Drosophila Stat92E Signaling Following Pre-exposure to Ethanol

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ABSTRACT: Repeated exposure to alcohol alters neuromolecular signaling that influences acute and long-lasting behaviors underlying Alcohol Use Disorder (AUD). Recent animal model research has implicated changes in the conserved JAK/STAT pathway, a signaling pathway classically associated with development and the innate immune system. How ethanol exposure impacts STAT signaling within neural cells is currently unclear. Here, we investigated the role of *Drosophila* Stat92E in ethanol-induced locomotion, signaling activity, and downstream transcriptional responses. Findings suggest that expressing *Stat92E-RNAi* causes enhanced ethanol-induced hyperactivity in flies previously exposed to ethanol. Furthermore, alternative splicing of *Stat92E* itself was detected after repeated ethanol exposure, although no changes were found in downstream transcriptional activity. This work adds to our growing understanding of altered neuromolecular signaling following ethanol exposure and suggests that STAT signaling may be a relevant target to consider for AUD treatment.

KEYWORDS: Alcohol, addiction, STAT signaling, transcription factor, transcript usage

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Introduction

AUD is a debilitating health condition characterized by uncontrollable drinking or seeking of alcohol.[1] Animal model research can aid AUD prevention and therapy efforts by identifying underlying genetic risk factors and biomarker-based treatment approaches. Ethanol does not act on a single molecular target, but it does directly and indirectly elicit biological effects by disrupting various cellular processes such as neuroimmune signaling and transcription factor activity.[2-4] Many immunomodulatory drugs are under consideration for treating AUD[5], but there remain many unexplored pathways in which alcohol causes lasting neurobiological changes. Recently, the Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling pathway was implicated in both *Drosophila* and mammalian models of AUD.[6-8] JAK/STAT signaling is widely studied in the context of development and innate immune signaling,[9] but it is unclear how STAT activity in the brain and alcohol-associated behavior are impacted after repeated ethanol exposure.

The JAK/STAT pathway is an evolutionary conserved pathway discovered for its role in interferon responses.[10-12] Upon activation by extracellular cytokines or growth factors, JAK receptor tyrosine kinases activate latent cytoplasmic STAT transcription factors. Activated STATs translocate into the nucleus and bind palindromic target DNA binding sites ($TTCN_{2\text{-}4}GAA$) to regulate gene transcription.[11,13] Mammals have 4 JAKs and 7 STATs, whereas *Drosophila* (hereafter "flies") have only 1 JAK, *hopscotch*, and 1 STAT, *Stat92E*.[14] The fly Stat92E is most functionally similar to mammalian STAT3, which was recently implicated in both rodent and human alcohol withdrawal.[7,8] Specifically, withdrawal increased STAT activation in astrocytic glia of the hippocampus and pharmaceutical reduction of STAT signaling relieved withdrawal-associated anhedonia behavior. *STAT3* is alternatively spliced into different functional isoforms, some with altered C-terminal transactivation domains and opposing impacts on signaling.[15,16] It is unclear how alcohol exposure impacts *STAT3* transcript usage, signaling activity, and ultimately animal behavior.

Previous studies have demonstrated that acute and repeated ethanol exposure cause transcriptomic changes in adult fly brains,[6,17-21] and various fly Stat92E transcript isoforms can impact signaling activity.[22,23] We recently found that flies that formed alcohol-associated memories show altered *Stat92E* transcript usage in their mushroom body (MB) neurons,[6] which are famously important for making associative olfactory memories. Importantly, knockdown of *Stat92E* in MB neurons results in long-term memory deficits.[24-26] *Stat92E* has 11 alternatively spliced transcript isoforms, with a notable delineation that 5 transcripts begin at exon 1, while the other 6 transcripts use exon 1a. Use of exon 1a can result in N-terminal truncation ($Stat92E^{\Delta N}$), producing a negative regulator of full length Stat92E.[22] However, another study found that Stat92E lacking both N- and C- termini ($Stat92E^{\Delta N \Delta C}$) produced a dominant-active effect in vivo.[23]

Various genetic tools have been developed in flies to investigate the spatiotemporal control of Stat92E signaling. For instance, a *10XSTAT-GFP* reporter line was created that contains 10 Stat92E binding sites upstream of enhanced GFP[27] and a line expressing a dominant-active Stat92E under the

control of a UAS promoter (*UAS-Stat92E$^{\Delta N \Delta C}$*).[23] Here, we used these, and other genetic tools and an established locomotion analysis to further characterize the role of Stat92E in flies previously exposed to ethanol.

