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# Genetic deletion of c-Jun Kinase-3 (JNK3) modestly increases disease severity in a mouse model of Multiple Sclerosis

**Mercedes Priego**[1,§], **Lorena Noriega**[1,§], **Sergey Kalinin**[2,§], **Lisa M Hoffman**[1], **Douglas L Feinstein**[2,3,*], **Gerardo Morfini**[1,*]

[1]Department of Anatomy and Cell Biology, University of Illinois, Chicago, IL 60612

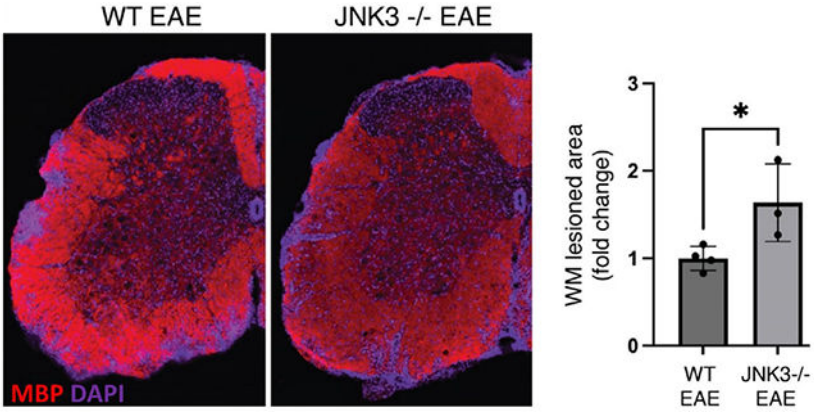
[2]Department of Research, Jesse Brown VA Medical Center, Chicago, IL 60612

[3]Department of Anesthesiology, University of Illinois, Chicago, IL 60612

## Abstract

The c-Jun amino terminal kinases (JNKs) regulate transcription, and studies suggest they contribute to neuropathology in the EAE model of MS. To examine the role of the JNK3 isoform, we compared EAE in JNK3 null mice to wild type (WT) littermates. Although disease severity was similar in female mice, in male JNK3 null mice the day of onset and time to reach 100% incidence occurred sooner, and disease severity was increased. While glial activation in spinal cord was similar, white matter lesions were increased in JNK3 null mice. These results suggest JNK3 normally limits EAE disease in a sex-dependent manner.

## Graphical Abstract




[*]Correspondence: Dr. Gerardo Morfini, PhD, Dept of Anatomy and Cell Biology, University of Illinois at Chicago, 808 S Wood Street., Rm 578, MC 512, Chicago, IL, 60612, gmorfini@uic.edu; Dr. Douglas Feinstein, Department of Anesthesiology, University of Illinois at Chicago, 835 South Wolcott Street, MC514, Room E720, Chicago, IL, 60612, dlfeins@uic.edu.
[§]These authors contributed equally.

**Keywords**

EAE; Multiple Sclerosis; JNK3; spinal cord; astrocytes; microglia

## 1. Introduction

The c-jun N terminal kinases (JNKs) are a family of serine/threonine mitogen activated stress kinases (MAPK) consisting of 3 isoforms: JNK1, JNK2, and JNK3, which are encoded by 3 different genes (*MAPK8*, *MAPK9*, and *MAPK10*, respectively). All JNKs have been shown to bind to, phosphorylate, and increase the activity of transcription factors, including c-Jun, ATF2 and ELK-1 in various cell types (Gupta et al. , 1996). JNK1 and JNK2 are ubiquitously expressed throughout the body. In contrast, JNK3 tissue expression is primarily limited to brain, although lower levels are also found in heart, testis, and pancreatic β-cells. In the CNS, immunostaining shows that JNK3 is expressed in neurons in several adult brain regions including the cerebral cortex, hippocampus, cerebellum and spinal cord (Martin et al. , 1996). Phenotypic analysis showed that mice featuring genetic JNK3 deletion (JNK3 null mice) have fewer neural progenitor cells in the hippocampus (Castro-Torres et al. , 2019), reduced neuritogenesis in dorsal root ganglion neurons following sciatic nerve damage (Barnat et al. , 2010), and abnormalities in axonal regeneration after facial nerve axotomy (Ruff et al., 2012) suggesting roles of this kinase in the context of adult neurogenesis and regeneration. Additionally, siRNA-based JNK3 knockdown was shown to decrease neurite outgrowth and cell viability in vitro (Tönges et al. , 2011), whereas its deletion in vivo protects neurons against kainic acid-induced excitotoxicity (Barnat, Enslen, 2010). Collectively, the available data indicates that JNK3 is involved in diverse roles relevant to survival, regenerative and degenerative actions.

