and induction of MOG-EAE

All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at the University of California San Francisco. For these studies, we used 10-week old, C57BL/6 female mice. EAE was induced following the protocol supplied by Hooke Laboratory (Hooke Kit™ MOG35-55/CFA Emulsion PTX; #EK-2110). Control females received the same dose of Pertussis toxin (PTX), Complete Freund's Adjuvant (CFA) or a PTX/CFA combination, but no MOG peptide. The mice were weighed, examined and scored daily for disease. Neurological deficits were graded on a 5 point scale (0 = No clinical symptoms; 1.0 = Tail paralysis; 2.0 = Unsteady gait; 3.0 = Complete hindlimb paralysis; 4.0 = Complete hindlimb and partial forelimb paralysis; 5.0 = Moribund). At least 3 mice per group were killed at the following stages: 5, 7 and 9 days post-MOG injection (for pre-onset analyses), one day before onset (identified by loss of 1 g), at onset (score 0–1), during disease progression (score 2) and at peak EAE (score 4). Three control females were killed at 5 and 3 at 15 days after adjuvant/vehicle injection.

2.2. Antibodies and immunohistochemistry

Animals were anesthetized with Avertin (250 mg/kg; 2, 2, 2-Tribromoethanol, Sigma) and then perfused transcardially with 10 ml phosphate buffer (PB: 0.1 M, pH 7.4) followed by 10 ml of 10% formalin (3.7% formaldehyde, Fisher Scientific) in PB at room temperature (RT). Tissues were dissected out and cryoprotected in 30% sucrose PB over-night at 4°C (with no post-fixation). Fourteen µm DRG/TG, 25 µm spinal cord and 35 µm brainstem cryostat sections were preincubated for 30 min at RT in PB saline (PBS-0.9%) containing 0.3% Triton X-100, 10% bovine serum albumin (BSA) and 10% serum (Normal goat or normal horse) and then immunostained overnight at RT in PBS containing 0.3% Triton X-100, 1% BSA, 1% serum and the primary antibodies. After 3 washes in PBS, sections were incubated for 1 h with secondary antibodies (Alexa 488- or 594-conjugated IgG; 1:1000, Invitrogen), washed in PBS, mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL) and coverslipped. We used the following antibodies: rabbit anti-ATF3 (1:2000, Santa Cruz), rat anti-CD4 (1:1000, eBiosciences), mouse anti-NF200 (1:20,000; Sigma), guinea-pig anti-TRPV1 (1:7000, gift from D. Julius, UCSF), mouse anti-Oligo2 (1:500, Millipore), mouse anti-PO.7 (1:1000, gift from J. Chan, UCSF), rabbit anti-neuropeptide Y (1:2000, gift from J. Allen), goat anti-CSF1 (1:1000, R&D Systems), rabbit anti-serotonin (1:500, Protos Biotech Corp.) and mouse anti-tyrosine hydroxylase (1:5000, RBI). Images were captured with a Zeiss LSM confocal microscope and ZEISS software (Zen Lite), then processed in ImageJ.

3. Results

In the present study, we monitored mice for ~20 days after MOG administration, so as to include both asymptomatic and symptomatic periods of the disease. Although we observed differences in the time course and severity of the signs, in most animals the first onset signs (loss of 1 g and tail paralysis) occurred between days 10–12; mice reached a maximal disease score of 4–5, ~7 days post-onset (Fig. 1A).

3.1. ATF3 and CD4: biomarkers of EAE progression?

In part our interest in the asymptomatic period was motivated by reports that some sensory abnormalities occur in MOG-treated mice at early stages of the disease, i.e. preceding the appearance of motor deficits (Chin et al., 2009; Olechowski et al., 2009; Thibault et al., 2011; Tian et al., 2013). These sensory disturbances include mechanical hypersensitivity of the hindlimbs, a potential indicator of ongoing pain. Given that EAE results from CNS demyelination, these sensory disturbances could be harbingers of the central pains that are common in patients with multiple sclerosis (Khan and Smith, 2014; O'Connor et al., 2008). On the other hand, because of the earlier manifestation of the disease in the lumbar spinal cord, we considered the possibility that topographically localized changes to sensory neurons also occur. For this reason, in addition to assessing CNS alterations during the asymptomatic period, we monitored induction of ATF3, a sensitive cellular marker of nerve injury. To determine whether there is a temporal association between neuronal pathology and immune cell infiltration, we also immunostained for infiltration of CD4⁺ T-cells, a cardinal feature of EAE.