Methods

Fly husbandry and ethanol pre-exposure paradigm

Flies were maintained in standard polypropylene vials containing the Nutri-Fly® Bloomington recipe, with final concentrations of 0.1% tegosept and 0.1% propionic acid and kept on a 12 h light/dark cycle at 25°C 40% to 60% humidity throughout experiments. Around 20 to 35 adult male flies were collected per vial by CO_2 anesthesia 2 to 4 days after eclosion and allowed 1 to 2 days to recover prior to testing. Male flies were used throughout the entirety of this work to compare findings with previous work and reduce variability caused by mating status. The following genetic strains were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN, USA): *10XStat92E-GFP* (II) (BDSC#26197), *10XStat92E-DGFP* (II) (BDSC#26199). The *UAS-Stat92E-RNAi^{GD4492}* (VDRC#43866) and *UAS-Stat92E-RNAi^{KK106980}* (VDRC#106980) strains were obtained from the Vienna Drosophila Resource Center (Vienna, Austria). The GAL4 and Tub-GAL80^{ts} lines were acquired from BDSC or gifted by Dr. Karla Kaun (Brown University, RI). All lines were maintained as homozygous or balanced stocks, although extensive backcrossing was not performed following recombination with other transgenes such as Tub-GAL80^{ts}. Unless otherwise specified, genetic crosses were performed to produce heterozygous transgenic offspring for analysis. Although the GAL80^{ts} transgene was recombined into the background with Act5c, elav, and repo drivers, all stocks were maintained at 25°C during development and 20°C to 25°C during collection and experimentation. This decision was made to potentially assess adult-specific requirement of Stat92E in the event that constitutive *Stat92E-RNAi* expression produced significant results. As shown in Figure 1A, treatment groups throughout this work consisted of either naïve, mock-treated, or pre-exposed flies. Humidified air was delivered throughout 15 min acclimation and subsequent exposures of 3 approximately 25% ethanol vapor (bubbling of 1 part 50% ethanol liquid mixed with 1 part 100% humidified air) 10 min epochs spaced by 1 h rests, similar to previous addiction-associated spaced learning and memory designs. Roughly 30 min after the final exposure flies were transferred back into food vials and stored in the incubator overnight.

Ethanol-induced locomotor challenge

Locomotion in response to ethanol exposure was measured like previously used methods in the field.[28] Briefly, flies were gently aspirated into a custom-made 3D-printed (PLA material) behavior arena with a clear plexiglass cover and white plexiglass floor (Supplemental Figure 1) (.STL file available on GitHub: https://github.com/epetrucc/Stat92E-paper-Wilson-et-al-2022-). The arena was placed on a shadowbox containing evenly spaced IR LED light strips and diffuser paper on top, then housed within a dark "behavior box" made of erected black corrugated boards. Genotypes were systematically rotated throughout the 4 circular regions of interest (ROIs), each 35 mm in diameter to reduce any location-specific effects. A fish tank bubbler (Tetra Whisper Air Pump, 60 Hz) was used to constantly stream humidified air via tygon tubing to the arena enclosed in the behavior box. Roughly 10 flies were transferred into each ROI and allowed to acclimate for 5 to 10 min, during which time humidified air was continuously perfused. Following acclimation, locomotor activity was recorded for 10 min using an overhead USB-based infrared (IR) camera (ELP HD Digital Camera, 6 mm IR 1/2.5″ 5MP lens): 2 min with humidified air (baseline), followed by 8 min of 100% ethanol vapor. After recording 1920 × 1080 pixel .mp4s at 30 frames per second, videos were trimmed, cropped, and converted using FFmpeg.[29] Code available on GitHub: https://github.com/epetrucc/Stat92E-paper-Wilson-et-al-2022-.

Tracking analysis

MATLAB-coded FlyTracker software[30] was used for tracking with manually adjusted settings to ensure each fly was detected (foreground threshold ~0.90, body threshold ~0.60). FlyTracker-generated trackfeat files with "per fly" features were analyzed in a custom RStudio script, which capitalized on the "trajr" R package.[31] Small portions of "missing" data points (due to slow video input processing) were linearly interpolated to allow seamless comparisons among multiple videos. (Code available on GitHub: https://github.com/epetrucc/Stat92E-paper-Wilson-et-al-2022-). Individual FlyTracker x,y coordinates were compiled and analyzed in RStudio. Path speed (mm/s) and distances traveled (m) per fly were averaged into 20 s time bins. To prevent confounding identity switches, data is presented and statistically assessed as n = 1 being the average of each ROI of ~10 flies, however, individual flies are also displayed for distributional consideration.

