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AP-1 and the injury response of the *GFAP* gene

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Abstract

Increased *GFAP* gene expression is a common feature of CNS injury, resulting in its use as a reporter to investigate mechanisms producing gliosis. AP-1 transcription factors are among those proposed to participate in mediating the reactive response. Prior studies found a consensus AP-1 binding site in the *GFAP* promoter to be essential for activity of reporter constructs transfected into cultured cells, but to have little to no effect on basal transgene expression in mice. Since cultured astrocytes display some properties of reactive astrocytes, these findings suggested that AP-1 transcription factors are critical for the upregulation of *GFAP* in injury, but not for its resting level of expression. We have examined this possibility by comparing the injury response in mice of *lacZ* transgenes driven by human *GFAP* promoters that contain the wild type AP-1 binding site to those in which the site is mutated. An intact AP-1 site was found critical for a *GFAP* promoter response to the three different injury models used: physical trauma produced by cryoinjury, seizures produced by kainic acid, and chronic gliosis produced in an Alexander disease model. An unexpected additional finding was that the responses of the *lacZ* transgenes driven by the wild type promoters were substantially less than that of the endogenous mouse *GFAP* gene. This suggests that the *GFAP* gene has previously unrecognized injury-responsive elements that reside further upstream of the transcription start site than the 2.2 kb present in the *GFAP* promoter segments used here.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, M.B.; *Methodology*, M.B, A.M., M.L.O.; *Investigation*, M.B, A.M., M.L.O.; *Formal Analysis*, M.L.O.; *Resources*, M.B, A.M., M.L.O.; *Writing – Original Draft*, M.B., M.L.O.; *Writing – Review & Editing*, M.B, A.M., M.L.O.; *Visualization*, M.B., M.L.O.; *Supervision*, M.B, A.M., M.L.O.; *Funding Acquisition*, M.B, A.M., M.L.O.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Keywords

Mice; Transgenic; Transcription; Genetic; Brain Injuries; Gliosis; Seizures; Kainic Acid; Cold Injury; Alexander disease; Glial Fibrillary Acidic Protein; Transcription Factor AP-1; STAT3 Transcription Factor; Aging; Female; Sex Characteristics; RRID:IMSR_TAC:fvb; RRID:IMSR_JAX:003487

INTRODUCTION

Transcription of the *GFAP* gene is strongly increased by most forms of CNS injury. Identification of the regulatory elements in the *GFAP* promoter required for this upregulation has been pursued to obtain information about signaling pathways occurring in the injured brain. This study evaluated the participation in the injury response of a consensus binding site for the AP-1 family of transcription factors that is located 1592 bp upstream of the transcription start site of the human *GFAP* gene. The presence of a similarly located AP-1 binding sequence in the mouse and rat *Gfap* genes suggests that this site has a regulatory role. The family of AP-1 transcription factors includes c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2 (reviewed in Rylski & Kaczmarek, 2004). These act as dimers, usually to enhance transcription, but can also serve as inhibitors. To perform this study we compared the activity of a wild type *GFAP* promoter to one in which the AP-1 site was mutated. Transgenic mice were used instead of cultured cells, as we have consistently observed that the activity of the *GFAP* gene in culture does not accurately report its activity *in vivo* (de Leeuw et al., 2006; Lee et al., 2006; Yeo et al., 2013). In particular, such a discrepancy has been observed for the role of the AP-1 site. Previous studies in human glioma cell lines using reporter genes driven by a human *GFAP* (h*GFAP*) promoter indicated that the conserved AP-1 binding site is essential for transcription of the gene (Masood et al., 1993; Gopalan et al., 2006a). This was supported by the observation of Gopalan et al. (2006b) that expression of a dominant negative c-Jun in the U373 glioma cell line abolished endogenous *GFAP* expression. However, it was subsequently found that mutation of this site in a h*GFAP* promoter-driven *lacZ* transgene had little effect on its basal expression in mice (Yeo et al., 2013). These seemingly disparate findings could be reconciled if the AP-1 site is

important for upregulation of the *GFAP* gene, as occurs in cultured astrocytes, but not for its basal expression in the CNS. To determine if the response of either the wild type h*GFAP-lacZ* transgene or the AP-1 mutated form depends on the nature of the injury, three different forms of injury were used: excitotoxicity produced by kainic acid (KA) induced seizures, physical trauma resulting from cryoinjury, and genetic injury involving chronic gliosis produced by transgene-mediated over-expression of human *GFAP*, which has been used as an Alexander disease (AxD) model (Messing et al., 1998; Messing et al., 2001; reviewed in Brenner et al., 2009).

METHODS

Transgenic animals

All experimental protocols were approved by the Institutional Animal Care and Use Committees of the University of Alabama at Birmingham (protocol 07859) and the University of Wisconsin, Madison (protocols G00199, G00321). Mice were maintained in AAALAC approved housing with standard food and water available ad libitum. Previously described lines are the wild type h*GFAP-nlacZ* lines tg7-2 (Tg(X:GFAP,lacZ)Mes3) (RRID:IMSR_JAX:003487) (Brenner et al., 1994), gfa2-nlac2 and gfa2-nlac3 (Su et al., 2004). In this paper the more descriptive names WT-1, WT-2 and WT-3 will be used for these three lines, respectively. Each of these lines carries the same h*GFAP-nlac* transgene, which consists of h*GFAP*bp –2163 to +47 (RNA start site is +1), with the initiating ATG at +15 mutated to TTG (the gfa2 promoter), placed in front of a nuclear-targeted *E. coli lacZ* gene, which in turn is followed by a fragment of the mouse protamine-1 gene (*mPI*). The latter supplies an intron, stabilizing 3' UTR, and a polyadenylation signal. Also previously described is line 73-7 (TgN(GFAP)Mes10) (Messing et al., 1998). Newly made lines for this study are WT-4, AP1m-1, AP1m-2 and AP1m-3. Each of these lines carries the same gfa2-nlac transgene just described, except that the promoter starts at –1757 instead of –2163. The shorter 5' extension was used for the mutant transgene because a long polyA stretch immediately upstream of –1757 compromised accurate amplification when inserting the AP-1 mutation using PCR-based methods. This difference in promoter start point was not expected to have any effect on the promoter injury response based on previous data (Besnard et al., 1991; Lee et al., 2008), and the fact that most of the deleted region, –2070 to –1758, is occupied by a primate-specific *alu* sequence (Roy-Engel et al., 2001). As will be noted below, this assumption was verified by the similar expression properties of WT-4 and WT-2. In addition to the shorter 5' extension, the transgene in the AP1m lines has the consensus AP-1 binding site at –1592 mutated from TGACTCA to TTCAGAA by transversion of the five internal nucleotides, using methods similar to those described previously (Masood et al., 1993; Yeo et al., 2013). The presence of the intended mutation was confirmed by DNA sequencing. Diagrams of each of the transgenes are presented in Fig. 1.

