PARTIAL CHANGE IN EPHA4 KNOCKOUT MOUSE PHENOTYPE: loss of diminished GFAP upregulation following spinal cord injury

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ABSTRACT

In a previous study we found that the EphA4 receptor inhibits regeneration following spinal cord injury by blocking regrowth of axons and regulation of astrocyte reactivity. In our original studies using EphA4 null mice (Goldshmit et al., J. Neurosci. 2004) we found attenuated astrocyte reactivity following spinal cord injury. Several other studies have now supported the role of EphA4 in regulating neural regeneration but a recent study (Herrmann et al., Exp. Neurol. 2010) did not find an effect of EphA4 on astrocyte reactivity. Re-examination of astrocytic gliosis following injury in our current cohort of EphA4 null mice revealed that they no longer showed attenuation of astrocyte reactivity, however other EphA4 null mouse phenotypes, such as decreased size of the dorsal funiculus were unaltered. We hypothesised that long-term breeding on the C57Bl/6 background may influence the EphA4-mediated astrocyte phenotype and compared astrocytic gliosis at 4 days following spinal cord injury in wildtype and EphA4 null mice on the C57Bl/6 background and backcrossed C57Bl/6x129Sv(F2) mice, as well as wildtype 129Sv mice. 129Sv mice had increased GFAP expression and increased numbers of reactive GFAP astrocytes compared to C57Bl/6 mice. There was no significant effect of EphA4 deletion on GFAP expression in C57Bl/6 mice or the F2 crosses other than a moderately decreased number of EphA4 null astrocytes in C57Bl/6 mice using one of two antibodies. Therefore, there has been an apparent change in EphA4-mediated astroglial phenotype associated with long term breeding of the EphA4 colony but it does not appear to be influenced by background mouse strain.

KEY WORDS: EphA4, astrocyte reactivity, proliferation, GFAP, C57Bl/6, 129SVJ

ABBREVIATIONS:

129, 129X1/SVJ; B6, C57Bl/6 mice; B6x129(F2), F2 progeny of a C57Bl/6x129X1/SVJ cross; DAB, diaminobenzidine; GFAP, glial fibrillary acidic protein; PBS, phosphate buffered saline; SEM, standard error of the mean

INTRODUCTION

Injury to the adult mammalian spinal cord leads to long lasting functional disability due to an inability of the severed axons to regenerate and connect with their target cells, at least partly due to astrocyte reactivity. Following neural injury, astrocytes proliferate, become hypertrophic and form a barrier around the injury site preventing expansion into nearby uninjured tissue [21, 22].

A subfamily of tyrosine kinase receptors, the Ephs and their ligands, the ephrins, also play a role in neural injury responses, with variable upregulation of their expression on astrocytes, neurons and oligodendrocytes [9]. In particular, EphA4 is expressed by reactive astrocytes following central nervous system injury in mice, rats and primates [7, 8, 18] and on severed axon stumps rostral to the injury site [6] causing axonal growth cones to retract upon contacting cells expressing ligands to the EphA4 receptor. EphA4 can regulate astrocyte reactivity *in vitro* [18, 19] and *in vivo* [10] and deleting or blocking EphA4 in mice or rats with spinal cord injury promotes axonal regeneration and functional recovery [5, 8, 10], although partial reduction in EphA4 expression is ineffective [1, 2]. One of the hallmarks of astrocyte reactivity is glial fibrillary acidic protein (GFAP) upregulation. In our original studies examining regeneration in EphA4 knockout mice following spinal cord injury, upregulation of GFAP and the increase in GFAP-expressing astrocyte number was attenuated in EphA4 knockout compared to wildtype mice [8]. However, a recent study examining spinal cord injury in another EphA4 knockout mouse line found no reduction in GFAP expression at the injury site [11]. Several possibilities may explain the discrepancy between the two studies, including use of different injury models and EphA4 knockout mice of different derivation. The mice used in our 2004 study were derived by homologous recombination [4] while those used by Herrmann and colleagues were derived by gene trap [13]. However, these factors are unlikely to be the only or primary cause of the discrepancy. Another possibility is the background strain of the mice used and the current study investigated whether the background strain of EphA4 knockout mice influences astrocytic gliosis.