Analysis was refined to the distance traveled during the 2 to 6 min into ethanol challenge exposure. The first 2 min of ethanol exposure were not analyzed to avoid olfactory-based startle activity.[32] Similarly, the last minutes of ethanol exposure were not analyzed to avoid the impact of sedation. Finally, ethanol-stimulated locomotion was not directly compared to baseline locomotion.[33]

Reverse Transcriptase – Quantitative PCR

Total RNA was extracted from 25 to 35 whole bodies using TRIzol® (Ambion, Life Technologies). RNA was resuspended in DEPC-treated RNase-free H_2O, treated with DNase (Invitrogen DNA-free™ Kit), and nanodropped. Samples were

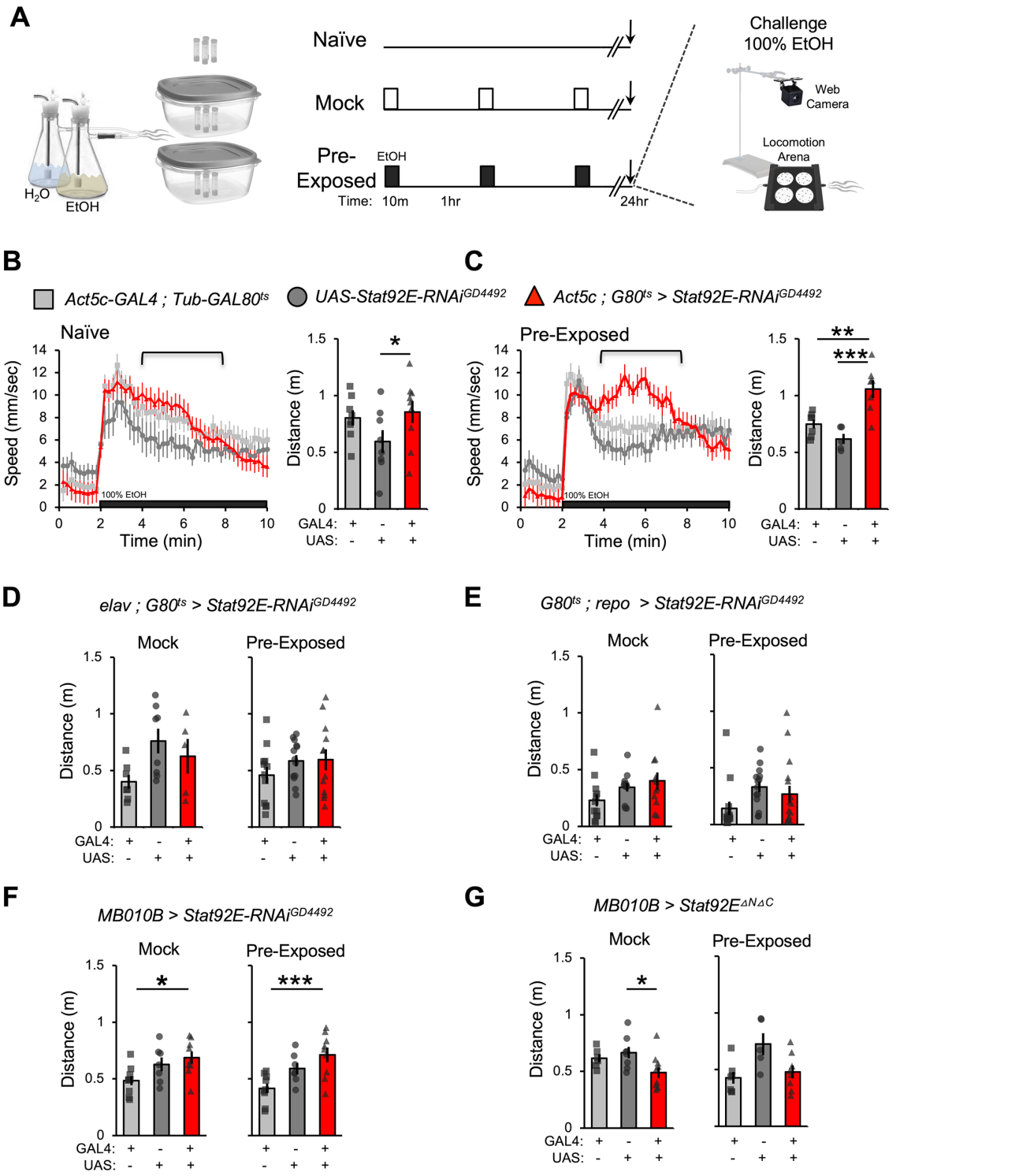

Figure 1. Role of Stat92E in ethanol-induced locomotion after pre-exposure treatment. (A) Paradigm for naïve, mock-treated, or ethanol pre-exposure prior to locomotor challenge. Flasks of water and 50% ethanol were connected to vapor tanks. Naïve flies were never introduced to vapor tanks. Mock treatment consisted of 10 min epochs of only humidified air and pre-exposure treatment consisted of 10 min epochs of ~25% ethanol vapor, each spaced by 60 min intervals. Flies were challenged 24 h later with 100% ethanol in a custom locomotion arena. (B and C) Mean speed (mm/s) across 20 s bins and mean distance traveled (m) during 4 to 8 min interval in naïve (B) and pre-exposed (C) flies. Genotypes from left to right: GAL4/+;Tub-GAL80^{ts}/+, *UAS*/+, and Act5c-GAL4;G80^{ts} > Stat92E-RNAi^{GD4492} naïve (n = 8, 8, 9) (B) and pre-exposed (n = 7, 5, 7) (C). (D–G) Mean distance traveled (m) during 4 to 8 min interval of pan-neuronal (D), pan-glial (E), and mushroom body (F) expression of *Stat92E-RNAi* or mushroom body *Stat92E*$^{\Delta N\Delta C}$ overexpression (G) in naïve (left) and pre-exposed (right) flies. Genotypes from left to right: GAL4/+;Tub-GAL80^{ts}/+, *UAS*/+, and elav;G80^{ts} > Stat92E-RNAi^{GD4492} mock (n = 7, 8, 5) and pre-exposed (n = 12, 13, 12) (D), Tub-GAL80^{ts}/+;GAL4/+, *UAS*/+, and G80^{ts};repo > Stat92E-RNAi^{GD4492} mock (n = 12, 12, 12) and pre-exposed (n = 14, 14, 16) (E), *GAL4*/+, *UAS*/+, and MB010B > Stat92E-RNAi^{GD4492} mock (n = 9, 7, 9) and pre-exposed (n = 9, 7, 9) (F), *GAL4*/+, *UAS*/+, and MB010B > Stat92E$^{\Delta N\Delta C}$ mock (n = 6, 8, 10) and pre-exposed (n = 7, 5, 8) (G). One-way ANOVA statistics, post hoc Tukey test (*$P < .05$, **$P < .01$, ***$P < .001$).