The transgenic mice were made at the University of Wisconsin-Madison in an FVB/N background and then bred to C57BL/6 for at least 10 generations. However, just prior to initiating this study, Bai et al. (2013) reported that h*GFAP*-driven transgenes lost activity in the C57BL/6 background, but regained activity after 1 to 2 backcrosses to FVB/N. Although it had been our experience that most of our h*GFAP-lacZ* lines retained activity on C57BL/6,

a few had indeed stopped expressing, and had been discontinued. All mice used in this study were thus backcrossed to FVB/N for at least 2 generations using breeders obtained from Taconic (RRID:IMSR_TAC:fvb), with the exception of line 73-7, which was continuously maintained on an FVB/N background. Despite the backcrossing to FVB/N, a few mice showed more than a 10-fold reduction in *lacZ* expression compared to other mice of the same line, prompting exclusion of those samples. This occurred for 1 of 35 WT-2 mice, 6 of 35 WT-3 mice, 1 of 47 WT-4 mice and 4 of 31 AP1m-1 mice, but for none of the 41 WT-1 mice, 32 AP1m-2 mice and 34 AP1m-3 mice used for this study. Ages of mice used were 2.5-9.6 months for the kainic acid (KA) experiments, 2.1-7.4 months for the Alexander disease (AxD) injury model, and 1.9-12.1 months for the cryoinjury experiments. Mice were assigned to injury or control groups to match as closely as possible the age and sex among the animals available, and had no other distinguishing phenotype. Our intention was to use mice without regard to their sex. However, following discovery of the female-predominant seizure syndrome described below, we preferentially used male mice for the remaining experiments (primarily cryoinjury) in order to minimize the frequency with which such seizures would artifactually elevate GFAP promoter activity. The numbers of male (M) and female (F) mice used were as follows (see Tables S1 and S2): KA and AxD controls, 73M, 51F; KA injury, 22M, 30F; AxD model, 21M, 18F; cryoinjury controls, 9M, 2F; cryoinjury, 41M, 14F.

After most of the mice needed for this project had been born, a marked increase in mortality was noted among both wild type and transgenic female mice that had been back-crossed to FVB/N and were four months of age or older. The female death rate increased over five-fold, from about 0.4% per month to about 2.2%, whereas the average male death rate increased only about 1.5-fold, to about 0.6%. In addition, some female mice were observed to be highly aggressive and hyperactive, to adduct rather than extend laterally all four limbs when picked up by the tail, to have seizures and to stare straight ahead. From these observations and results of an autopsy of a female mouse that had seizures, the UAB veterinary staff diagnosed a sudden death and seizure syndrome previously reported for FVB/N mice (Goelz et al., 1998; Rosenbaum et al., 2007; Donnelly, 2007). That this syndrome exists in some FVB/N colonies does not appear to be widely appreciated; since its original description, only a few publications have reported either its presence (Tennant et al., 2001; Silva-Fernandes et al., 2010) or absence (Buckmaster et al., 2017), and it is not mentioned on the website of Taconic, the source of our FVB/N breeders.

Injury protocols

The excitotoxic injury was produced by IP injection of KA (Tocris Bioscience) dissolved in PBS at 1 mg/ml, and was performed blinded to genotype. A multiple low dose protocol was used since we confirmed the finding of Tse et al. (2014) that this produces more consistent seizures with lower mortality than a single large bolus. The initial dose was 9 mg/kg, followed an hour later (if needed) by 2.25 mg/kg, and after another hour (if needed) by 4.5 mg/kg. These booster injections were given only if a seizure score of 5 on the scale of McLin and Steward (2006) had not been reached. In most instances this seizure level occurred after the first or second dose. All surviving animals were used for experiments. Nearly all of these reached a seizure score of at least 4, and met the requirement we set for

sample inclusion for each of the injury models of at least a 3-fold increase in endogenous *Gfap* mRNA compared to uninjured controls.

Acute physical trauma was produced by a widely used cryoinjury procedure (e.g., see Oury et al., 1993), and was performed blinded to genotype. The mouse was anesthetized with isoflurane, hair removed from the incision site, the site cleaned three times with betadine and 70% isopropyl alcohol, and a skin incision made over the dorsal midline of the skull. A 3 mm diameter brass rod probe cooled in isobutane in dry ice was placed in contact with the skull over the right cerebral hemisphere centered between bregma and lambda for 3 sec. If a transient white spot was not noted upon removal of the probe, another 3 sec contact was performed with a backup cooled probe. The skin incision was then closed with 2 surgical staples, coated with lidocaine 2% and the mouse returned to the animal facility.

A chronic gliosis model was produced by crossing WT and AP1m transgenic mice with 73-7 transgenic mice. The latter carry a *hGFAP* transgene that extends from -6,156 to +11,228, and includes all exons and introns (Brenner et al., 1990, and unpublished results)(Fig. 1). This transgene causes over-expression of *hGFAP*, resulting in formation of astrocytic protein aggregates and chronic gliosis, and serves as a model for AxD (Messing et al., 1998; Messing et al., 2001; reviewed in Brenner et al., 2009). The 73-7 transgenic mice weigh about 40% less than wild type mice when young, but only slightly less as adults. About 7% die before 5 weeks of age of unknown cause, but the rest live a normal life span.