Fig. 1B–I illustrates that at peak EAE (score 4–5), ATF3 is, in fact, substantially induced in dorsal root (DRG; Fig. 1D) and trigeminal ganglia (Fig. 1C), as well as in neurons of trigeminal mesencephalic nucleus (MesV), which is the CNS location of trigeminal proprioceptive primary afferent cell bodies that innervate muscles of mastication (Fig. 1B). Triple labeling with antibodies against NF200 (a marker of sensory neurons with myelinated axons) and of TRPV1 (a marker of subpopulation of unmyelinated C-fibers) showed that a heterogeneous population of neurons expressed ATF3 (Fig. 1E). At peak EAE, we also detected CD4⁺ T-cells in sensory ganglia (Fig. 1B, C).

Having observed extensive ATF3 induction and CD4⁺ T-cell infiltration, we next examined their time course. We first detected ATF3-immunoreactive sensory neurons at 5 days (the earliest time investigated) after immunization, i.e., well before disease onset (Fig. 1F, G). In contrast, and in agreement with Thorburn et al. (Thorburn et al., 2016) we did not detect CD4⁺ T-cells in sensory ganglia until disease onset (~10–11 days; Fig. 1H and 2). The number of ATF3⁺ and CD4⁺ T-cells increased over time, paralleling the progression of the disease (1G, H). At late stages of the disease (Score 4), we also detected ATF3⁺ nuclei in an undefined population of cells distributed along the nerve fibers that course within the ganglia (data not shown). The identity of these ATF3⁺ profiles remains to be determined. Some may correspond to satellite glial cells that surround DRG cell bodies and that are activated during EAE (Warwick et al., 2014). This pattern of ATF3⁺ labeling is somewhat reminiscent of the ATF3 expression seen in Schwann cells located at the distal stump of injured sciatic nerve axons (Hunt et al., 2004). It is, thus, possible that at late stages of EAE, non-neuronal cells within the DRG also contribute to the pathophysiology and symptoms of the disease.

The fact that the ATF3 induction in sensory ganglia preceded CD4⁺ immune cell infiltration was unexpected and suggests that the sensory neurons' response in the EAE model (namely ATF3 induction) is not secondary to T-cell infiltration. In fact, and surprisingly, we recorded ATF3⁺ sensory neurons in mice that only received adjuvant (CFA, PTX or the combination of CFA + PTX), i.e., without MOG (Fig. 3A). The magnitude of ATF3 induction at 5 days post-injection was comparable for PTX (11.3 ± 3.5 ATF3⁺ neurons/section, $n=3$), CFA (10.0 ± 3.0

ATF3+ neurons/section, $n = 3$) or for the combined injection (11.4 ± 4.3 ATF3+ neurons/section, $n = 3$). The number of ATF3+ DRG neurons in control groups was also not significantly different from that observed 5 days after injection of MOG with the adjuvants (11.0 ± 1.6 ATF3+ neurons/section, $n = 3$). However, at 15 days a combined adjuvant injection, without MOG, provoked a significant increase of ATF3+ DRG neurons (32.3 ± 12.4 ATF3+ neurons/section, $n = 3$; Fig. 3J) compared to either CFA (11.1 ± 2.6 ATF3+ neurons/section, $n = 3$) or PTX alone (10.3 ± 2.0 ATF3+ neurons/section, $n = 3$). Conceivably, the increase produced by the combination reflects a delayed additive effect of the adjuvants. In contrast, we never detected CD4+ T-cells in sensory ganglia from the control mice (Fig. 3C, D, G, H, K, L). We conclude that the induction of ATF3 in sensory ganglia is MOG-independent. In contrast, the CD4+ T-cell infiltration requires MOG administration.