In addition to neurons, JNK3 is also expressed in glial cells. In primary astrocytes, knockdown studies showed a role of JNK3 on chemokine secretion (Wang et al., 2007); while in BV2 microglial cells, the selective JNK3 inhibitor azelastine reduced the LPS-induced production of TNFα, IL6, and nitric oxide (Nguyen et al. , 2021). JNK3 is also expressed in oligodendrocytes, where its expression increases upon exposure to tumor necrosis related apoptosis inducing ligand (TRAIL) or TNF, and is required for induction of oligodendrocyte death (Jurewicz et al. , 2006, Jurewicz et al. , 2003). Similarly, activation of both JNK1 and JNK3 was required for neurotrophin p75-mediated apoptosis of oligodendrocytes (Harrington et al. , 2002) and in JNK3 null mice, the number of TUNEL+ oligodendrocytes decreased 2-to-3-fold following spinal cord injury (Li et al. , 2007). In a mouse model of spinal cord injury, treatment with the estrogen receptor ligand 17β-estradiol, which inhibited JNK3, reduced oligodendrocyte apoptosis, suggesting potential gender-dependent effects of this kinase (Lee et al. , 2012). Collectively, these findings point to JNK3 roles in inflammatory activation and in oligodendrocyte damage and death.

The above findings suggest that JNK3 may modulate neuropathology in mouse models of multiple sclerosis (MS), and several studies have examined the roles of specific JNK isoforms in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS. In the MOG peptide-based chronic EAE model, disease development in JNK2 null mice was

similar to wild type mice. Although JNK2 reportedly participates in T cell differentiation, the authors concluded that it is not necessary for the induction or effector phases of EAE (Nicolson et al. , 2002). Examination of the expression of several MAPKs including ERK1, p38, and non-specific JNKs, showed that phosphorylation of each of these classes of MAPKs was increased at the peak of EAE, followed by a decline (Shin et al. , 2003). The same study documented phosphorylated (active) forms of JNK kinases in T cells and in some glial cells, but the experiments did provide distinguish specific JNK isoforms (Shin, Ahn, 2003). A potential contribution of JNKs on EAE-induced neuropathology was shown by use of SP600125, a pan-JNK isoform inhibitor, which suppressed EAE disease progression (Bagnoud et al. , 2020, Ichiyama et al. , 2011). A contribution of JNK kinases to EAE-induced neuropathology is also supported by studies using mice with knockdown of JKAP, a phosphatase that dephosphorylates and inactivates JNKs. In JNKAP null mice, which have greater JNK activity, EAE disease was worsened (Li et al. , 2014). Other agents, including several natural products that reduce JNK phosphorylation have also been shown to ameliorate EAE disease severity (Chen et al. , 2021, Yang et al. , 2021). Taken together, the existing studies show that JNK1 suppression can reduce EAE symptoms, while inhibiting JNK2 does not seem to play a significant role. Despite this knowledge, specific contributions of JNK3 to EAE-induced neuropathology have not yet been reported.

In the current study, we monitored the progression of EAE disease severity and spinal cord inflammation in JNK3 null mice and wild type littermates. In contrast to results involving JNK1 and JNK2 inhibition, we find that JNK3 null male, but not female mice, show a modest increase in disease severity. This effect was associated with a small, but significant increase in the lesioned white matter area of the spinal cord.

## 2. Materials and Methods

### 2.1. Animals and induction of EAE

Male and female wildtype (WT) and JNK3 homozygous null mice (JNK3 null mice) (Jackson laboratory C6.129S1-Mapk10tm1Flv/J, RRID:IMSR_JAX:004322) The mice were immunized to develop chronic demyelinating experimental autoimmune encephalomyelitis (EAE) using reagents purchased from Hooke Laboratories (catalog #EK-2110) as we previously described (Kalinin et al. , 2020). Both JNK3 null and the WT mice were originally generated in C67BL/6 blastocytes, and were backcrossed several generations to C57BL/6. Therefore, immunization with $MOG_{35-55}$ peptide in these mice is expected to be chronic disease (Moreno et al. , 2013, Voskuhl and MacKenzie-Graham, 2022). Briefly, 10-week old mice were injected with 200 μg $MOG_{35-55}$ peptide emulsified in CFA (one 100 μl s.c. injection into the midline of the upper and one into the lower back). Two hours later, mice received an i.p. injection of pertussis toxin (PT; 125 ng in 100 μl PBS), followed by a second PT injection 24 hours later. Sham mice received the same injections as EAE mice, but the $MOG_{35-55}$ peptide was omitted. Clinical signs were scored as: 0, no signs; 1, limp tail; 2, impaired righting; 3, paresis of one hind limb; 4, paresis of two hind limbs; 5, death. Scoring was performed every other day at the same time by the same investigator blinded to treatment allocation.