diluted to 100 ng/ul for One-Step RT-qPCR (Bio-Rad iTaq™ Universal SYBR® Green One-Step Kit) and performed in 10 µl reactions with biological (⩾4) and averaging technical (⩾2) replicates on a Bio-Rad CFX Connect Real-Time PCR Detection System. PCR conditions were performed as specified by kit instructions: 10 min 50°C; 5 min 95°C; 10 s 95°C, 30 s 60°C × 39 cycles; 10 s 65°C; melt curve 65°C to 95°C (0.5°C increments for 5 s); hold 95°C (lid 105°C). The following primers (Integrated DNA Technologies, Coralville, IA) were used:

Stat92E_Exon1-F	GGTAGTCGCGTTCGCAAAAA
Stat92E_Exon1-R	GCAGGTGTTGGGGGAAAAAC
Stat92E_Exon1a-F	TGCGCAACCAGTTGAATTCTT
Stat92E_Exon1a-R	CATTACACACACGACGCAGT
Stat92E_Exon2-F	CGCATGTATGCGAGTGCATTT
Stat92E_Exon2-R	GTGACAGCTGAATGTGTATGGTG
Stat92E_Exon4-F	CAACAATCCACCCACAGTCGAG
Stat92E_Exon4-R	GATACTCCATAGTGCTAGAGG
Stat92E_Exon8-F	CACCGCATCATGCTCAGGAAA
Stat92E_Exon8-R	AGCGCCTATCACAATTCTCTC
GFP-F	CTGGACGGCGACGTAAAC
GFP-R	CGGTGGTGCAGATGAACTT
RpL32-F	CCCACCGGATTCAAGAAGTTC
RpL32-R	AAACGCGGTTCTGCATGAG

Cycle threshold (Ct) values were determined in Bio-Rad CFX Maestro Software (version 4.1.2434.0124), using single threshold mode and baseline subtracted curve fit settings. The $2^{-\Delta\Delta Ct}$ method was used for comparative analysis whereby target expression was first normalized to *RpL32* (ΔCt), a gene that is not a known target of Stat92E nor expected to change expression levels in response to ethanol exposure,[34] or to total *Stat92E* isoforms (ΔCt), and then normalized target expression was relativized to the average of a control genotype or condition (ΔΔCt) to assess fold enrichment changes and standard error of the mean. In template dilution series experiments, primers reached >90% efficiency and all CT values had expected relative abundances—that is, the ubiquitous P4 Stat92E amplicon was greater in abundance than isoform-specific amplicons.