Interestingly, the CD4+ T-cells in sensory ganglia of the experimental, MOG-treated animals, observed at early stages were concentrated around other cells that immunostained for oligo2, a marker of oligodendrocytes (Fig. 2G, H). This finding suggests that the invasion of peripheral ganglia by T-cells occurs via the central branch (CNS) of the sensory afferents, namely those myelinated by oligodendrocytes rather than Schwann cells. Taken together, our results suggest that sensory neurons respond to and likely are “activated” (ATF3+) soon after administration of the CFA/PTX adjuvant and that this is followed by a central to peripheral movement of CD4+ T-cells, along the central branch of the sensory neurons. Conceivably, stressed sensory neurons that induce ATF3 release a signal that attracts CD4+ T-cells to the peripheral ganglia, but only when MOG is present (presumably due to blood brain barrier disruption). To what extent the ATF3 induction, coupled with CD4+ T-cell infiltration into sensory ganglia, contributes to the sensory abnormalities that characterize the early stages of EAE remains to be determined.

3.2. ATF3-induction in sensory neurons of EAE mice is nerve injury-independent

Although ATF3 induction in sensory ganglia is a reliable marker of nerve damage (Braz and Basbaum, 2010; Tsujino et al., 2000), ATF3 can be induced in neurons in response to a host of conditions, including ischemia, toxicity and seizures. In vitro, ATF3 has been detected in cells after exposure to cytokines, serum factors or adenoviral proteins (Hai et al., 1999). To determine whether the ATF3 induction in sensory neurons of EAE mice is secondary to nerve damage produced by the CFA/PTX vehicle, we monitored expression of two other peripheral nerve injury markers: neuropeptide Y (NPY), a peptide induced in a mixed population of primary afferent neurons following peripheral nerve injury (Noguchi et al., 1993; Zhang et al., 1993) and the cytokine, colony-stimulating factor 1 (CSF1), which we recently demonstrated is de novo up-regulated in axotomized sensory neurons (Guan et al., 2016). Fig. 4 illustrates, as expected, that a peripheral nerve injury induces ATF3 (red) in DRG neurons, most of which co-express CSF1 (green). In sharp contrast, we found no induction of CSF1 in EAE (score of 4) or control (CFA+PTX) animals, despite the large number of ATF3+ DRG neurons. Similarly, there was no induction of NPY (Fig. 4H, I). Taken together, these results demonstrate that the induction of ATF3 in sensory neurons, in both experimental and control mice, is nerve injury-independent and likely reflects a more generalized cellular stress response to the PTX/CFA adjuvant.

3.3. CD4 infiltration and ATF3 expression in the spinal cord of EAE mice

In contrast to the early ATF3 induction in peripheral ganglia (in MOG-treated mice), we rarely detected expression of ATF3 in the lumbar spinal cord prior to the appearance of clinical symptoms (Fig. 5). In general, the first signs of ATF3 induction occurred at pre-onset (when mice lose 1 g) and predominated in the ventral horn, in motoneurons and presumptive interneurons and in ventral white matter (lateral and ventral funiculi). This pattern persisted through the intermediate EAE stages. Only when EAE signs peaked (score 3–4) did we detect ATF3 immunoreactive neurons in the dorsal horn (data not shown). CD4+ T-cell infiltration into the lumbar spinal cord was also first detected around onset, which interestingly paralleled its detection in peripheral ganglia. And as for the sensory neurons, the number of CD4+ T-cells increased over time. Although we observed CD4+ T-cells in dorsal and ventral spinal cord, greater numbers were recorded in the ventral horn, in both white and gray matter (Fig. 5C). Substantially fewer ATF3+ and CD4+ T-cells were observed in more rostral segments of the spinal cord (data not shown). Most importantly, we never recorded ATF3+ or CD4+ T-cells in control mice, indicating that the spinal cord neuronal and white matter response in the EAE mice is MOG-dependent.

3.4. Motor and pre-cerebellar nuclei of the brainstem express ATF3 before EAE onset

In contrast to our observations in the spinal cord, we found that brainstem somatic and visceromotor neurons of MOG-treated mice expressed ATF3 well before onset of the disease. For example, we detected ATF3+ neurons in the VIIth (facial) nucleus as early as 5 days post immunization (Fig. 6A) and in the Xth (dorsal motor) and XIIth (hypoglossal) nuclei at 7 days post immunization (Fig. 7B). The presence of ATF3 expression in brainstem neurons prior to their appearance in neurons of the lumbosacral spinal cord was particularly surprising as the major, observable EAE symptoms (motor impairment), first appear in the tail and hindlimbs. Rostral disease progression only occurs at very late stages. Interestingly, and similar to the sensory ganglia, ATF3 expression in the brainstem preceded immune cell infiltration. In fact, we first detected CD4+ T-cells around onset of the disease, predominantly in white matter of the ventral medulla, notably in the pyramidal tract (Fig. 7E). The number of CD4+ T-cells increased with time, slowly invading gray and more dorsal white matter throughout the brainstem. As noted above, we never detected ATF3+ neurons or CD4+ T-cells in mice that only received the adjuvant (CFA and/or PTX). We conclude that different mechanisms underlie the ATF3 induction in CNS neurons and in primary afferent neurons.