### 2.2. Tissue processing and analysis

Mice were transcardially perfused with PBS, followed by PBS containing 4% paraformaldehyde (PFA). Spinal cords were dissected, post-fixed in PBS with 4% PFA for 16 hrs, then cryoprotected through washes in sucrose solutions of increasing concentration (5%,10%,20% and 30% sucrose in PBS) and stored at 4 °C. Lumbar sections of the spinal cord were dissected using a metal matrix, mounted in OCT compound, fast frozen in dry ice plus isopentane and then stored at −80°C until sectioning. Spinal cord coronal sections (40 um) were prepared using a Leica Cryostat from rostral to caudal of the lumbar cord segment and stored in tris-buffered saline (TBS) containing 0.02% sodium azide.

### 2.3. Immunohistochemistry (IHC)

IHC was carried by incubating free-floating sections with the following primary antibodies (Abs): anti-Iba1 rabbit polyclonal Ab (Wako #019-19741; RRID:AB_839504) at 1:500 to visualize microglial cells; anti-glial fibrillary acidic protein (GFAP) rat monoclonal Ab at 1:500 to visualize astrocytes (Trojanowski et al. , 1986); and anti-myelin basic protein (MBP) rabbit polyclonal Ab at 1:700 to visualize oligodendrocytes (Reidl et al. , 1981). After incubation at 4°C overnight, tissue sections were washed in PBS, then incubated at 4°C for 2 hr with appropriate secondary antibodies (Alexa-568 conjugated goat anti-rabbit IgG, 1:500, Invitrogen #A11011, RRID:AB_143157; FITC-conjugated donkey anti-rat, 1:250, Jackson ImmunoResearch 712-095-153, RRID:AB_2340652). After 2 hr incubation, sections were washed 3 times in PBS, counter-stained with DAPI, mounted onto glass slides, and coverslips applied. Negative control sections were prepared where primary antibodies were omitted. Images were collected on a Zeiss Axioplan 2 microscope equipped with an MRm camera. Axiovision version 4.7 software parameters were set to define positive staining versus background values, obtained from the same regions in negative control sections.

### 2.4. Data analysis

Average daily clinical scores were compared using a repeated measures mixed-effects model. The progression of daily incidence (# of ill mice divided by total mice in each group) was tested for normality using D'Agostino & Pearson test, then compared by Wilcoxon matched-pairs signed rank test. Daily cumulative scores were calculated for each mouse as the sum of all clinical scores up to that day, and groups were compared by repeated measures mixed-effects model. Images were quantified for % area displaying anti-GFAP and anti-Iba1 immunoreactivity above background (no primary Ab). Fluorescence intensity values were obtained from 3-4 mice/experimental group (6-9 sections/mouse). Lesion volume was defined as the total area occupied by DAPI staining in white matter, which was delineated by anti-MBP antibody staining. Bars show mean ± s.e.m. Data were tested for normality, then compared by 1-way parametric ANOVA, with Sidak's post hoc comparisons. Analyses were done using GraphPad Prism Version 9.5.1 (GraphPad Software, San Diego, CA, USA).

# 3. Results

## 3.1. EAE disease severity is increased in JNK3 null male mice.

In male mice, the incidence of disease increased more quickly in JNK3 null mice reaching 100% by day 14, compared to WT mice which reached 100% at day 19 (Fig 1A). The average day of onset (Fig 1B) was earlier in the JNK3 null mice than WT mice (12.0 ± 0.33 versus 14.0 ± 0.86 days, p=0.036). Comparison of the daily average clinical scores (Fig 1C) showed that JNK3 null mice tended to exhibit higher clinical scores than the WT mice, reaching a maximum of 3.2 ± 0.3 on day 17, compared to 2.6 ± 0.5 on day 17 in WT mice. Although daily disease severity between groups did not reach statistical significance, average daily cumulative scores (Fig 1D) were statistically different (p=0.023 2-way repeated measures ANOVA). In contrast to males, there was no effect of genotype on incidence of disease, average day of onset in female mice, average daily clinical scores, or cumulative scores in female mice (Fig 2).

## 3.2. JNK3 depletion does not increase neuroinflammation in EAE spinal cords.

Spinal cord sections from male mice were stained using antibodies that recognize well-established markers of microglial (Iba1) and astrocyte (GFAP) activation to determine whether increased glial cell activation was associated with increased disease severity. As expected, EAE-treated WT mice showed significant increases in both GFAP (Figs. 3A, A') and Iba1 (Figs. 4A, A') staining, compared to sham-treated mice. The increase in GFAP immunostaining predominantly localized to gray matter, while increased Iba1 immunoreactivity was primarily observed in white matter. However, both GFAP (Figs. 3B, B') and Iba1 (Figs. 4B, B') immunostaining were similar between WT and JNK3 null EAE mice, suggesting that JNK3 depletion did not influence the extent of EAE-induced glial cell activation.