Immunohistochemistry, confocal imaging and analysis

Flies were lightly anesthetized with CO_2 and brains dissected in 1x phosphate buffered saline (PBS). Brains were then fixed in 2% paraformaldehyde in PBS overnight at 4°C and continuously rocked on a nutator throughout the remainder of the protocol. Brains were washed 4 × 15 min with PBS plus 0.1% Triton®-X (PBST) at room temperature and blocked in PBST with 5% normal goat serum (PBST-Goat) for 1 h at room temperature. Brains were then incubated in primary antibodies (rabbit anti-GFP (A11122 Invitrogen), mouse anti-repo (8D12 Developmental Studies Hybridoma Bank)) in PBST-Goat overnight at 4°C. Concentrations of primary ranged from 1:50 to 1:500. Brains were washed 4×15 min with PBST at room temperature and then incubated in secondary antibodies (goat anti-rabbit AlexaFluor 488 (A11008 Invitrogen), goat anti-mouse AlexaFluor 647 (A28181 Invitrogen)) in PBST-Goat for 1 h at room temperature or overnight at 4°C at 1:500 concentrations. Brains were washed 4×15 min with PBST at room temperature and mounted in DAPI Fluoromount-G® (Southern Biotech).

Images were obtained using an Olympus FluoView FV1000-IX81 confocal laser scanning microscope with Olympus FluoView software (FV10, version 4.2b). Non-saturating laser power and minimal offset settings were determined for each channel and held constant throughout each experiment. Z-sections acquired under a 20X or 60X oil objective were done at 2 and 0.5 µm depths, respectively. FIJI software[35] was used to quantify fluorescence intensity, count cells, and create max Z-stack images. Contrast and brightness settings were adjusted for visualization purposes in representative images.

Statistical analysis

Statistical analyses were performed in RStudio. For fly tracking data, one-way parametric analysis of variance (ANOVA), followed by post hoc Tukey analysis was used to compare means between groups of (not individual) flies. For RT-qPCR data, parametric t-tests were performed. For all figures, bars represent the mean with error bars representing standard error of the mean. All main and pairwise differences were considered statistically significant at $^{*}P \leqslant .05$, $^{**}P \leqslant .01$, $^{***}P \leqslant .001$.

Results

Ethanol-induced locomotion after tissue-specific changes in Stat92E expression

To determine if Stat92E has a role in ethanol-induced locomotion, ubiquitous GAL4-expressing flies (*Act5c-GAL4*) were crossed to a UAS line with RNAi targeting *Stat92E* mRNA (*UAS-Stat92E-RNAi*^{GD4492}). As others have demonstrated with null alleles and knockdown, Stat92E was essential for survival and ubiquitous expression of *Stat92E-RNAi* caused lethality. To overcome this, a ubiquitously expressed temperature-sensitive GAL80 inhibitor of GAL4 (*Tub-GAL80*^{ts}) was recombined into the background of GAL4 driver lines (See material and methods for further information). When maintaining crosses at 25°C, without performing temperature shifts, enough adults were produced for analysis. For genetic controls,

each transgenic line was crossed to a *w-* control background. Hemizygous offspring, naïve to ethanol, were then exposed to a 100% ethanol vapor challenge (Figure 1B). An initial odor-induced startle response was observed upon ethanol vapor exposure, followed by activity associated with the absorption and rapid metabolism of ethanol. Taking a conservative approach to focus our analysis, the distance flies traveled during 2 to 6 min into the ethanol exposure was quantified (bracket in Figure 1 line graphs). Although genotype had a significant effect on the distance traveled ($F = 3.535$, $P = .045$*), post hoc analysis did not reach a statistical difference between the experimental and both control genotypes (P-adj = 0.94 and = 0.055 to GAL4 and UAS controls, respectively).

Since Stat92E had been implicated in neuronal changes after previous spaced exposures to ethanol,[6] the same experiment was performed with ubiquitous expression of *Stat92E-RNAi* in pre-exposed flies (Figure 1C). Again, genotype was a significant effect ($F = 16.34$, $P < .001$***), but here post hoc analysis reached statistical significance between the experimental and both control genotypes (P-adj < .002** and < .001*** to GAL4 and UAS controls, respectively). An alternative RNAi line (*UAS-Stat92E*^{KK106980}) was also tested with similar, albeit less drastic, results (Supplemental Figure 2B). Both RNAi lines target RNA sequences found in all *Stat92E* isoforms. These findings indicate that expression of *Stat92E-RNAi* throughout the fly increases ethanol-induced locomotor activity in pre-exposed animals.