In addition to the early induction of ATF3 in motor nuclei of the brainstem, we observed somewhat delayed, but nevertheless pronounced expression in many precerebellar nuclei, (Fig. 7D, E), including the inferior olive (IO) and lateral reticular nucleus (LRN) of the medulla (data not shown) and extensively throughout the pons. The first appearance was around onset, and the number increased with time. In these precerebellar loci the CD4+ T-cell infiltration occurred concurrently with the appearance of ATF3+ neurons. On the other hand, despite significant immune cell infiltration in the cerebellum (appearing once motor deficits were apparent, score of 1 and higher), we never detected cerebellar ATF3 expression (Fig. 7F). The T-cells in the cerebellum were concentrated in white matter, rarely penetrating folia of the cerebellar cortex.

3.5. ATF3 expression and CD4+ T-cell infiltration in other brainstem nuclei

In addition to the brainstem regions that have a clear association with motor function, and presumptive EAE-associated dysfunction, Fig. 7 shows that at peak EAE, many other areas in the brainstem contained ATF3+ neurons and significant invasion by CD4+ T-cells. Among these regions were the trigeminal nucleus caudalis (TNC, Fig. 7G), the area postrema (Fig. 7B, H), nucleus of the solitary tract (Fig. 7H) and the midline raphe nuclei (Fig. 8). In contrast to Thorburn et al. [13], who found extensive CD4+ T-cell invasion of all subregions of the trigeminal nucleus, we found very limited invasion outside of nucleus caudalis. Particularly striking was the extensive neuronal ATF3 expression and CD4+ T-cell infiltration in the ventral, but not dorsal cochlear nucleus (Fig. 7I). Interestingly, the ventral cochlear induction occurred at pre-onset. As we observed a progressive ventral to dorsal pattern of pathology in the brainstem manifestation of the disease, it appears that the lumbosacral spinal cord and brainstem share some common susceptibility, with dorsal structures being affected only when signs of disease are more profound.

3.6. Serotonergic and catecholaminergic systems of the brainstem are differentially affected in EAE

Given our interest in the ongoing pain and hypersensitivity that are often comorbid with the motor dysfunction in the EAE model, we turned our attention specifically to the brainstem monoaminergic systems, which are at the origin of major descending systems that regulate pain and motor processing networks of the spinal cord (Basbaum and Fields, 1984; Braz and Basbaum, 2008; Ossipov et al., 2014). As noted above we observed significant ATF3 expression in neurons of the medullary raphe. To determine the neurochemistry of these neurons, we performed double immunolabeling experiments with antibodies against ATF3 and serotonin (5HT), to label the serotonergic neurons, or tyrosine hydroxylase (TH), a marker of catecholaminergic neurons. Fig. 8A to C show that one day prior to onset, many of the ATF3+ raphe neurons contained for 5HT. Surprisingly, however, we found no ATF3 induction in TH-immunoreactive cells of the brainstem (Fig. 8D to F), despite the large number of ATF3+ cells found in the vicinity of catecholaminergic neurons of the A1 and A5 cell groups. Interestingly, most 5HT+ neurons that co-expressed ATF3 were located in the caudal medulla (in raphe pallidus, obscurus and caudal aspect of the lateral paragigantocellularis), but rarely in the nucleus raphe magnus and none in the dorsal raphe. As the most caudal raphe nuclei regulate spinal cord motoneurons, more so than do the rostral nuclei (e.g. raphe magnus), which target dorsal horn pain transmission circuitry, our results suggest that the motor dysfunction in the EAE model may, in part, arise from alterations in these descending serotonergic systems. Furthermore, it does not appear that the pain-associated deficits in the EAE model reflect dysfunction of raphe magnus-derived descending serotonergic or noradrenergic antinociceptive pathways.