mRNA analysis

For both the KA and cryoinjuries, tissues were harvested two days after the insult, a time when pilot studies found that the levels of both the endogenous *Gfap* mRNA and reporter *lacZ* mRNA had reached a peak that then slowly decayed over several days (data not shown). In the AxD model mice, the elevation of endogenous and reporter mRNAs extends throughout adulthood (Messing et al., 1998). Mice were anesthetized with CO₂, decapitated, and whole brains removed. Tissues were either processed immediately to isolate RNA, or stored in RNA Later (Thermo Fisher Scientific) at 4° C for 1 day and then at -20° C until used. Total RNA was isolated using STAT-60 (Tel-Test). RNA was prepared from whole brain for the KA and AxD experiments, but for the cryoinjury it was isolated from a cortical tissue punch centered on the injury site. Two coronal slices were made at bregma and lambda, and a 1.5 mm tissue punch used to obtain cortical tissue from the remaining cryoinjured right hemisphere. Visualization under a dissecting scope was used to ensure the tissue punch obtained did not contain underlying white matter. A tissue punch from the corresponding position in the contralateral (uninjured) hemisphere served as the control for cryoinjury; controls for the KA and AxD treatments were uninjured mice carrying the same transgene. In initial experiments these controls were littermates, but since it was found that non-littermates did not yield a greater variation in values than littermates, non-littermates were often used as controls in order to greatly reduce the number of mice required and the associated time and expense.

RNA was reversed transcribed using the TaqMan Gold RT-PCR kit with random hexamer primers (Thermo Fisher Scientific), and quantitative real time PCR was performed on a StepOne Real-Time PCR machine (Thermo Fisher Scientific) using Master Mix and probe/

primer sets from Thermo Fisher Scientific. The probe/primer sets were 4352339E for mouse *Gapdh*, Mm01253033_m1 for mouse *Gfap* and Hs00909238_g1 for human *GFAP*. A custom set was used for *lacZ*, consisting of forward primer AAGATGTCGACAGACGGAGGA, reverse primer GACTTGCTATTCTGTGCATC, and 6FAM labeled probe GCCGCTCATACACCATAA. These primers amplify an *mPI* sequence at the 3' end of the *lacZ* transgene, and flank the *mPI* intron (see Fig. 1). *Gapdh* and *Gfap* mRNA assays were multiplexed, whereas *lacZ* mRNA was assayed singly (trial experiments showed that *Gapdh* amplification interfered with that of *lacZ*, perhaps due to the relatively low level of *lacZ* mRNA). mRNA levels are expressed in arbitrary units normalized to *Gapdh* obtained by subtracting the *Gapdh* mRNA CT value for a sample from the CT value for the given target, using the difference as the negative exponent of 2, and multiplying by 1,000. Responses of m*Gfap* and the *lacZ* transgene to injury were assessed by comparison to controls run on the same plate.

Sample exclusion criteria

Samples were excluded if injury did not produce at least a 3-fold increase in endogenous m*Gfap* mRNA. They were also excluded if the *Gapdh* CT value was more than 2 units higher than the median for a given assay set, thereby indicating a poor RNA preparation (15 of 551 samples were excluded by this criterion); or if the *lacZ* mRNA level was less than 1/10th the median for that line, indicating a partial shut-off of transgene expression (12 samples excluded). As noted above, the seizure syndrome was presumably responsible for some of the uninjured control mice having highly elevated m*Gfap* transcript levels (Table S1). However, such samples could not be identified for exclusion because of a continuum of high to low values (Table S1), probably due to the seizures having occurred at different times prior to sacrifice, and the elevated levels of *Gfap* mRNA having decayed for variable times. Thus, samples were excluded solely on the basis of being statistical outliers, resulting in removal of just 7 of the 124 controls. As a result, some of the included KA and AxD controls almost certainly had elevated m*Gfap* mRNA levels due to the seizure syndrome. The effect of these elevated control values on interpretation of the results is described in the Discussion. In contrast to m*Gfap*, no significant seizure syndrome effect was observed for the *lacZ* mRNA values for the controls (Table S1). This is likely due to the *lacZ* mRNA half-life being just a minute or two (Pedersen et al., 2011), whereas that for *Gfap* mRNA is 10 to 20 hours (Pedersen et al., 2011; Neary et al., 1994).

Statistical analysis

After all data were accumulated, a modest increase in controls of m*Gfap* RNA level with mouse age was noted of about 7% per month (Table S1). Accordingly, when comparing the m*Gfap* mRNA level of an experimental mouse to that of the controls for the KA and AxD analyses, each control m*Gfap* value was adjusted for any age difference between it and the injured mouse sample before determining the m*Gfap* injury response for that sample. Since the ages of the control and injury mice were generally closely matched for each experiment, most corrections were quite small compared to the mouse-to-mouse variation in injury response; the median adjustment was 6%, only 9 of 111 samples had a correction > 20%, and the maximum correction was 40%. No correlations were observed among the controls between age and *lacZ* mRNA level, or between m*Gfap* level and backcross number, sex or *lacZ* level.

Two-tailed *t*-tests and ANOVA's were performed using Graphpad Prism software (San Diego, CA) and exact value of *N*, degrees of freedom, test value and exact *P*-value when >0.001 are reported in the text. For non-parametric data, Kruskal Wallace was used for ANOVA and Mann Whitney for T-tests, a Welch's corrected T-test was utilized when two groups being compared displayed unequal variances. The test used for individual data sets are stated in the text. Unless otherwise stated, all values are reported as means $\pm$ SE with *n* indicating the number of animals sampled. To calculate the significance of the effect of the AP-1 mutation on both the basal level and injury response of *lacZ* expression, pooled results for the WT lines were compared to those for the AP1m lines. The number of animals varied among the lines within each group, and the lines in each group differed in their responses; therefore, using the pooled individual values might not yield an accurate comparison. To address this possibility, statistical analyses were also performed using the median values for each line, so that the contribution of each line to the comparison would have equal weight. The *P*-values reported in Results are those obtained using these median values; however, the two methods yielded similar results with respect to significance. Both the response of the four WT lines to the three injury models and their difference from the aggregate response of the three AP1m lines was significant at $P < 0.001$ using both methods of analysis (WT response, individual values: $U = 732$, $P < 0.001$, $n = 58,92$; median values: Welch $t_{11} = 4.8$, $P = 0.001$, $n = 9,12$; WT compared to AP1m using individual values: $U = 757$, $P < 0.0001$, $n = 92,54$; median values: $U = 6.0$, $P = 0.0002$, $n = 12,9$). Similarly, using both the individual values and the median data points yielded *P*-values > 0.05 for the difference between the WT and AP1m lines for basal *lacZ* expression (individual values: $U = 497$, $P = 0.287$, $n = 47,25$; median values: $t_5 = 0.3163$, $P = 0.765$, $n = 4,3$); and for the aggregate response of the AP1m lines to injury (individual values: $U = 930.5$, $P = 0.735$, $n = 36, 54$; median values: Welch $t_8 = .74$, $P = 0.479$, $n = 7,9$).