To potentially narrow down a tissue-specific role for Stat92E in ethanol-induced locomotion, RNAi was expressed pan-neuronally (*elav-GAL4*), pan-glially (*repo-GAL4*), or in memory-associated MB neurons (*MB010B-GAL4*). Speed line graphs and the breakdown of distance traveled by individual and groups of flies can be found in Supplemental Figure 2B-F, but for simplicity, distance traveled by groups of flies in the 2 to 6 min ethanol time period are shown (Figure 1D–G). Due to the significant impact of pre-exposure conditioning, subsequent experiments were performed using mock-treated instead of naïve control flies. When maintaining crosses at 25°C, genotype was not found to be significant with the distance traveled when *Stat92E-RNAi* was expressed in all neurons (Figure 1D; $F = 2.812$, $P = .08$ mock and $F = 1.34$, $P = .28$ pre-exposed) or glia (Figure 1E; $F = 2.35$, $P = .11$ mock and $F = 1.52$, $P = .23$ pre-exposed). Given these negative results, we did not pursue temperature shifts to restrict RNAi expression to adulthood.

Since broad tissue knockdown approaches may obscure prominent circuitry-specific effects on ethanol-induced behavior,[36] we did attempt MB-specific modulation of Stat92E. When *Stat92E-RNAi* was expressed in MB neurons, there was a significant effect of genotype in mock-treated flies (Figure 1F; $F = 4.94$, $P = .016$*), but post hoc analysis did not reach significance between the experimental and both control genotypes (P-adj = .0161* and =.077 to GAL4 and UAS controls, respectively). Similarly, a significant effect of genotype was observed in pre-exposed flies with *Stat92E-RNAi* expression in MB neurons (Figure 1F; $F = 8.54$, $P = .002$**; P-adj = 0.001*** and =0.27 to GAL4 and UAS controls, respectively). We also considered the potential impact of overexpressing a dominant active form of Stat92E, Stat92E$^{\Delta N \Delta C}$, in MB neurons (Figure 1G). There was a significant effect of genotype in mock-treated flies, but again post hoc analysis failed to be significant between experimental and both control genotypes ($F = 5.25$, $P = .012$*; P-adj = 0.077 and =0.011* to GAL4 and UAS controls, respectively). Similarly, there was a significant effect of genotype in pre-exposed flies with *Stat92E*$^{\Delta N \Delta C}$ overexpressed in MB neurons (Figure 1G; $F = 5.29$, $P = .014$*), but post hoc comparisons did not reach significance (P-adj = 0.7 and =0.074 to GAL4 and UAS controls, respectively). Despite these statistically negative results, there was still a notable bidirectional trend where *Stat92E-RNAi* expression tended to increase locomotion and overactivation tended to decrease locomotion.

These findings suggest that altering Stat92E ubiquitously, or potentially in MB neurons, but not broadly in all neurons or glia, influences ethanol-induced locomotion and that this effect is exaggerated in animals previously exposed to ethanol. Caution should be taken when interpreting these results, as using a more liberal binning approach, subtracting basal locomotion, or using individual fly rather than group data altered statistical findings. We also calculated the overall effect of pre-exposure as a variable by measuring the difference to naïve or mock-treated animals, but only found statistical significance in the ubiquitous *Stat92E-RNAi* experiments.

STAT signaling activity following ethanol pre-exposure

Since *10XSTAT-GFP* reporter flies are a measure of downstream transcriptional activation by Stat92E, but not its phosphorylation state or inhibitory transcriptional modulation, we broadly refer to reporter activity with the term "STAT" signal. To determine the basal state and potential impact of ethanol on STAT activity, mock-treated and pre-exposed *10XSTAT-GFP* reporter flies[27] were examined via immunohistochemistry and confocal microscopy (Figure 2). As expected from previously published works, STAT activity in control flies was relatively low and diffusely detected throughout the adult fly brain (Figure 2A). There was prominent reporter activity in a brain structure we suspect to be the anterior optic tubercle (AOTU), a region that controls vision-guided behavior.[37] STAT signal was also observed in the olfactory lobe, as has been previously documented.[38,39] In the posterior fly brain, STAT activity was observed in the Kenyon cells, also known as the MB soma, region.

Stat92E is known to function in both neurons and glia of the adult fly brain. Thus, to determine whether the STAT activity was occurring in glia, we examined the overlap of GFP + reporter signal with repo, a glial marker (Figure 2). In