4. Discussion

Here we show that the stress-associated activation transcription factor, ATF3, in addition to CD4+ T-cell infiltration, is a reliable CNS biomarker of EAE progression. To our knowledge, ours is the first description of extensive ATF3 induction in brainstem neurons. Most importantly, its induction occurs soon after MOG injection, always preceding T-cell

infiltration. In peripheral sensory ganglia, ATF3 induction also preceded T-cell infiltration, but here the ATF3 induction was MOG-independent.

4.1. Peripheral nerve changes in MOG-EAE

Molecular changes have been reported in peripheral sensory ganglia in EAE (Begum et al., 2013; Warwick et al., 2014; Zhu et al., 2013). For example, in myelin basic protein-induced EAE, TNF α and fractalkine expression in the DRG correlates with motor symptom severity. In this latter model, the onset of neuropathic pain symptoms coincided with increased DRG cytokine levels. In contrast, studies in animals with MOG-induced EAE, following the same protocol used in the present study, observed a much earlier onset of mechanical allodynia (Olechowski et al., 2009). In fact, this index of pain hypersensitivity occurred within 5 days of the MOG injection, well before the appearance of motor deficits and strikingly coincident in time with our finding of neuronal ATF3 induction. To what extent the very early molecular changes in sensory and CNS neurons contribute to the sensory abnormalities remains to be determined. Our findings, however, may be relevant to an earlier report that demonstrated profound axonal pathology in postmortem tissue from MS patients in areas with active lesion (Trapp et al., 1998). These authors concluded that the pathophysiology of MS should take into account a likely contribution of axonal pathology, in addition to demyelination, a conclusion entirely consistent with our new findings.

Here, we show that sensory neurons are, in fact, “activated” (manifest as ATF3 induction) very soon after inoculation. Most surprisingly, the early response in DRG and trigeminal ganglion neurons did not require MOG. In fact, injection of CFA, PTX or the CFA/PTX combination induced ATF3 expression in peripheral ganglia, but these mice never exhibited objective signs of sensory or motor impairment. To our knowledge, this is the first report of ATF3 induction in response to a systemic injection of PTX. The induction occurred in all sensory ganglia, and importantly, included cell bodies of MesV, which are the only sensory ganglion cells located within the CNS. As we did not observe ATF3 induction in any other CNS location (without MOG), unless there is some unique receptor on sensory neuron cell bodies to which PTX binds, we conclude that the PTX acts via peripheral axons of the sensory neurons. It is not clear, however, whether adjuvant-induced ATF3 contributes to the sensory abnormalities observed when MOG is included, i.e., when EAE is induced.

The ATF3 induction in response to subcutaneous CFA was particularly unexpected. Even hindpaw injections of much higher doses of CFA do not induce ATF3 in sensory neurons of lumbar DRG (Allchorne et al., 2012; Braz and Basbaum, 2010; Tsujino et al., 2000). In fact, the lower dose systemic injections produced a widespread response in DRG and trigeminal ganglion neurons, topographically unrelated to the injection sites. As CFA also induced ATF3 expression in MesV cell bodies, we presume that its mode of action is also via the peripheral branch of the sensory neurons. Although there are reports of ATF3-induction in some CFA-induced osteoarthritis models, this latter response is likely secondary to peripheral nerve damage (Ivanavicius et al., 2007; Nascimento et al., 2014). Importantly, we found no evidence that damage to the peripheral nerve was a prerequisite. Specifically, DRG induction of ATF3 in EAE mice, or in response to the PTX or CFA, was not associated with the expression of CSF1 or NPY, two proteins induced in DRG neurons following peripheral

nerve injury (Guan et al., 2016; Noguchi et al., 1993; Zhang et al., 1993). We conclude that ATF3 induction in sensory neurons in the EAE model reflects a more generalized cellular stress state produced by systemic injection of the adjuvants (CFA/PTX). Whether this phenomenon is a common feature among various EAE models remains to be determined but our results suggest that adjuvants may contribute more to the overall EAE phenotype than a mere enhancement of the pathophysiology provoked by the myelin immunogen.