Results

Response of wild type hGFAP promoter to injury

To determine the contribution of the *GFAP* consensus AP-1 binding site to its response to injury, we compared the fold increase in expression of a transgene carrying a wild type h*GFAP-lacZ* reporter to that of one with a mutated AP-1 site. In order to determine the success of the injury protocols, the fold increase in expression of the endogenous m*Gfap* gene was also measured. Unexpectedly, the wild type h*GFAP-lacZ* transgenes were found to respond much less to injury than the endogenous m*Gfap* gene. The injury-induced increase in *lacZ* mRNA as a percentage of that for the endogenous m*Gfap* mRNA ranged from a statistically insignificant 0.1% (cryoinjury of line WT-3) to a maximum of 43.2% (AxD model of line WT-2) (Table 1). The data in Table 1 suggest that the response of the h*GFAP-lacZ* transgenes to injury is line-dependent, and this was confirmed by statistical analysis. When the *lacZ* response data for the three injury models were combined, no statistical difference was found between the responses of lines WT-1 and WT-3 or between lines WT-2 and WT-4 (Dunn's post hoc comparison, $P > 0.999$); but there was a significant difference between the responses of both WT-1 and WT-3 compared to that of both WT-2 and WT-4 ($P < 0.0001$).

The data in Table 1 also suggest that the response of the wild type *hGFAP-lacZ* transgenes relative to that of the endogenous *mGfap* gene differed among the three injury models, being strongest for the AxD model, intermediate for KA treatment, and lowest for cryoinjury. Statistical analysis comparing the injury responses for a given wild type line found significant differences in the responses to different injuries for some comparisons, but not for others. For example, post hoc comparisons using the Tukey HSD test indicated that the response of WT-1 to AxD was significantly different from that to KA ($P = 0.034$) or cryoinjury ($P = 0.002$), but the difference between the responses to KA and cryoinjury were not significant ($P = 0.349$). When the data were pooled by injury for all four WT lines, no significant difference was found between the responses to KA and AxD ($P = 0.338$), but the responses to both of these injury models was significantly greater than that to cryoinjury ($P = 0.002$ and $P < 0.0001$, respectively).

Basal activity of the AP-1 mutated *hGFAP* promoter

It was previously reported that mutation of the AP-1 site had no effect on the basal activity of a derivative of the *hGFAP* promoter shorter than that used here (Yeo et al., 2013). That promoter, denoted ABC1D (Lee et al., 2008), contains bp -1757 to -1256 linked to bp -132 to $+47$. A similar lack of effect of the AP-1 mutation was observed in this study for the longer *hGFAP* promoters used: lines WT-1 to WT-4 had median *lacZ* mRNA levels of 1.35, 0.66, 1.15 and 2.02, respectively (average = 1.29); whereas lines AP1m-1, AP1m-2 and AP1m-3 had median values of 1.06, 3.37 and 0.26 (average = 1.56) (Table S1). Statistical analysis found no significant difference between the wild type and AP-1 mutant lines for basal *lacZ* expression ($t_5 = 0.3163$, $P = 0.765$, $n = 4,3$).

Injury response of the AP-1 mutated *hGFAP* promoter

Despite the relatively low injury response of the four WT *hGFAP-lacZ* transgenes compared to *mGfap*, each displayed a statistically significant ($P < 0.05$) increase in *lacZ* mRNA for each of the three injury models, except for the response of line WT-3 to AxD and cryoinjury (Table 1). In contrast, among the three AP-1 mutant lines, the only statistically significant responses were that of AP1m-2 to KA and AxD. Combining the data for the three injury models for each of the four WT lines showed that their aggregate response was significant (Welch $t_{11} = 4.8$, $P = 0.001$, $n = 9,12$), whereas the combined results for the AP-1 mutant lines was not (Welch $t_8 = .74$, $P = 0.479$, $n = 7,9$). In addition, the response of the WT lines significantly differed from that of the AP-1 mutant lines ($U = 6.0$, $P = 0.0002$, $n = 12,9$). Thus, presence of the AP-1 site contributes to the injury response of the *hGFAP* promoter.

Injury response of an extended *hGFAP* promoter

The weaker response to injury of the wild type *hGFAP* transgenes compared to the endogenous *mGfap* gene raises the possibility that the *hGFAP* promoter used lacks some injury-responsive elements. The *hGFAP* 73-7 transgene present in the AxD model mice includes considerably more *hGFAP* sequence than the promoters used here for *lacZ* expression (Fig. 1), and thus might contain additional injury response elements within its expanse. Whereas the *hGFAP* sequence used in WT-1, WT-2 and WT-3 promoters extends from nucleotide -2173 to $+47$, that in the 73-7 transgene extends from $-6,156$ to $+11,228$. This sequence includes all exons and introns (Brenner et al., 1990, and unpublished results),

allowing the h*GFAP* transcript to be used as the transgene reporter. To determine its injury response, h*GFAP* mRNA expression in young pups, for which gliosis is not yet significant, was compared to that in adults. The data, presented in Fig. 2, show a high signal-to-noise ratio for detection of the h*GFAP* mRNA in the AxD mice (compare the h*GFAP* values for the negative control pups to those for the 73-7 pups). The data also show only a slightly higher level of m*Gfap* mRNA in the eight day old pups with the 73-7 transgene compared to those without the transgene (1.14-fold increase), confirming little if any gliosis at this age. Comparison of expression levels in the adult 73-7 mice and pups yielded a 4.7-fold increase for m*Gfap* mRNA and a 2.8-fold increase for h*GFAP* mRNA, corresponding to a transgene response that is 49% that of the endogenous m*Gfap* gene.