EXPERIMENTAL PROCEDURES

Mice and spinal cord injury

Experiments were performed on 8-12 week old male and female wildtype (EphA4$^{+/+}$) and sex-matched EphA4 null (EphA4$^{-/-}$) mice on the C57Bl/6 (B6) background, and on the F2 progeny of a C57Bl/6x129X1/SVJ cross (B6x129(F2)) and compared with 129X1/SVJ (129) wildtype mice. As the original EphA4-/- mice used by us for gliosis analyses were on a mixed B6x129 background [2], use of F2 progeny was designed to mimic the original mix of backgrounds. Mice were anaesthetised with Ketamine/Xylazine (I.P. 100mg/kg and 16mg/kg, respectively) and a lateral hemi-lesion made in the spinal cord at T10, essentially as previously described [8]. In sham-operated animals the spinal cord was uncut. All experiments were approved by the University of Melbourne Animal Experimentation Ethics Committee, in accordance with the "*Code of practice for the care and use of animals for scientific purposes*" of the National Health and Medical Research Council of Australia.

Tissue preparation and immunohistochemistry

Four days after spinal cord injury animals were perfused intracardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Spinal cords were post-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, frozen in OCT (Sakura) and longitudinal serial cryostat sections (10μm) were cut. Immunohistochemistry was carried out on all sections from each group simultaneously. Sections were blocked in 2% goat/2% fetal calf serum/0.2% TritonX100 (blocker) for 45 minutes, then incubated overnight at 4^{o}C in monoclonal rat anti-GFAP (1:250 in blocker; cat# 13-0300 Invitrogen (Zymed)) or polyclonal rabbit anti-GFAP (1:500 in blocker; cat# Z0334 Dako). After washing, sections were incubated at room temperature for 2 hours in biotinylated anti-rat or anti-rabbit secondary antibodies (1: 200 and 1:800 respectively in PBS; Vector Laboratories), then washed, incubated in

Vectastain ABC solution for 45 minutes and GFAP labelling then visualised using diaminobenzidine (DAB; Invitrogen).

Astrocyte cell counts and GFAP expression analysis

Since the core of a mouse spinal cord hemi-section is fibronectin positive and GFAP negative [11], grey matter in sections stained for GFAP were analysed approximately 100μm rostral and caudal to the injury site core, as well as 2.5mm rostral and caudal to the injury site (5 sections per animal). Images were taken at x100 magnification with the same exposure time and light intensity, imported into ImageJ (Wayne Rasband, NIH, Maryland, USA), converted to 8 bit greyscale and set thresholding was used to obtain the area fraction of pixels positive for GFAP labelling. In addition, as basal levels of GFAP expression were higher in 129 mice, for each section the mean area fraction obtained 2.5mm away from the injury was subtracted from the mean area fraction obtained next to the injury site to give amount of GFAP upregulation adjacent to the injury site.

To analyse the number of GFAP positive reactive astrocytes 6 micrographs per section in the grey matter immediately rostral and caudal to the injury site at x40 magnification were imported into ImageJ. The Cell Counter plugin was used to determine the total number of GFAP positive cells and the number of reactive astrocytes (defined as GFAP+ cells with thicker primary processes and amorphous cell body [22]). Only cells which contained a nucleus were counted. For each section, the total number of astrocytes and the number of reactive astrocytes was averaged and calculated per $0.1mm^2$ and the number of reactive astrocytes was calculated as a percentage of the total number of astrocytes.

Dorsal funiculus area measurements

Using ImageJ the area of the dorsal funiculus at T10-T12 in uninjured mice was measured in coronal spinal cord sections (10μm; *n*=4/animal) stained with haemotoxylin and eosin.

Statistics

Homogeneity of data was analysed using Levene's test and statistical comparisons were made using unpaired t-tests in SPSS (v11.0.1), where results with $p<0.05$ were considered significant. Data in figures is expressed as mean +/- SEM.