4.2. Infiltration of CD4+ T-cells into sensory ganglia

In contrast to the very early adjuvant-only induced ATF3 in sensory ganglia, CD4+ T-cell infiltration of DRG neurons required MOG and occurred around pre-onset. Based on the distribution of the T-cells, which were initially concentrated proximal to the root entry zone, we conclude that the T-cells infiltrate sensory ganglia via their central branch. It follows that the T-cells must gain access to the CNS before accessing the PNS (Barkauskas et al., 2015; Reboldi et al., 2009). Based on localized demyelination at this transition zone, Thorburn et al. came to a comparable conclusion (Thorburn et al., 2016). Our results raise the possibility that MOG-associated disruption of the brain blood barrier in EAE mice (Engelhardt and Ransohoff, 2005; Wang et al., 2013) facilitates reactive CD4+ T-cell spread to peripheral sensory neurons. Conceivably the efferent migration of these T-cells occurs in response to a signal released by ATF3-expressing (PTX/CFA-derived) sensory neurons that target the spinal cord and brainstem. A comparable T-cell invasion of the trigeminal ganglion may contribute to the very high prevalence of trigeminal neuralgia in MS patients. Interestingly, increased permeability of the blood brain barrier (BBB) has been associated with PTX administration in EAE mice (Linthicum et al., 1982) suggesting that in this model PTX both “activates” peripheral neurons and contributes to the early (prior to the development of clinical symptoms) CNS infiltration of T-cells and macrophages (Murphy et al., 2010). On the other hand, other studies reported that PTX has no direct effect on BBB permeability (Blankenhorn et al., 2000; Muller et al., 2005). Rather, PTX increases the incidence and severity of EAE by stimulating inflammatory responses and increasing Th1-associated cytokine production and signaling (Andreassen et al., 2009; Marcondes et al., 2014).

4.3. CNS neurons induce ATF3 in response to MOG

In contrast to peripheral ganglia, induction of ATF3 in brainstem neurons required MOG. But once again we found that the neuronal response to the immunogen precedes T-cell invasion and paralysis. Interestingly, the ATF3 neuronal and CD4+ T-cell infiltration often occurred in neighboring, but unrelated structures. For example, we observed profound ATF3 induction in neurons of the inferior olive, which target the cerebellum, but the T-cell infiltration occurred in the ventrally located pyramids (i.e. around axons of the corticospinal tract). In other instances, notably in several precerebellar nuclei of the brainstem (pontine nuclei and lateral reticular nucleus), we observed extensive ATF3 neuronal expression that was followed days later by T-cell infiltration of the same regions. And curiously, the T-cell infiltration that reliably occurred in the cerebellar cortex was never preceded by neuronal induction of ATF3.

These findings indicate that the response to the MOG is far more complex than previously assumed. The neuronal cell body response is manifest early (by ATF3 induction) and this is

followed by CD4+ T-cell infiltration, which presumably triggers demyelination. In other words, the EAE model is not a disease limited to demyelination and consequent axonal dysfunction, but may reflect temporally distinct neuronal and demyelinating pathologies. The difficulty in attributing particular changes to the disease manifestation (namely motor dysfunction) is underscored by the fact that the earliest motoneuron changes that we observed were in the brainstem, in the VII and XIIth motor nucleus, and only later in lumbar spinal cord motoneurons, which target the affected muscle groups of the hindlimbs.

Also surprising was the differential responsiveness of descending monoaminergic systems. Specifically, we found that serotonergic neurons of the medullary raphe nuclei expressed ATF3 in response to MOG, but the catecholaminergic neurons did not. The majority of the 5HT+/ATF3+ neurons were located in the caudal medulla, including the raphe pallidus and obscurus, which are at the origin of descending axons that regulate ventral horn motoneurons and sympathetic preganglionic neurons. Thus, the motor impairment in the EAE model may result not only from demyelination of major descending motor control pathways but also from more subtle dysfunctional serotonergic controls. Whether the report that sympathectomy can exacerbate EAE (Pal et al., 1999) is at all related to dysregulated sympathetic preganglionic neurons remains to be determined.