## 3.3. JNK3 deletion increases spinal cord white matter lesion volume in EAE mice.

Quantitation of DAPI staining, an index of leukocyte infiltration, showed that spinal cord lesion areas in white matter (delineated by anti-MBP immunoreactivity) were significantly increased in spinal cord sections of EAE WT mice, compared to sections obtained from sham WT mice (Figs. 5A, 5A'). Remarkably, DAPI-stained areas were increased approximately 50% in EAE JNK3 null mice, compared to EAE WT mice (Figs 5B, B'), suggesting that JNK3 depletion increases cellular infiltration into the CNS.

# 4. Discussion

Findings presented here that JNK3 deletion increased EAE disease severity in association with increased lesion volume contrast with studies indicating that JNK3 is associated with an increase in cell damage and death. JNK3 null mice show reduced responses to epileptogenic injections of kainic acid (Yang et al. , 1997); and show reduced loss of dopaminergic neurons in response to MPTP (Hunot et al. , 2004). Similarly, *in vitro* studies have shown that cortical neurons prepared from JNK3 null mice show less cell damage when exposed to β-amyloid (Morishima et al. , 2001), and other revealed toxic effects of JNK3 on axonal transport, a major cellular process sustaining axonal health (Morfini

et al. , 2009). Similarly, in acutely isolated adult human oligodendrocytes, it was shown that cell death induced by TNFα was associated with phosphorylation of JNK, correlated most strongly with JNK3 activation (Jurewicz, Matysiak, 2003). The same authors later showed that JNK3 was the primary MAPK activated upon exposure to the tumor necrosis related apoptosis inducing ligand (TRAIL), and that treatment with inhibitors of calpain, p38, or serine proteases did not rescue the cells from apoptosis. Oligodendrocyte cell death following spinal cord injury has also been shown to involve JNK3 activation, since death was reduced in JNK3 null mice (Li, Tep, 2007). Since we did not directly assess cell death, it is possible that oligodendrocyte death was reduced in the JNK3 null EAE mice. However, worse EAE in those mice could be due to JNK3 depletion from other cell types, for example by increasing leukocyte infiltration into the CNS.

Despite numerous studies linking JNK3 activation to toxic effects, other studies in different context support protective ones. For example, exposure of human Jurkat T cells to a JNK2/3 inhibitor induced apoptosis, an effect associated with prometaphase arrest, Cdk1 activation, and Bcl-2 phosphorylation (Jang et al. , 2014), making it conceivable that JNK3 depletion could increase survival of activated, infiltrating T cells in EAE. Precedents from work in other cell types appear consistent with this possibility. For example, JNK3 depletion reportedly limits apoptosis in insulin secreting beta-cells (Abdelli and Bonny, 2012). Further, JNK3 was shown to maintain activation of the protein kinase Akt2, a specific molecular component of a PI3K pathway known to promote cell survival (Abdelli and Bonny, 2012). Also, JNK3 is also required for beta-cell resistance to IFNa (Roca-Rivada et al. , 2023), and its knockdown increases cytokine-induced apoptosis in these cells (Abdelli et al. , 2009, Ezanno et al. , 2014). Taken together, these findings again suggest the possibility that JNK3 null mice display increased cytokine-induced apoptosis, or reduced survival of cells in which Akt2 increases survival.

JNK3 roles in neurogenesis and regeneration have also been reported. For example, JNK3 null mice have fewer neural progenitor cells (Castro-Torres, Landa, 2019), reduced neuritogenesis following sciatic nerve damage (Barnat, Enslen, 2010), and deficits in axonal regeneration (Ruff, Staak, 2012). In addition, *in vitro* studies showed that JNK3 knockdown decreases neurite outgrowth and cell viability (Tönges, Planchamp, 2011). Although in EAE there is limited neurogenesis, proliferation and maturation of oligodendrocyte progenitor cells occurs in attempts at remyelination; an event which could conceivably be reduced in the JNK3 null mice. Finally, our results do not rule out a contribution of neuronal JNK3 to the worsening of EAE clinical scores observed in the JNK3 KO mice. For example, any attempts at recovery of axonal damage due to EAE could be compromised due to alterations in JNK3-depleted neurons. Additional experiments featuring cell-type specific JNK3 knockdown would help evaluate the extent to which neuronal and/or glial JNK3 contributes to the worsening of EAE clinical scores associated with ubiquitous JNK3 deletion.