RESULTS

Confirmation that astrocytic gliosis was inhibited in the original cohorts of EphA4$^{-/-}$ mice

Before progressing with the analysis of astrocytic gliosis in mice of different background strains, the original slides from mice analysed in our early studies [8] were re-examined. As expected, the extent of GFAP upregulation in EphA4$^{-/-}$ mice was attenuated compared to wildtype mice at 48hrs, 4 and 7 days post-injury (Supp. FIG. 1). Assessment of the total number of astrocytes in coronal sections from an additional cohort of mice at 7 days post-injury confirmed these results (Supp. FIG. 2).

EphA4 does not regulate astrocyte GFAP expression in spinal cords of non-injured mice

To compare basal levels of GFAP expression between different strain backgrounds, GFAP immunostaining was measured in the spinal cord of sham-operated wildtype 129 mice (*n*=5) and wildtype and EphA4$^{-/-}$ B6 (*n*=6 both genotypes) and B6x129(F2) (*n*=3 both genotypes) mice (FIG. 1). To assess whether antibodies raised against different epitopes would give different results two different anti-GFAP antibodies were used: a monoclonal rat antibody (Invitrogen) which primarily detects reactive astrocytes (FIG. 1A-F) and a polyclonal rabbit antibody (Dako) which detects normal and reactive astrocytes (FIG. 1G-L). Similar levels of GFAP expression were observed in wildtype and EphA4$^{-/-}$ mice on either genetic background (FIG. 1F&L), using either antibody. However, wildtype 129 mice expressed more GFAP than B6 or B6x129(F2) mice (FIG. 1L).

EphA4 does not regulate astrocyte GFAP expression after spinal cord injury in current cohorts of mice

Analysis of GFAP expression following injury using either the Dako polyclonal (FIG. 2) or Invitrogen monoclonal (Supp FIG. 2) antibodies revealed no differences in GFAP expression between genotypes on either B6 (FIG. 2A,E,C,G) or B6x129(F2) (FIG. 2 B,F,D,H) backgrounds at the injury site or in

non-reactive tissue 2.5mm away from the injury site (FIG. 2K). Similarly, no differences were found between genotypes in GFAP upregulation at the injury site (FIG. 2L).

In non-reactive areas of the spinal cord after injury and at the injury site wildtype 129 mice expressed more GFAP compared to both B6 and B6x129(F2) mice (FIG. 2K and FIG. Supp 2K). However, since B6 and B6x129(F2) mice had less GFAP expression in 'non-reactive' spinal cord compared to 129 mice, they had a greater apparent upregulation of GFAP at the injury site compared to 129 mice (FIG. 2L and Supp FIG. 2L).

Effect of EphA4 on astrocyte number at the injury site

To determine whether EphA4 deletion affects astrocyte number at the injury site independently of GFAP upregulation, the total number of astrocytes and the number of reactive astrocytes were counted rostral and caudal to the injury site. Differences were observed between groups, depending on the antibody used to visualise GFAP (FIG. 3). Using the Invitrogen monoclonal antibody EphA4$^{-/-}$ mice on the B6 background had a lower density of astrocytes at the injury site compared to wildtype mice (FIG. 3A), however there was no difference in the number of reactive astrocytes (FIG. 3B) or percentage of reactive astrocytes (FIG. 3C). No such differences between genotypes were detected using the polyclonal Dako anti-GFAP antibody on either the B6 or B6x129(F2) background (FIG. 3D-F). However, fewer reactive astrocytes were detected with the Dako antibody than the Invitrogen antibody and with both antibodies, the percentage of reactive astrocytes was higher in the B6x129(F2) cross than on the B6 background alone. Therefore there are strain differences in induction of astrocyte reactivity.

The increased GFAP expression in the spinal cord of 129 mice was reflected in the number of reactive astrocytes at the injury site, as 129 mice also had more reactive astrocytes, detected using both

anti-GFAP antibodies (FIG. 3B,C,E&F) and when using the Invitrogen monoclonal antibody 129 mice also had a greater density of astrocytes (FIG. 3A&D).