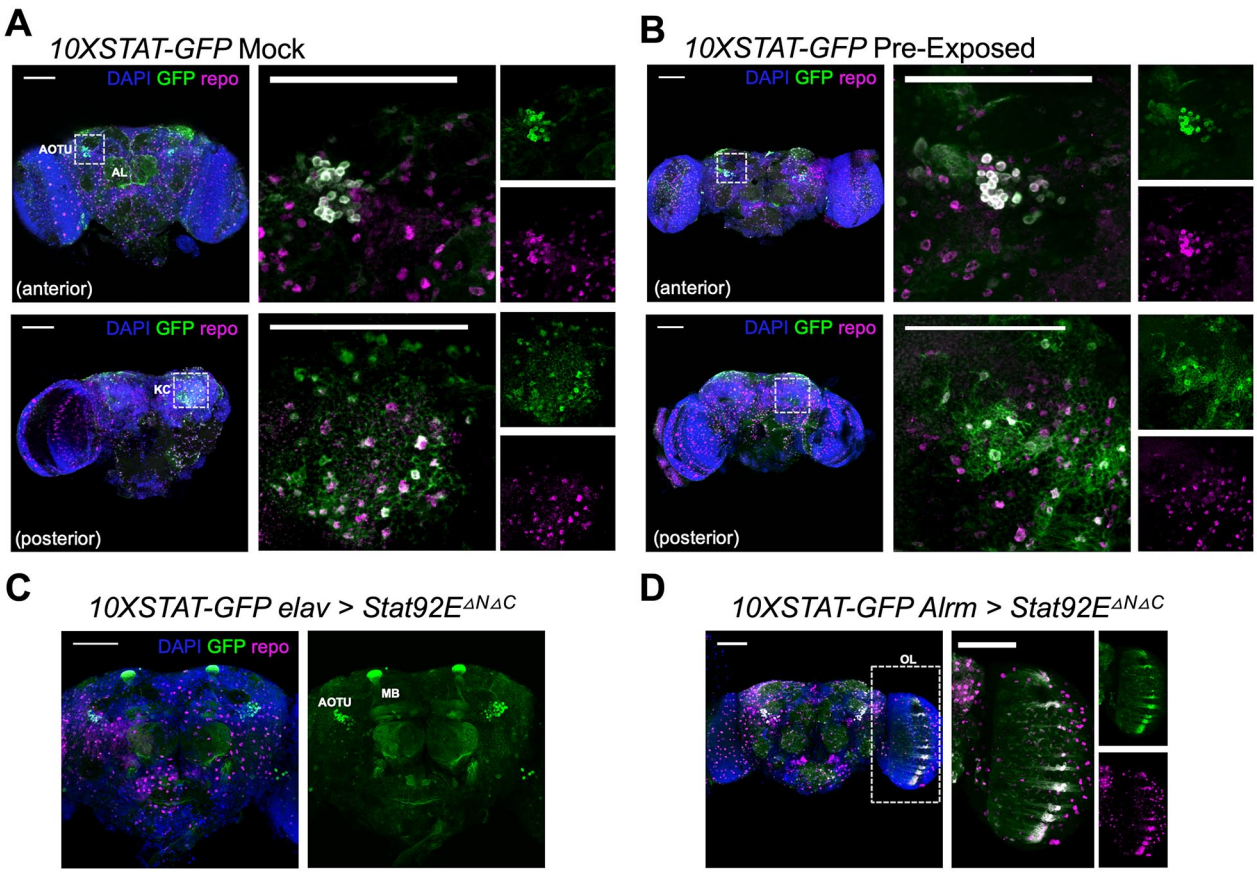

Figure 2. STAT reporter activity in adult brains. (A and B) Representative anterior (top) and posterior (bottom) confocal stack images of *10XSTAT-GFP* adult brains after Mock (A) or Pre-exposure (B) treatment. (C and D) Representative anterior confocal stack images of *10XSTAT-GFP elav* >Stat92E$^{\Delta N\Delta C}$ (C) and *10XSTAT-GFP Alrm* >Stat92E$^{\Delta N\Delta C}$ (D). For all images DAPI (blue) labels DNA, anti-GFP (green) reporter of STAT activity, and anti-repo (magenta) identifies glia. White indicates GFP + repo+ cells; dashed squares denote inset regions for closer analysis of STAT activity; scale bars 50 μm. AOTU, anterior optic tubercle; KC, Kenyon cells; MB, mushroom body; OL, optic lobe.

general, many of cells in the AOTU region were co-labeled, whereas cells in the Kenyon cell region were singularly-labeled. These findings suggest that the cells in the AOTU region with high STAT activity were likely glia generated by the DALv2 neuroblast lineage[37] and that STAT activity occurs in neurons within the Kenyon cell region. Together, these findings demonstrated that in a basal state STAT signaling is active, or was recently active, in both adult neurons and glia.

Next, we pre-exposed reporter flies to determine if repeated ethanol had a lasting impact on STAT activity in the adult brain (Figure 2B). No consistent changes were observed in pre-exposed reporter flies, anteriorly or posteriorly. Both the AOTU and Kenyon cell regions in pre-exposed reporter flies showed similar GFP + repo+ patterns as mock-treated controls. These findings suggest our pre-exposure paradigm did not alter the level or pattern of STAT activity in the adult brain.