Interestingly, our data show increased cellular infiltrates in spinal cord white matter of JNK3 null, EAE mice. Leukocyte infiltration is regulated by numerous factors including the expression of chemoattractants, the degree of neuroinflammation, as well as the integrity of the blood brain barrier (Legroux and Arbour, 2015). Interestingly, blood vessel pruning is

reduced the absence of JNK3, leading to vasculature defects (Salvucci et al. , 2015). Further, JNK3 has been shown to promote vascular remodeling (Kant et al. , 2019) and angiogenesis (Ebelt et al. , 2013), which occurs in both MS (Girolamo et al. , 2014, Lengfeld et al. , 2014) and EAE (Fujita and Yamashita, 2017), potentially to increase blood supply to lesion sites. Whether effects on brain vasculature contribute to increased damage in JNK3 null EAE mice remains to be determined.

### Gender bias in EAE and JNKs

In the current study we did not observe any differences due to JNK3 deletion on the course of EAE in female mice. As reported for MS, gender-related effects on neuropathology have also been reported in EAE (Alvarez-Sanchez and Dunn, 2023), in most cases showing a bias towards worse disease in females. These differences may be due to sex-specific features of the immune system under the influence of specific hormones (Ryan and Mills, 2022). Gender bias has also been reported with regard to the biological actions of MAPKs. For example, genetic studies have shown that depletion of the MAPK isoform p38α from microglial cells worsened EAE only in males; while depletion from microglia and peripheral macrophages reduced severity in females (McGill et al., 2021, McGill et al. , 2020). JNK activation also shows gender bias: JNK expression is greater in female P301L transgenic tau mice, and its levels were correlated with greater tau pathology (Buccarello et al. , 2018). In a formalin-based model of nociception, the anti-nociceptive effects of a pan-JNK inhibitor were more potent in male than female mice, although the inhibitor reduced inflammatory expression only in females (Blanton et al., 2021). Finally, oligodendrocyte-specific effects of 17β-estradiol have been reported (Lee, Choi, 2012). Further studies aimed to illuminate mechanisms underlying contributions of JNK3 to MS neuropathology, as well as its potential role on gender bias are therefore warranted.

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## Data availability

Data will be made available upon request

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## Highlights

- Roles for c-Jun amino terminal kinase (JNK) isoforms in EAE are not well known.
- EAE onset was sooner and severity increased in JNK3 male mice compared to WT mice
- In spinal cord, astrocyte and microglial activation was similar
- White matter lesions were increased in JNK3 EAE mice
- These findings suggest JNK3 normally plays protective roles in EAE