EphA4 regulates dorsal funiculus size

To investigate whether other phenotypes originally reported in the EphA4$^{-/-}$ line had also altered, we measured the area of the dorsal funiculus, which was previously shown to be reduced in size in EphA4$^{-/-}$ mice [4]. The dorsal funiculus in EphA4$^{-/-}$ B6 mice was approximately 1.8 fold smaller than that of B6 wildtype mice and retained the characteristic shallow morphology originally reported (SUPP FIG. 3).

Other changes in EphA4 knockout mouse phenotype

Another change in EphA4 knockout mouse phenotype has been a decrease in body size. When the mice were originally produced, there was no obvious difference in body size (M. Dottori, personal communication). Measurement of their stride length showed that wildtype mice had a stride length of 6.35 ± 0.11cm (n=7) and EphA4$^{-/-}$ mice had a stride length of 5.9 ± 0.18cm (n=9), p=0.15. They were also healthy, viable and bred well at normal Mendelian ratios [4]. The current mice are often runted, unhealthy and do not breed well: the EphA4$^{-/-}$ mice used in the current study were 15-18% smaller than wildtypes (measured at 8-12 weeks of age, just prior to SCI surgery). Weights of mice on C57Bl/6 background were: wildtype males 29.08±2.439g (n=3), EphA4$^{-/-}$ males 23.98±1.133g (n=9), p=0.05 and wildtype females 23.50±1.202g (n=9), EphA4$^{-/-}$ females 19.87±1.160g (n=6) p=0.05. Weights of mice on the B6x129(F2) background were: wildtype males 29.93±2.14g (n=9), EphA4$^{-/-}$ males 25.19±0.48g (n=4), p<0.05 and wildtype females 24.33±0.50g (n=5), EphA4$^{-/-}$ females 20.405±0.78g (n=5) p<0.01.

DISCUSSION

We previously showed that deletion of EphA4 reduces astrocyte proliferation and reactivity [8] while a recent study found no effect of EphA4 on GFAP expression after spinal cord injury [11]. We have reconfirmed our original results by assessing histology slides from the original cohort, indicating a change in phenotype since the mice were first produced. We proposed that strain background may have influenced the phenotype. Our EphA4$^{-/-}$ mice were generated by implanting homologously recombined ES cells of the 129 strain into B6 recipients [4] and were only partially backcrossed to B6 mice when used in our original analyses of astrocyte reactivity. Indeed, a difference in astrocyte reactivity has already been demonstrated between wildtype B6 and 129 strains following spinal cord contusion injury [14] while strain differences in potential EphA4-associated effects on ventral root axon morphology and motor behaviour has also been reported [3].

To investigate possible strain effects on EphA4 mediated astrocyte reactivity we crossed our line of EphA4$^{-/-}$ mice (which has now been backcrossed for approximately 12 years onto the B6 line) to mice of the 129 strain and used the F2 progeny to compare to B6 mice. We found that in the current EphA4$^{-/-}$ mice there is little or no effect of EphA4 genotype on levels of GFAP expression, independent of background strain, although we confirmed the previously demonstrated strain difference in astrocyte reactivity between B6 and 129 mice [14]. We now add that after injury 129 mice have a greater number of total astrocytes and reactive astrocytes at the injury site, as well as an apparent increased global expression of GFAP throughout the spinal cord. There was a modest decrease in total astrocyte number in EphA4$^{-/-}$ mice particularly on the B6 background strain using a monoclonal anti-GFAP antibody from Invitrogen but not a polyclonal antibody from Dako. This effect on total cell number is consistent with other studies, as EphA4 is a reported regulator of proliferation of GFAP positive cell populations [17, 20]. This was also evident using primary cultured astrocytes from wildtype and EphA4$^{-/-}$ mice [19] and

observed in other cell types such as neural progenitor cells [17]. EphA4 also affects the ability of astrocytes to rearrange their cytoskeleton and close scratch wounds [18, 19], while administration of EphA4 blockers (decoy EphA4 receptor or soluble ephrinA5-Fc ligand) to wildtype mice following spinal cord injury attenuates astrocyte reactivity, as assessed by GFAP expression [10].