Effect of age on the injury response

The broad age range for the mice used in these experiments provided both an opportunity to determine if the responses of the endogenous m*Gfap* gene and the *lacZ* transgenes changed with age, and a possible confound if a large age effect were present. As shown in Fig. 3A, there is indeed a significant age-dependence of the endogenous m*Gfap* response to the AxD injury ($P = 0.0002$), with the injury-induced increase falling from about 9-fold at 2 months to about 6-fold at 7 months. There was also a trend toward a decrease in the m*Gfap* KA response with age ($P = 0.062$), but no effect of age for the cryoinjury response ($P = 0.123$). However, the parameter of interest for this study, the *lacZ* transgene response compared to that for the endogenous m*Gfap* gene, showed no apparent effect of age for the injury responses of either the WT or AP1m mice (Fig. 3B & 3C). Thus no corrections of the injury responses were required for the age differences among the animals.

Effect of sex on the injury response

There was no difference between male and female mice in their m*Gfap* response to either the AxD model ($U = 181$, $P = 0.833$, $n = 21, 18$) or to cryoinjury ($U = 262.50$, $P = 0.643$, $n = 41, 14$). However, female mice responded significantly less strongly to KA than male mice, the median fold-increases in m*Gfap* being 9.5 and 14.9, respectively ($U = 194.50$, $P = 0.012$, $n = 22, 30$). Similar to the effect of age, the critical parameter of the KA-induced increase in *lacZ* mRNA as a percent of that for the endogenous m*Gfap* gene showed no apparent effect of sex (Welch $T_{29} = 1.757$, $P = 0.090$, $n = 12, 20$); indicating that sex similarly affected the responses of both m*Gfap* and the transgenes (data for these statistics are in Table S2). Thus no corrections were needed for differing numbers of male and female mice in the experimental groups.

Female mice were previously suggested by Twele et al. (2015) to be less sensitive to KA in certain conditions than male mice. They observed that following intrahippocampal KA injection under choral hydrate anesthesia, female mice did not display the generalized convulsions during status epilepticus observed for male mice. This difference was not present when isoflurane was the anesthesia. Our mice received KA systemically by IP, in the absence of anesthesia.

DISCUSSION

The reactive response of astrocytes is complex, including both changes that limit damage and contribute to recovery and others that exacerbate injury (reviewed in Sofroniew et al., 2009). The ability to understand and control gliosis is thus of considerable clinical interest. Due to its almost universal upregulation in response to CNS injury, transcription of the *GFAP* gene has been a common subject of these investigations. In this study, we investigated the role of AP-1 transcription factors in the injury-induced increase in *GFAP* gene activity by determining the effect of mutation of the consensus AP-1 site in the *GFAP* promoter. This work was prompted by the seemingly contradictory results that the AP-1 site was required for *GFAP* promoter activity in transfected glioma cells, but not in mice (Yeo et al., 2013). Since cultured glioma cells share properties with reactive astrocytes (Nakagawa & Schwartz, 2004; Cahoy et al., 2008), but basal promoter activity was measured in the mice, this discrepancy could be explained if AP-1 transcription factors were critical for *GFAP* upregulation in the reactive response but unimportant for its basal expression. Our observations support this conclusion. The average basal (uninjured) level of *lacZ* mRNA expression of the three AP1m transgenic lines did not differ significantly from that of the four WT lines (1.42 vs 1.31, respectively). In contrast, whereas the group response of the WT lines to the three injuries was significant ($P = 0.001$), the group response of the AP1m lines was not ($P = 0.479$). These data thus strongly suggest that the AP-1 binding site in the *GFAP* promoter is critical for the increased transcription of the gene that occurs in response to several different CNS injuries, but is not involved in its basal level of expression. A caveat is that since entire brain hemispheres were used for these measurements, it is possible that basal expression could be affected in a subpopulation of brain astrocytes or in the spinal cord.

Several previous reports have suggested that AP-1 transcription factors contribute to the increased *GFAP* mRNA and protein levels in reactive astrocytes, but did not distinguish between direct and indirect effects. For example, correlations between activation of AP-1 proteins and *GFAP* transcription were observed by Pennypacker et al. (1994), Pozas et al. (1999), Hashimoto et al. (2005), Cole-Edwards et al. (2006), Tang et al. (2006) and Zeng et al. (2011). Consistent with the results reported here, Raivich et al. (2004) found that knockout of *c-jun* in the mouse had no effect on basal expression of *Gfap*, but attenuated its increase in response to facial nerve axotomy.

Other investigations have suggested a link between AP-1 activity and *GFAP* expression by interfering with signaling. Using cultured rat astrocytes, Gadea et al. (2008) observed that treatment with endothelin led to an increase in GFAP protein that was accompanied by increased *c-jun* mRNA and protein, as well as phosphorylation of JNK and c-Jun. Treatment of the cells with either a MEK inhibitor or JNK kinase inhibitor had no effect on the GFAP increase, but when added together the increase was completely blocked. Since the MEK pathway leads to phosphorylation of c-Fos and the JNK pathway to phosphorylation of c-Jun, this finding suggested that AP-1 is required for the upregulation of the *Gfap* gene in response to endothelin. Using a cell culture scratch model for astrogliosis, Gao et al. (2013) found the increase in *Gfap* mRNA and protein dependent on an influx of calcium, which increased phosphorylation of JNK and c-Jun. Inhibition of JNK activity prevented both c-

Jun phosphorylation and the GFAP increase. Inhibitors of other mitogen activated protein kinases or JAK-STAT were without effect, indicating primacy of AP-1 in this injury model.

Our experimental manipulation was limited to mutation of the consensus AP-1 site in the *GFAP* promoter, thereby providing the first evidence for AP-1 transcription factors acting directly on the *GFAP* gene to increase its activity in response to injury. Our findings also confirm and extend a prior observation that mutation of the AP-1 binding site in a truncated h*GFAP* promoter had no significant effect on basal expression (Yeo et al., 2013), despite such mutations blocking AP-1 binding (Masood et al., 1993) and strongly inhibiting activity in transfected cells (Masood et al., 1993; Gopalan et al., 2006a & 2006b). This is consistent with suggestions that cultured astrocytes better model reactive astrocytes than resting astrocytes (Nakagawa & Schwartz, 2004; Cahoy et al., 2008).