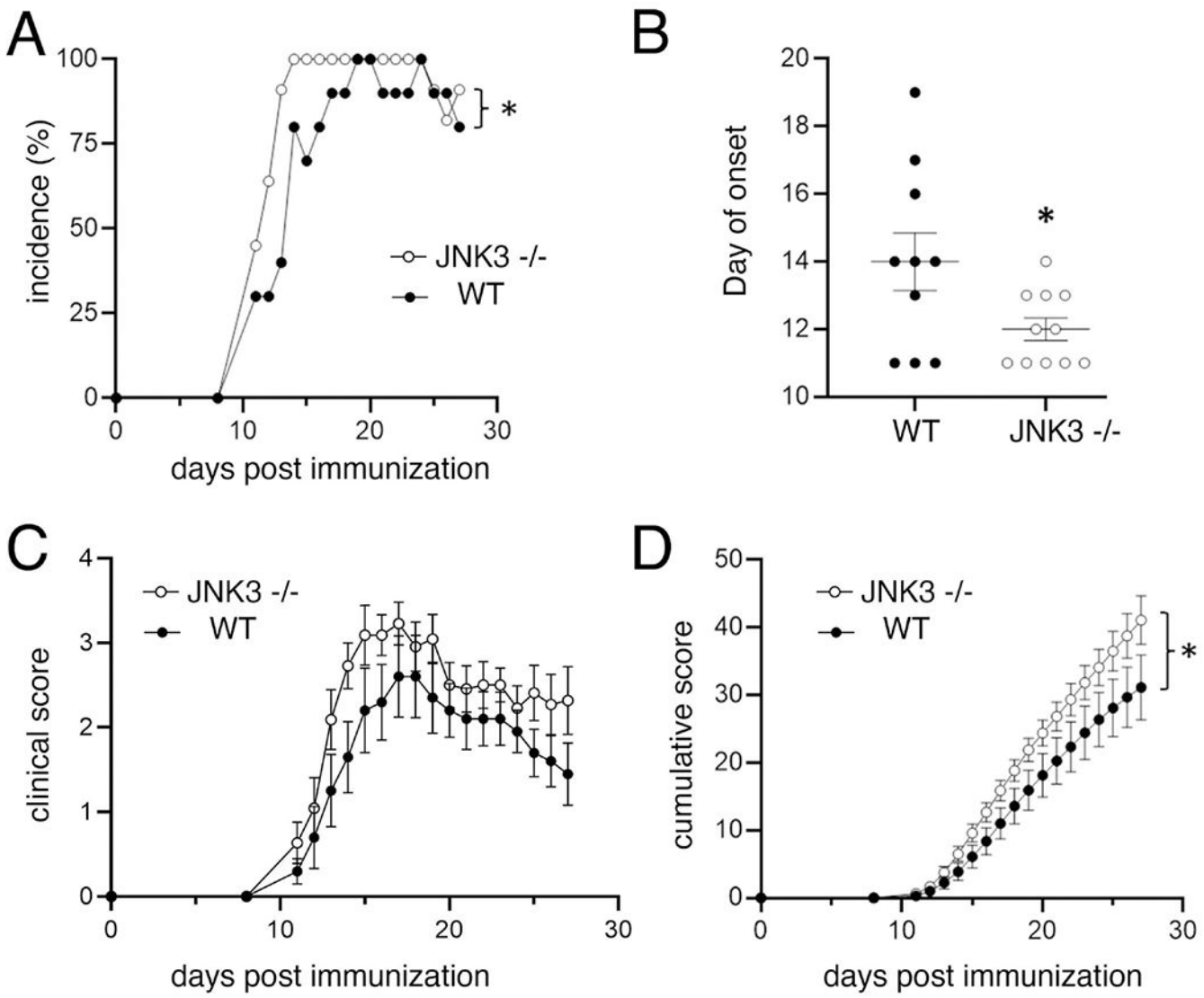


**Figure 1. EAE disease is modestly worsened in JNK3 null male mice.**

>Male WT (n=10, ●) and JNK3 null (n=11, ○) mice were immunized with MOG $_{35-55}$ peptide to develop EAE. (**A)** The daily incidence of disease was significantly greater in the JNK3 null mice (p=0.0004, Wilcoxon matched-pairs signed rank test). (**B**) The average day of disease onset was shorter in the JNK3 null mice (p<0.05, unpaired T-test). (**C**) Compared to WT mice, average daily disease severity was slightly higher in JNK3 null mice, but this difference did not reach statistical significance. (**D**) Cumulative disease scores were significantly higher in the JNK3 null mice (mixed-effects model).