As yet, we have been unable to account for the differences in astrocyte reactivity observed in our original and current cohorts of mice. There is intrinsic variability in GFAP immunoreactivity and we cannot exclude that there was a genetic background component that contributed to the original gliosis phenotype, for example if some mice had a higher 129/Sv contribution than others. However data presented in the original paper was from 8 wildtype and 9 EphA4 null mice across 3 timepoints and data from a further 4 wildtype and 3 EphA4 null mice were presented here in Supp. Fig. 2, suggesting that other factors may be playing a role. A number of phenotypic changes to the EphA4 mouse line have occurred with continuous breeding over the intervening years. Originally EphA4 mice were not distinguishable from wildtypes, other than a hopping gait resulting from corticospinal tract and central pattern generator defects [4, 12]. They were of normal size and bred well, with no apparent health problems. The current line of EphA4 null mice does not breed well; the homozygous mice are runted, have balance problems and occasional seizures (unpublished observations) with decreased viability. The reasons for this shift in phenotype are not clear but may be related to the shift in environment from being housed under specific pathogen free conditions on a high fat (9%)/high nutrient diet (Barastoc Irradiated WEHI mouse cubes, Ridley Agriproducts Melbourne, Australia) to a standard animal facility, initially on standard mouse chow (Barastoc). Further, EphA4$^{-/-}$ mice have alterations in thymus and T cell development [15] and a general attenuation of many inflammatory pathways following spinal cord injury [16] which may alter how animals thrive in different environments and may also influence the inflammatory response of astrocytes to injury. Not all phenotypes related to EphA4 signalling have been altered, as evidenced by maintenance of differences in dorsal funiculus morphology in the current

EphA4$^{-/-}$ colony and maintenance of a hopping gait, consistent with corticospinal defects as originally identified [4]. This may reflect a constitutive developmental effect of loss of EphA4 expression (dorsal funiculus and hopping gait) versus an interaction of genetics and environment for the inflammatory astroglial response to injury.

In summary, the current study shows that EphA4 has the potential to regulate astrocyte proliferation but not GFAP reactivity in the injured spinal cord of our current cohort of EphA4 null mice. However, the regulation is complex and likely involves several independent factors. While the background strain of the mouse affected the astrocytic upregulation of GFAP, with 129 mice showing greater global expression of GFAP compared to B6 mice, this was independent of EphA4 expression.

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FIGURES

FIGURE 1: EphA4 does not regulate GFAP expression in the spinal cord before injury

GFAP expression in sham-operated spinal cord visualised using Invitrogen monoclonal anti-GFAP antibody (WT (A-C), EphA4$^{-/-}$ (D-E)) or Dako polyclonal anti-GFAP antibody (WT (G-I), EphA4$^{-/-}$ (J-K)). Graphs shows percent of pixels positive for (F) Invitrogen monoclonal GFAP antibody or (L) Dako polyclonal GFAP antibody in each mouse strain. WT; wildtype. Scale bar in (A), representative of all panels = 20µm. Results in F and L show mean+/-SEM.

FIGURE 2: EphA4 does not regulate GFAP expression after spinal cord injury (Dako Ab)

GFAP expression in the injured spinal cord of WT and EphA4$^{-/-}$ B6, B6x129(F2) and 129 mice: WT mice at the injury site (A,B&I), WT mice 2.5mm away from the injury (E,F&J), EphA4$^{-/-}$ mice at the injury site (C&D) and EphA4$^{-/-}$ mice 2.5mm away from the injury site (G&H). (K) Graph compares percent of pixels positive for GFAP antibody at the injury site and 2.5mm away from the injury site. (L) Graph compares percentage of GFAP upregulation at the injury site. White arrowheads in C and D are examples of reactive astrocytes. White arrows in F and H are examples of non-reactive astrocytes. Scalebar in (A), representative of all panels = 20µm; * p<0.05. Results in K and L show mean+/-SEM.