The injury response of the four WT h*GFAP-lacZ* lines was consistently less than that of the endogenous m*Gfap* gene, ranging from an insignificant 0.1% of that of m*Gfap* for cryoinjury of line WT-3, to a high of 43% for the AxD injury model of line WT-2. The median response of the transgenes for all four lines for the three injury models was 14.8% that of the endogenous m*Gfap* gene. This value might even be an overestimate of the relative response of the transgenes, due to the presence of the seizure syndrome in our colony described in Methods. The presence of this syndrome almost certainly resulted in some of the KA and AxD controls having elevated m*Gfap* mRNA levels. This would result in the calculated m*Gfap* mRNA fold-increases in those injury models being lower than their true value. Since the transgene responses are compared to that of the endogenous m*Gfap* gene, these lower values for the m*Gfap* response would cause the relative injury response of the transgenes to KA and AxD to be overestimated. The seizure syndrome is not an issue for the cryoinjury data, since the uninjured hemisphere of the same animal was used as the control.

The blunted upregulation of *lacZ* compared to the endogenous m*Gfap* could be due to several factors, including a human/mouse species difference, cryptic negative elements in the *lacZ* reporter, or response elements residing outside of the h*GFAP* promoter used. A species difference appears unlikely from results of Mucke et al. (1991) using a transgene in which a *lacZ* reporter was embedded in the first exon of a mouse *Gfap* genomic clone. This mouse clone started at -1980 (Johnson et al., 1995), which is similar to ours (-2163), and extended downstream to about +11,000, which is similar to the termination site of our 73-7 transgene (+11,228). In response to a needle stab wound, *lacZ* mRNA encoded by this transgene reached a peak 3.4-fold higher than its basal level, whereas that for m*Gfap* increased 9.8-fold (data obtained from Fig. 4 of Mucke et al., 1991). Thus the response driven by this mouse *Gfap* promoter was about 35% that of the endogenous *Gfap* gene.

The possibility of negative elements in the *lacZ* reporter or the presence of injury-responsive elements lying further upstream of -2163 is suggested by findings of Zhu et al. (2004) using a mouse *Gfap* promoter that begins at about -12,000 and drives a luciferase reporter (the 3' end of the promoter was not specified, but presumably is near the RNA start site). They observed that KA intoxication yielded a 10.7-fold increase in luciferase mRNA compared to a 7.4-fold increase in m*Gfap* mRNA (data obtained from Fig. 2B). However, more equivocal results were obtained by Jany et al. (2013) using the same m*Gfap-luc* transgenic line when

examining the response in an AxD mouse model. This mouse model has phenotypic similarities to the 73-7 line used in our present study, but was made by inserting a human AxD-causing missense mutation into the mouse *Gfap* gene (Hagemann et al., 2006). Surprisingly, they found that the injury response of the reporter mRNA relative to m*Gfap* mRNA was highly dependent on the brain region examined. For example, the reporter increased only about 56% as much as the endogenous m*Gfap* mRNA in the olfactory bulb and hippocampus, but about 500% more than endogenous *Gfap* mRNA in the brain stem and spinal cord (data obtained from their Fig. 2). Since only a single reporter line was examined (as was also the case for the Mucke et al. (1991) and Zhu et al. (2004) studies), it is not known if this dependence on anatomical region is a consequence of the insertion site of the transgene or reflects profound regional differences in either promoter element activity or mRNA stability.

The 73-7 line of AxD model mice used as the genetic injury model for our study afforded another opportunity to examine the effect of a larger promoter segment while also providing a reporter gene, h*GFAP*, whose mRNA likely behaves similarly to the endogenous m*Gfap* mRNA. The h*GFAP* transgene in this line extends from -6,156 to +11,228 (Fig. 1). As disease developed in these mice, the level of reporter h*GFAP* mRNA increased 49% as much as the endogenous mouse *Gfap* mRNA (Fig. 2). This is a higher percentage than the response to AxD injury of any of the four WT h*GFAP-lacZ* lines studied (14.9%, 43.2%, 10.2% and 31.3% for WT-1 to WT-4, respectively), but is still well short of matching the endogenous mouse gene. This higher relative fold increase for the 73-7 line and the even greater increases for the m*Gfap*-luciferase transgene just described suggest that additional injury response elements lie outside the h*GFAP-lacZ* transgenes used in this study. This is also suggested by the observation that the responses of the h*GFAP-lacZ* transgenes relative to that of the endogenous m*Gfap* gene differed depending on the injury model. Limits for the location of such elements may be set by the presence of coding regions for unrelated transcripts. Upstream, the uncharacterized transcript JD128074 starts at -8,334; whereas downstream the coding sequence of the alternatively spliced proteins CDDC103 and FAM187A end within the 73-7 sequence at +10,110 (University of California Santa Cruz Genome Browser, <https://genome.ucsc.edu/>).

The necessity of the AP-1 site for the *GFAP* promoter response to injury appears to conflict with a large number of studies suggesting instead a prime role for STAT3 for both the increase in GFAP production and other aspects of the reactive response (reviewed in Ceyzeriat et al., 2016). Some of these reports are simple correlations, but others demonstrate that blocking the JAK/STAT pathway at various points prevents *GFAP* upregulation. These seemingly contradictory findings could have several possible explanations, although none appears entirely satisfactory.

One possibility is that the AP-1 binding site is indeed required for the upregulation, and the role of the JAK/STAT pathway is to activate the AP-1 transcription factors by cross-talk between the signaling pathways (reviewed in Rawlings et al., 2004). In one instance, a MEK1/2 inhibitor was used to ostensibly rule out a role for AP-1 (Leung et al., 2010), but its effect on AP-1 DNA binding was not monitored. This leaves open the possibility that AP-1 proteins were nevertheless activated.

A second explanation is that both the *GFAP* AP-1 and STAT binding sites are required, and act synergistically. Synergism between closely situated NF-1 and AP-1 transcription factor binding sites was noted by Gopalan et al. (2006a) for expression of the alpha1-antichymotrypsin gene in U373 glioma cells. A synergism mechanism also explains the finding that under certain circumstances JAK/STAT signaling can be strongly stimulated without increasing *GFAP* transcription, indicating that activation of STAT3 alone is insufficient (Damiani & O'Callaghan, 2007; O'Callaghan et al., 2014). However, there are several reasons why a synergy explanation is unlikely. Using a cell culture scratch model, Gao et al. (2013) observed that upregulation of *Gfap* was prevented by a JNK inhibitor, but unaffected by a JAK/STAT inhibitor. In this case the model would be tenable only if the JAK/STAT inhibitor had not been active, which was not tested. The synergism model is also inconsistent with results of Gopalan et al. (2006a) from transient transfection studies of U373 glioma cells with a *GFAP* promoter-driven CAT reporter. They observed little difference between the fold-increase induced by oncostatin M for the wild type construct (1.8 fold) and for the AP-1 mutant construct (1.7 fold) (these data were not discussed explicitly in the paper, but were obtained from their Fig. 6 bar graph). This suggests that in cultured U373 cells, at least, the STAT and AP-1 binding sites do not interact.