Interestingly, neurons of the nucleus raphe magnus, which regulate dorsal horn pain transmission circuits, were ATF3-negative, suggesting that the pain manifestations that often occur in the EAE model are not secondary to loss of descending inhibitory serotonergic controls. Finally, and completely unexpected, was the very early and profound ATF3 induction in presumptive auditory neurons of the ventral, but not dorsal, cochlear nucleus of the brainstem. As both ventral and dorsal cochlear nuclei receive primary afferent (VIIIth nerve) inputs, it is more likely that selective ATF3 induction in the ventral cochlear neurons reflects changes centrally rather than an induction from alterations in the VIIIth nerve itself. Whether our findings relate to the reports of retrocochlear hearing loss in several mouse models of autoimmune diseases (including EAE) (Matsuoka et al., 1999; Trune et al., 1999; Watanabe et al., 1996) remains to be determined.

5. Conclusions

The molecular consequences of administering MOG and its PTX/CFA adjuvants in the production of EAE are clearly far more complex than previously reported. Indeed the ability of PTX and CFA alone to induce ATF3 in peripheral sensory neurons may contribute to the development of the sensory abnormalities that precede or are concomitant with the motor dysfunction that characterizes EAE in mice. On the other hand, as CD4+ T-cell infiltration into the CNS only occurred when MOG is present and always followed ATF3 induction, we suggest that their trafficking may be influenced by a signal released by “stressed” PNS and CNS neurons. It will be of interest to determine whether ATF3 induction is a common feature of all EAE models and whether other adjuvants also have molecular consequences independent of and that may synergize with those provoked by the myelin immunogens.

Acknowledgments

These studies were supported by the NIH NS14627 (AIB), NS083688 (RD) and the Wellcome Trust (102645) (United Kingdom; AIB).

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Fig. 1.

ATF3 induction precedes CD4⁺ T-cell infiltration in sensory ganglia. (A) Distinct periods characterize the EAE model in mice: an asymptomatic phase (up to 10 days post-MOG injection), where there is no overt motor deficit; pre-onset, where there is a loss of 1 g, followed by onset of the symptomatic phase, where there is frank weakness and/or paralysis. The paralysis begins in the tail and hindlimbs and progresses to the forelimbs, with concurrent weight loss. The latter features allow for consistent clinical scoring of the animals on a 0 to 5 scale (see Materials and methods). (B–D) When motor deficits are severe (score of 3 or higher) sensory neurons of the trigeminal mesencephalic nucleus (B), the trigeminal ganglion (TG, C) and dorsal root ganglia (DRG, D) express ATF3 (red) and are invaded by large numbers of CD4-expressing T-cells (green). (E) Induction of ATF3 (red) can be detected in a mixed population of sensory neurons, including neurons with myelinated axons (NF200⁺, blue) and in others that express TRPV1 (green). (F–I) ATF3 induction (red) was detected as early as 5 days (A, F) post-MOG injection. In contrast, we observed CD4⁺ T-cell infiltration only at pre-onset (A, H) and at later stages (Score 1, I). Insets show higher magnifications of ATF3⁺ DRG neurons and CD4⁺ T-cells. Scale bar

equals 75 μm in B–D, 100 μm in E and 200 μm in F–I. 4 V: 4th ventricle, PB: parabrachial nucleus, LC: Locus coeruleus, Me5: mesencephalic trigeminal nucleus.

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Fig. 2.

Time course of CD4+ T-cell infiltration into sensory (trigeminal) ganglia. (A–F) Mice were killed at 5, 7 and 9 days post MOG injection, when they lose 1 g (pre-onset) and at different days post disease onset (Score 2 and Score 4). CD4+ T-cells (green) were first detected at pre-onset (around ~10–12 days post-MOG). The CD4+ T-cells appeared first along the nerve fibers of the trigeminal root (central branch in schematic in A). E, F: Only at later stages (Scores >2) did the T-cells surround the sensory neuron cell bodies. (G, H) Oligo2+, presumptive oligodendrocytes (red) concentrated at the central most (CNS) branch of the trigeminal root, where they normally myelinate CNS axons (G). Juxtaposition of CD4+ T-cells (green in H) and these oligo2+ cells suggests that the T-cells infiltrate sensory ganglia by migrating from the CNS via the trigeminal root. Scale bar equals 100 μ m.

Fig. 3.