FIGURE 3: Analysis of astrocyte numbers and reactivity after spinal cord injury

Graphs show the total number of GFAP positive astrocytes (A&D) and the number of reactive astrocytes counted (B&E), as well as the number of reactive astrocytes as a percentage of the total number of astrocytes counted (C&F), visualised using the Invitrogen monoclonal antibody (A-C) and the Dako polyclonal antibody (D-F). * p<0.05. Results show mean+/-SEM.

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Figure 1

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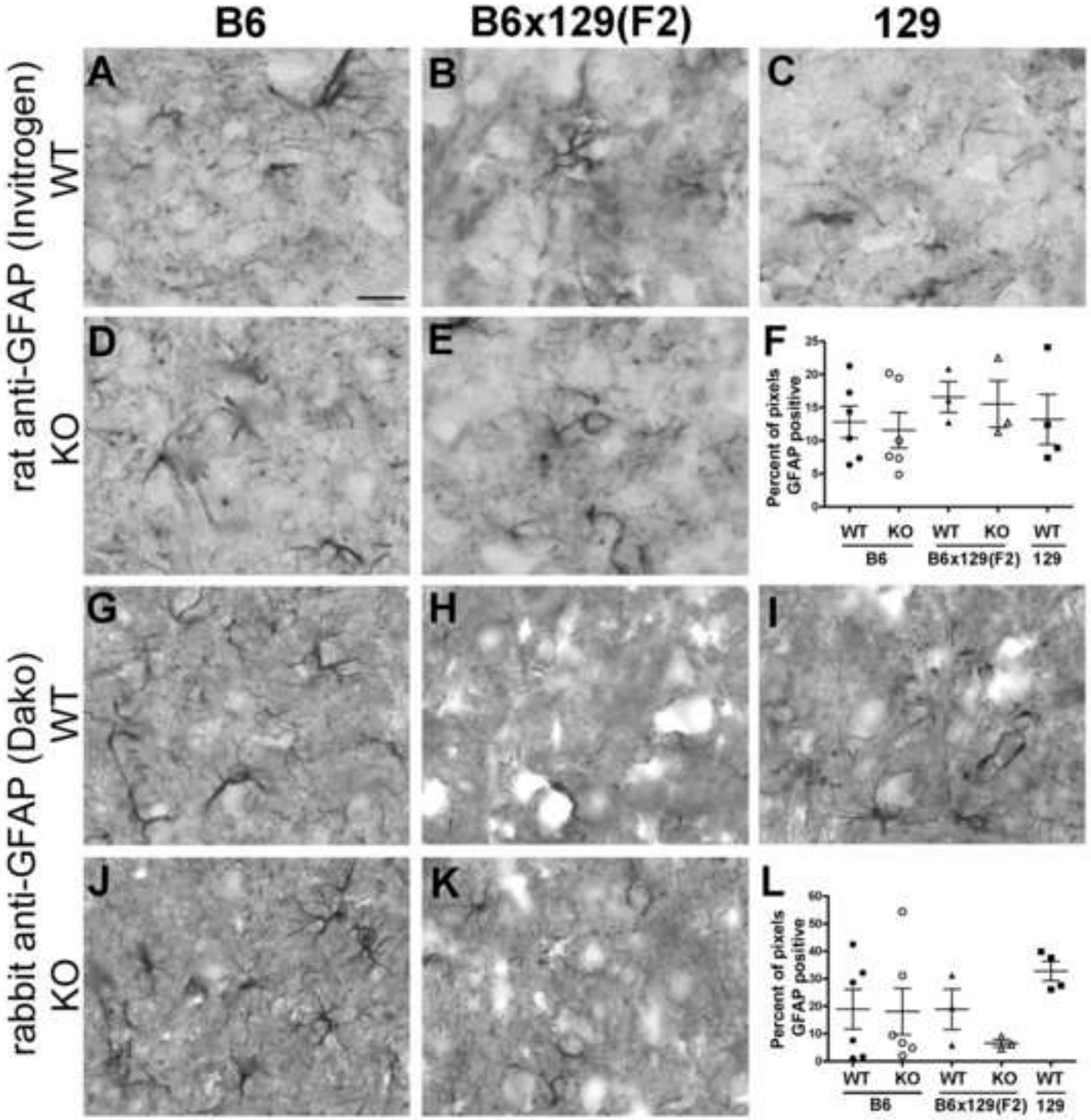