Similar to the analysis just discussed, a third possibility is that the conclusion that STAT3 is required for the *Gfap* injury response did not take into account that unlike AP-1, STAT3 and its *GFAP* promoter binding site are required for basal transcription of both the mouse and human *GFAP* genes (Herrmann et al., 2008; Yeo et al., 2013). The reduced level of promoter activity in response to injury when JAK/STAT signaling is inhibited could be due to a general effect on *GFAP* transcription, rather than to a reduction in the fold-increase in activity. Thus in evaluating the effect of STAT3 inhibition on the *GFAP* reactive response, the base line for comparison should be the uninjured state in the presence of STAT3 inhibition. This comparison is generally not made in reports addressing the role of STAT3 in the *Gfap* injury response, but in several instances the relevant data can be extracted from figures in the publications (Table 2). These data show that inhibition of JAK/STAT signaling actually had little effect on the injury-induced fold change in *Gfap* expression in some cases (entries 1 and 2), but in others the inhibition remained substantial, although less than that suggested in the publication.

A final possibility is that the *GFAP* promoter AP-1 binding site is required for the upregulation in response to some injuries, whereas STAT3 is required for others. This scenario seems unlikely because we found that the AP-1 site is critical for three very different modes of CNS injury—acute physical trauma (cryoinjury), acute chemical trauma (KA induced excitotoxicity), and chronic degeneration (AxD). Thus, although the pathways linking each initial injury to elevated AP-1 activity are not yet delineated, our findings indicate that they converge on activation of AP-1 transcription factors as a general feature of reactive gliosis. Similarly, activation of the JAK/STAT pathway has been considered a convergent and fundamental feature for the reactive response to a variety of CNS injuries (Ceyzeriat et al., 2016). Insults for which there is evidence that AP-1 and STAT3 activation are each essential for the reactive response include excitotoxicity (Pennypacker et al., 1994; O'Callaghan et al., 2014) and facial axotomy (Raivich et al., 2004; Tyzack et al., 2014).

Presently, none of the above explanations is entirely satisfactory to resolve the conflicting evidence for primacy of the AP-1 or JAK/STAT signaling pathways for the *GFAP* response to injury. Sorting out the distinctive contributions of each will require additional studies that include verifying the presumed effects of the pathway inhibitors used. Although the *GFAP* upregulation studied here is a universal feature of reactive gliosis, multiple other changes occur in reactive astrocytes, many of which are dependent on the particular conditions. Activation of STAT3 has been implicated in most of these other responses (reviewed in Ben Haim, et al., 2015b; Ceyzeriat et al., 2016). Given our observations for *GFAP* upregulation, it will be of interest to explore whether AP-1 transcription factors also have a role in these additional responses.

In summary, our results provide strong evidence for a critical and direct effect of AP-1 transcription factors for upregulation of the *GFAP* gene in response to injury, but that they are unimportant for its basal expression. Development of small molecule inhibitors of AP-1 protein activity is being pursued for treatment of a variety of diseases, including cancer and immune disorders (reviewed in Ye et al., 2014). Our findings suggest that such drugs could also be useful for controlling the reactive response of astrocytes. In particular, they may be therapeutic for AxS, where increased production of mutant and wild type GFAP is considered an important aspect of disease progression (Quinlan et al., 2007). In addition, our findings strongly suggest that an undiscovered regulatory site that contribute to the injury response of the *GFAP* gene resides in a region between 2.2 and 8.3 kb upstream of the h*GFAP* transcription start site. Lastly, we draw attention to the caveat of using FVB/N mice for neurological studies due to their propensity for spontaneous seizures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Astrocytes are abundant cells in the central nervous system (CNS). During CNS injury, astrocytes undergo a response called reactive gliosis, which alters their functions. Some of these changes are beneficial, but others lead to greater injury. Transcription of the *GFAP* gene increases during reactive gliosis. Understanding the mechanisms responsible for this increase will provide new ideas for how to manage CNS injury. In this study we show that a DNA binding site for AP-1, a transcription regulatory protein, is required for the injury-induced increase in GFAP. Inhibitors of AP-1 may thus prove useful for managing CNS injury.

FIGURE 1. Transgene diagrams.

hGFAP is the human *GFAP* DNA fragment used to generate the 73-7 line used as the AxD model (Messing et al., 1998). The WT-1, -2 and -3 transgene is the previously described gfa2-nlac (Brenner et al., 1994); its complete sequence is available from AddGene as part of pGfa2-nLac (Plasmid #53126). WT-4 is identical to WT-1, except that the promoter starts at bp -1757 instead of -2163. The AP1m-1, -2 and -3 transgene is identical to WT-4, except that the AP-1 site has been changed from TGACTCA to TTCAGAA. Nucleotide positions are relative to the RNA start site as +1. The positions for the AP-1 and STAT3 binding sites are the first (5') nucleotide. The diagrams are not drawn to scale.

Figure 2. Injury response of the 73-7 transgene.

The four transgene negative (control) and four 73-7 positive pups were all eight day old littermates. The adults (age in months) were each from a separate litter. The mRNA levels for *mGfap* and *hGFAP* were measured as described in Methods. The bar graph presents the average mRNA levels relative to the average for the 73-7 eight day old pups, with each individual value represented by a small circle. Vertical lines at the top of each bar represent the standard deviation. Within each mRNA type, the statistical differences between either the control or the adult group and the 73-7 eight day old pups was $P < 0.0001$ (Tukey post hoc), indicated by *** above the bar value, except that the difference for *mGfap* between the eight day control and 73-7 pups was not significant ($P = 0.402$). Note that the *hGFAP* mRNA levels for the controls were too low to be visualized. Actual numerical values are presented in the inserted table.

FIGURE 3. Effect of age on the injury responses of *mGfap* and *lacZ*.