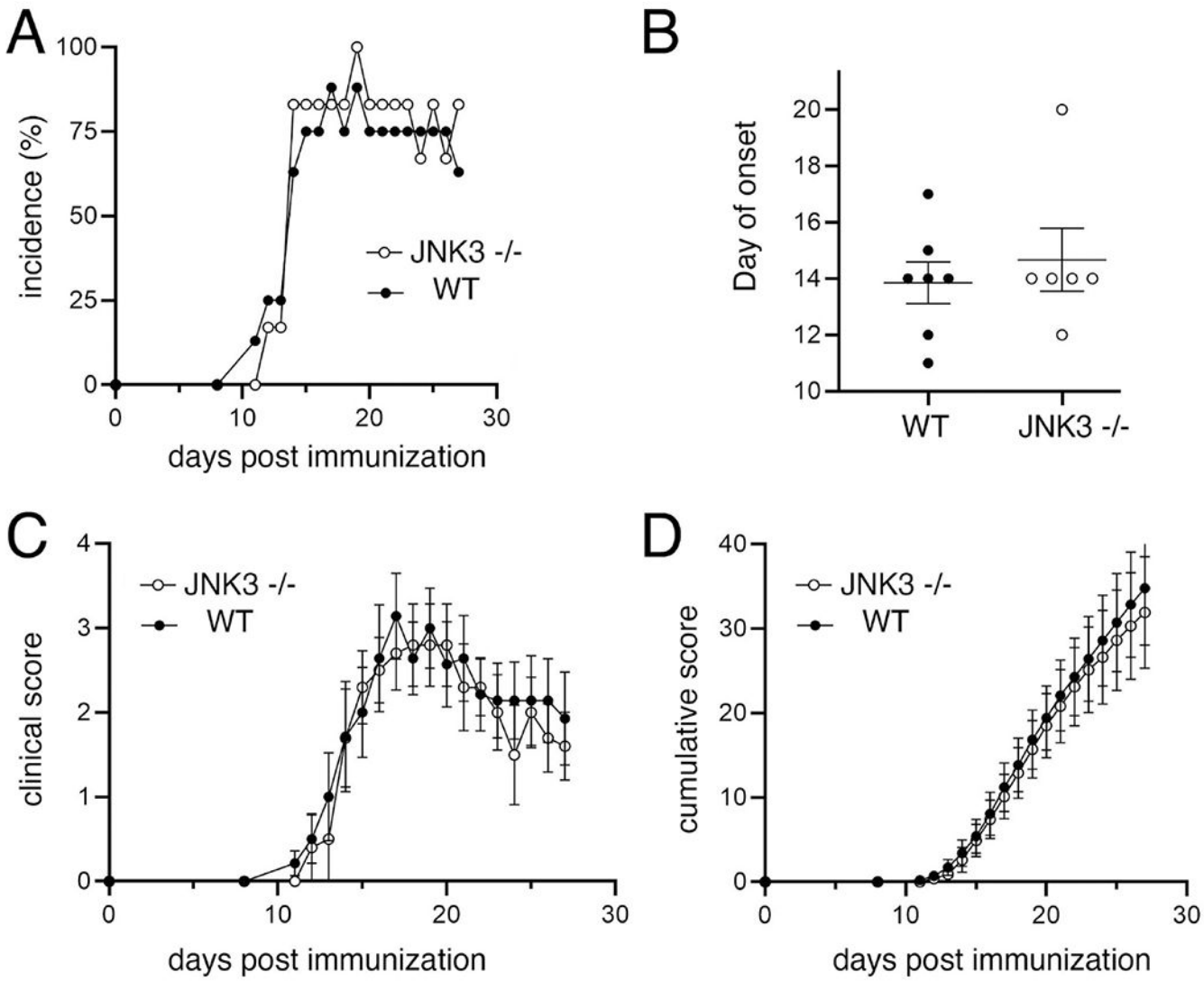


**Figure 2. EAE disease is not altered in JNK3 null female mice.**

Female WT (n=7, ●) and JNK3 null (n=5, ○) mice were immunized with MOG $_{35-55}$ peptide to develop EAE. There were no significant differences observed between the 2 groups for either (**A) D**aily incidence of disease; (**B**) Average day of disease onset; (**C**) Average daily disease severity; or (**D**) Cumulative disease scores.

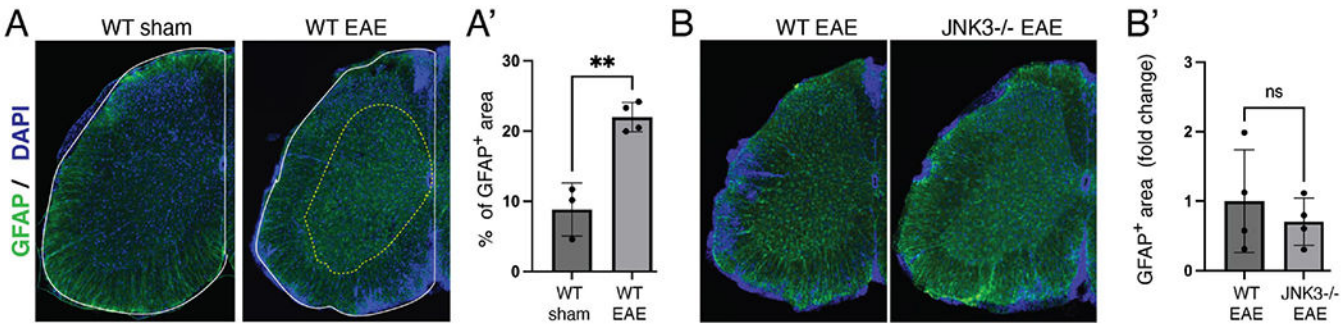


**Figure 3: Effect of JNK3 deletion on EAE-induced astrocyte activation.**

Representative images of spinal cord sections (lumbar level) from sham and EAE WT male mice double stained with the nuclear marker DAPI (blue) and with antibody recognizing the astrocytic marker GFAP. Total spinal cord areas used for quantitation are delineated by a white line. (**A**) Compared to sham mice, EAE mice showed an increase in the percentage of total spinal cord area displaying GFAP immunoreactivity. This increase was particularly prominent in gray matter (yellow dashed line). (**A**') Quantitative data confirms a significant increase in the percentage of GFAP-immunoreactive spinal cord area in EAE WT mice, compared to sham WT mice. (**B**, **B**') Quantitative analysis shows similar GFAP-immunoreactive areas in EAE WT, compared to EAE JNK3 null mice. For panel A, data is mean ± sem % area stained; for panel B, data is relative staining of JNK3 null EAE samples, compared to WT EAE samples. $n$=3-4 mice per genotype; 6-9 sections imaged per mouse. **, p<0.01; ***, p<0.001 (unpaired T-tests)