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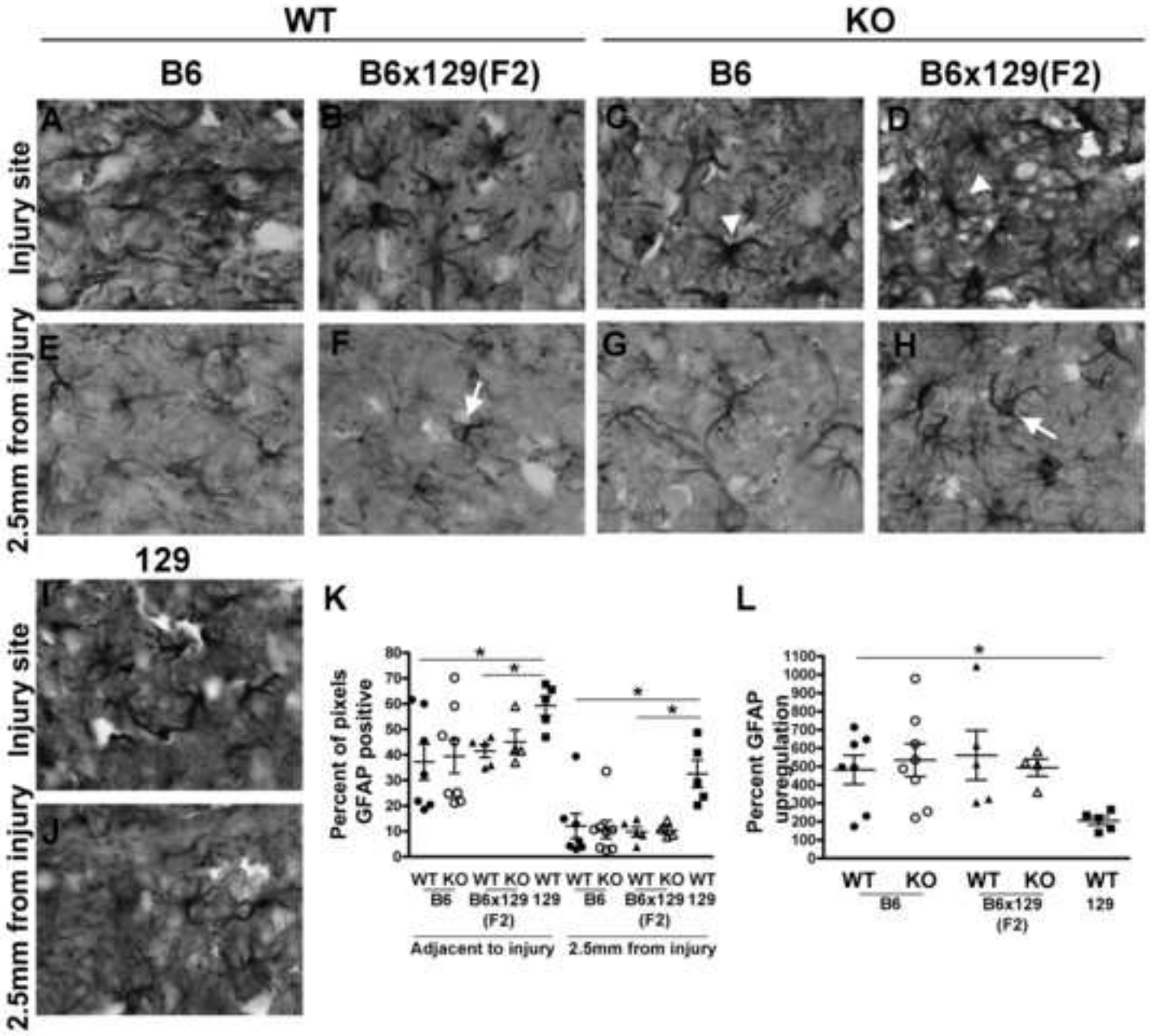
Fig. 2 Dixon et al