A. Responses of the endogenous *mGfap* gene. Data are pooled for all lines. The Y-axis is the fold increase of *mGfap* mRNA compared to controls. B. Responses of the *lacZ* transgene of the WT-1, WT-2, WT-3 and WT-4 lines. The Y-axis is the increase in *lacZ* mRNA as a percent of the increase in *mGfap* mRNA for the same animal. Since different lines respond to different extents, the responses for each line were divided by the average response for that line so the data could be pooled to achieve a higher n value. Thus the *lacZ* responses have an overall average of 1.0. C. Responses of the *lacZ* transgene of the AP1m-1, AP1m-2 and AP1m-3 lines. Data were normalized as described for panel B. Linear regression was performed using GraphPad Prism version 7.02.

Table 1.Responses of h*GFAP-lacZ* transgenes to injuries.

	WT-1	WT-2	WT-3	WT-4	AP1m-1	AP1m-2	AP1m-3
KA							
n	10,11	5,6	3,3	7,12	3,7	6,8	5,5
<i>mGfap</i> fold change	7.6	7.9	4.6	11.7	12.3	14.5	10.8
<i>lacZ</i> as % <i>mGfap</i>	9.3%	28.2%	8.8%	23.6%	-2.0%	4.1%	-3.3%
<i>P</i> -value	0.016	0.045	0.032	<0.0001	0.361 [†]	0.029	0.116 [†]
AxD							
n	9,7	7,7	3,4	6,5	4,4	5,6	5,6
<i>mGfap</i> fold change	6.0	5.3	6.2	11.3	7.4	6.2	6.6
<i>lacZ</i> as % <i>mGfap</i>	14.9%	43.2%	10.2%	31.3%	-4.7%	3.4%	-2.3%
<i>P</i> -value	0.008	0.002	0.057	0.008	0.310 [†]	0.035	0.313 [†]
Cryo-injury							
n	11,11	11,5	11,15	11,6	11,6	11,5	11,7
<i>mGfap</i> fold change	13.6	13.1	14.0	10.5	8.3	4.6	9.8
<i>lacZ</i> as % <i>mGfap</i>	0.9%	14.6%	0.1%	20.2%	-3.1%	2.3%	-2.7%
<i>P</i> -value	0.036	0.002	0.186	0.001	0.615 [†]	0.070	0.124 [†]

n is number of control samples, number of injury samples. For cryoinjury, two kinds of controls were used: the levels of *mGfap* and *lacZ* mRNAs present in the injured area were compared to the levels in the corresponding region of the uninjured area (same n for injured and this control), and the fold differences obtained then compared to the fold differences between similar hemispheric regions in 11 uninjured mice (this in the n listed in the table). *mGfap* fold change is the median value for the fold change in *mGfap* mRNA. *LacZ* as % *mGfap* is the injury-induced change in *lacZ* mRNA as a % of that for *mGfap*. This was calculated separately for each sample as $100(\text{lacZ fold change} - 1)/(\text{mGfap fold change} - 1)$ and the median value reported. *P*-value is for the significance of the change in *lacZ* mRNA following injury; *P*-values > 0.05 are in gray fill, and those marked with an (†) are for the significance of the observed decrease in *lacZ* mRNA following injury. Details for statistics are as follows (line/injury): WT-1/KA: U = 21, n = 10,11; WT-2/KA: WelchT₅ = 2.660, n = 5,6; WT-3/KA: T₄ = 3.247, n = 3,3; WT-4/KA: T₁₁ = 5940, n = 7,12; AP1m-1/KA: U = 6.0, n = 3,7; AP1m-2/KA: WelchT₉ = 2.585, n = 6,8; AP1m-3/KA: U = 4.5, n = 5,5; WT-1/AxD: U = 7.0, n = 9,7; WT-2/AxD: U = 0.00, n = 7,7; WT-3/AxD: U = 0.00, n = 3,4; WT-4/AxD: U = 0.00, n = 6,5; AP1m-1/AxD: U = 4.0, n = 4,4; AP1m-2/AxD: U = 3.5, n = 5,6; AP1m-3/AxD: U = 9.0, n = 5,6; WT-1/cryo: U = 28.00, n = 11,11; WT-2/cryo: U = 0.00, n = 11,5; WT-3/cryo: U = 56.5, n = 11,15; WT-4/cryo: U = 0.00, n = 11,6; APm-1/cryo: U = 27.5, n = 11,6; APm-2/cryo: U = 11.0, n = 11,6; APm-3/cryo: U = 21, n = 11,7. The underlying data for this table are presented in Table S2.

Table 2.

Effect of STAT inhibition on basal and injury-induced *Gfap* expression.

Injury	Basal Inhib [†]	Control Increase [‡]	Inhibition Increase [§]	Inhibitor	Reference
LPS	0.38	22.4	22.3	conditional STAT3 KO	Nobuta et al., 2012
MPTP	0.14	3.5	3.4	conditional STAT3 KO	O'Callaghan et al., 2014
peripheral nerve injury	0.37	2.1	1.6	conditional STAT3 KO	Kohro et al., 2015
Huntington's disease	0.61	2.4	1.3	SOCS3 overexpression	Ben Haim et al., 2015a
facial nerve axotomy (immunofluorescence)	ND	5.8	2.5	conditional STAT3 KO	Tyzack et al., 2014
facial nerve axotomy (immunoblot)	0.54	1.5	1.0	conditional STAT3 KO	Tyzack et al., 2014

All experiments were performed in mice. All measured *Gfap* mRNA levels except those of Tyzack et al., 2014, which measured protein.

[†] Fraction of basal (uninjured) *Gfap* mRNA or protein level in the STAT inhibitory condition relative to the non-inhibited state.

[‡] Injury-induced fold-increase in *Gfap* mRNA or protein in the absence of STAT inhibition

[§] Injury-induced fold-increase in *Gfap* mRNA or protein in the presence of STAT inhibition of both the basal and injured states

Sources of data were Fig. 2b of (Nobuta et al., 2012), Fig. 6A of (O'Callaghan et al., 2014), Supplemental Fig. 2B of (Kohro et al., 2015), Fig. 8J of (Ben Haim et al., 2015a), and Fig. 11 of (Tyzack et al., 2014) for the immunofluorescence data (explicitly discussed in the text) and Fig 1m for the immunoblot data (there was no comment concerning the discrepancy between these two analyses).