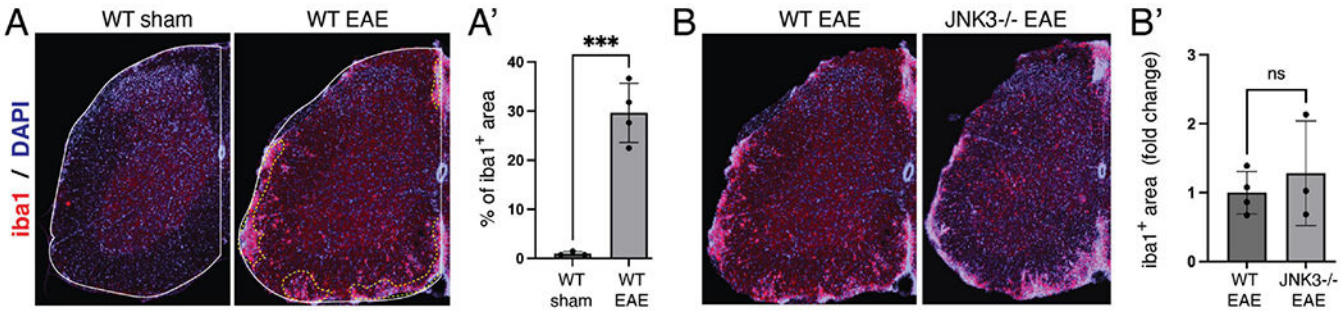


**Figure 4: Effect of JNK3 deletion on EAE-induced microglia activation.**

Representative images of spinal cord sections (lumbar level) from sham and EAE WT male mice double stained with the nuclear marker DAPI (blue) and with antibody recognizing microglial marker Iba1. (**A**) Compared to sham-treated mice, EAE WT mice showed an increase in the percentage of total spinal cord area displaying Iba1 immunoreactivity. (**A**') The percentage of Iba1-immunoreactive area for the entire spinal cord was significantly increased in EAE WT mice, compared to sham WT mice. As observed for GFAP, no differences in Iba1-stained areas were observed between EAE WT JNK3 null mice (**B**, **B')**.

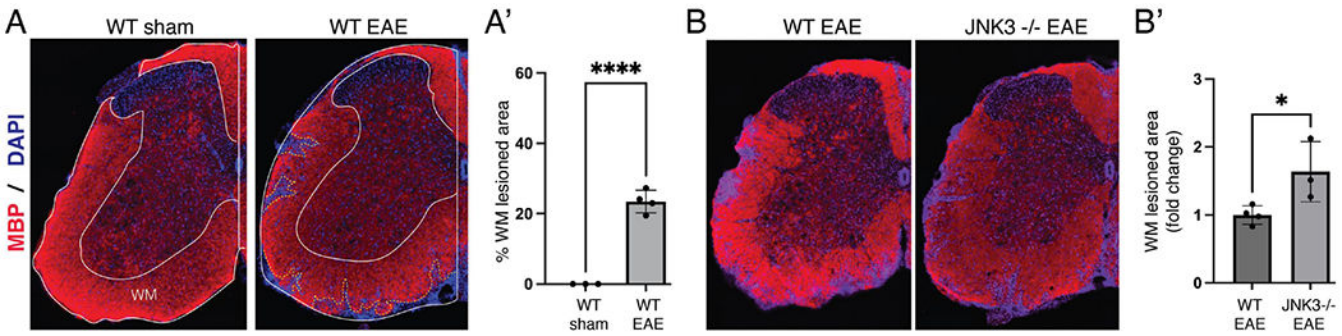


**Figure 5: Effect of JNK3 deletion on EAE-induced white matter spinal cord lesion size.**

Representative images of spinal cord sections (lumbar level) from sham and EAE WT male mice (**A**) and EAE WT and JNK3 null mice (**B**). Sections were double stained with the nuclear marker DAPI (blue) and with an antibody recognizing myelin basic protein to help delineate white matter (WM, dashed yellow line). (**A**, **A**') Compared to sham WT mice, EAE WT mice showed an increase in total white matter lesion areas. (**B**, **B**') Compared to EAE WT mice, the area covered by DAPI-positive lesions in white matter was significantly higher in EAE JNK3 null mice. For panel A', data is mean ± sem lesion volume. For panel B', data is relative lesion area of samples from EAE JNK3 null mice compared to EAE WT mice. $n$=3-4 mice per genotype; 6-9 sections imaged per mouse. *, $p<0.05$; ****, $p<0.0001$ (unpaired T-tests).