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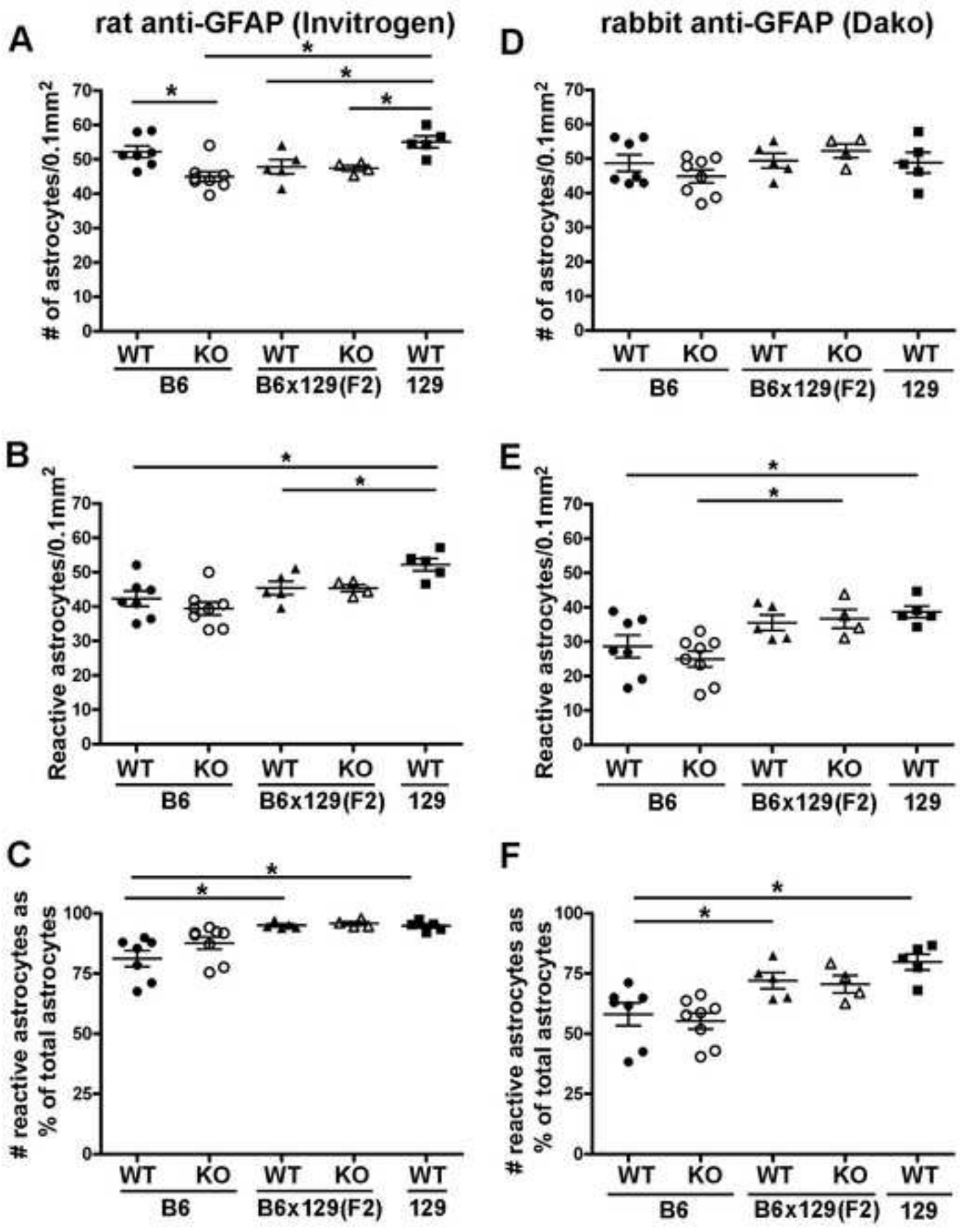

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