Adjuvant administration (CFA, PTX or CFA/PTX) induces ATF3 expression with no subsequent CD4⁺ T-cell infiltration in sensory ganglia. Injection of CFA (A–D), PTX (E–H) or the combination (I–L), induces similar levels of ATF3 (red) expression within 5 days (A, E and I). After combined injection of CFA/PTX (J), the number of ATF3⁺neurons increased dramatically 15 days post-injection, but did not increase substantially with injection of CFA or PTX alone (B, F). Injection of these adjuvants never induced infiltration of CD4⁺ (green) T-cells into sensory ganglia. Scale bar equals 100 μ m.

Fig. 4.

ATF3 induction in sensory neurons of EAE mice is nerve injury-independent. (A–F) In contrast to sciatic nerve transection (SN cut; A–C), which induces strong expression of CSF1 (green) in ATF3+ (red) sensory neurons, in EAE mice (D–F) ATF3 induction is not associated with CSF1. Similarly, whereas a sciatic nerve transection induces strong expression of neuropeptide Y (NPY; green) in axotomized DRG neurons (G), in EAE mice, there is ATF3 induction without concurrent NPY expression (H–I), regardless of disease score. Scale bar equals 100 μ m.

Fig. 5.

ATF3 induction and CD4+ T-cell infiltration in the spinal cord of EAE mice. (A–D) In contrast to sensory ganglia, expression of ATF3 (red) and CD4+ T-cell (green) infiltration occurred in the spinal cord of EAE mice only at the first appearance of motor deficits (Score 1). As the disease progressed there was a particularly large increase of ATF3 expression and CD4+ T-cells in ventral white matter (D). Inset shows a higher magnification of labeling in the white matter. Scale bar equals 100 μ m.

Fig. 6.

ATF3 induction precedes CD4+ T-cell infiltration in brainstem motor nuclei. (A–E) As for sensory ganglia, we detected ATF3 induction (red) in brainstem motor nuclei (VIIth nucleus; blue box in schematic) as early as 5 days post-MOG (A), i.e. prior to disease onset. ATF3 induction always preceded infiltration of CD4+ (green) T-cells. The latter cells were only detected when clinical signs first appeared (Score 1). Both ATF3 and T-cell infiltration increased greatly as disease progressed (E). Scale bar equals 100 μm. VII: Facial motor nucleus.

Fig. 7.

ATF3 induction and CD4+ T-cell infiltration in brainstem nuclei. In EAE animals at peak disease stages (Scores 3–4) we recorded large numbers of ATF3+ (red) neurons and invasion of CD4+ (green) T-cells in most cranial motor nuclei (A, B), in presumptive spinal motoneurons (C), in precerebellar neurons of the brainstem, including pontine nuclei, reticular tegmental nucleus (RtTg; D) and inferior olive (IO; E). Note concentration of ATF3+ neurons in the inferior olive and T-cells in the adjacent white matter of the pyramidal tract (pyr; E), with minimal overlap of the two markers. Contrasting the precerebellar nuclei of the brainstem, we never detected ATF3 expression in the cerebellum (F), but cerebellar invasion of CD4+ T-cells occurred when motor deficits appeared (Score 1) and only within

white matter ventral to the cerebellar cortex (F). Among others areas containing ATF3+ cells and CD4+ T-cell infiltration were the trigeminal nucleus caudalis (G), the intermediate reticular nucleus (IRt; G), the area postrema (AP; H) and surprisingly, the ventral (vCoch; I), but not dorsal (dCoch; I) cochlear nucleus. Note that more rostral subnuclei of the spinal trigeminal nucleus (Sp5; A, I) contained neither ATF3+ cells nor T-cells. Scale bar equals 100 μ m. DH: dorsal horn; cc: central canal; NTS: Nucleus of the solitary tract; RPa: Raphe pallidus; VH: ventral horn; VII: facial motor nucleus; X: dorsal motor nucleus; XII: hypoglossal nucleus.

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Fig. 8. Differential effects of EAE on brainstem monoaminergic systems. (A–C) Many serotonin (5HT, green)-immunoreactive neurons of the midline caudal raphe nuclei (obscurus-ROb and pallidus-RPa) and the neighboring lateral paragigantocellularis nucleus (LPGi) co-express ATF3 (red) in EAE mice. Arrows in B and C point to double-labeled neurons. (D–F) In contrast, ATF3+ neurons never double-labeled for tyrosine hydroxylase (TH, green). Scale bar equals 100 μ m in A, D–F and 200 μ m in B, C. A1: A1 adrenergic cell group; VIIth: Facial motor nucleus.