To verify that changes in STAT activity could indeed be detected in the reporter line, we overexpressed dominant active Stat92E$^{\Delta N\Delta C}$ in either neurons (*elav-GAL4*) or astrocyte-like glia (*alrm-GAL4*) (Figure 2C and D). Interestingly, neuronal overexpression produced more overall signal, but restricted GFP expression, particularly in alpha/beta lobes of the MB (Figure 2C). This suggests that various negative regulators of STAT signaling may reduce STAT activity throughout most neurons, but that alpha/beta lobes of the MB neurons are sensitive to STAT overactivation. Glial overexpression also produced more GFP signal, including within the glia of the optic lobe (Figure 2D), suggesting that many astrocyte-like glia are sensitive to STAT overactivation. These results ultimately demonstrate that the *10XSTAT-GFP* reporter was at least capable of modulation.

Real-time RT-PCR of Stat92E following ethanol pre-exposure

Although no detectable ethanol-induced changes in STAT activity were identified via immunohistochemical analysis, the question remained whether pre-exposure might impact *Stat92E* mRNA processing and downstream reporter activity. According to FlyBase.org, *Stat92E* is highly expressed in various adult fly tissues, such as the fat body, gut, and heart.[40] Therefore, we first sought to determine whether pre-exposure to ethanol would alter *Stat92E* isoform usage or reporter *GFP* levels in whole flies. We first created primer pairs to detect non-coding UTR, isoform-specific, and ubiquitously-included exons of *Stat92E*

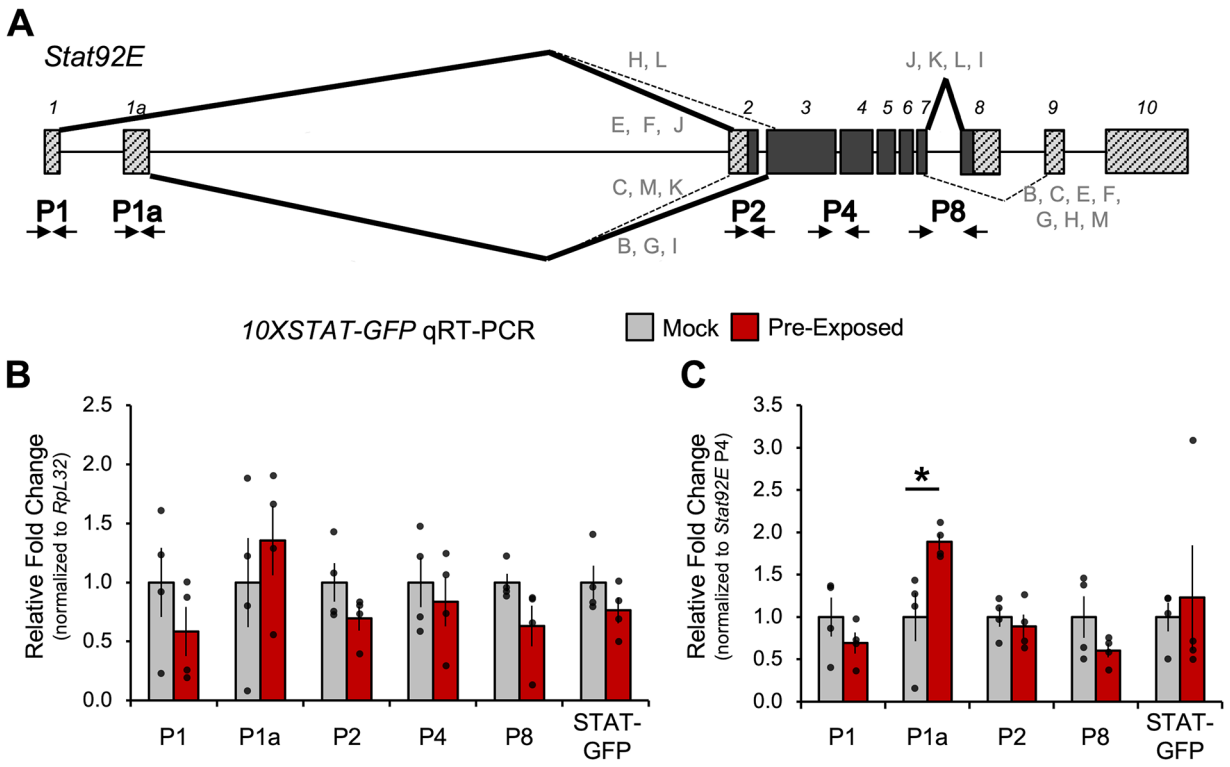

Figure 3. Quantification of *Stat92E* transcript isoforms. (A) Schematic of *Stat92E* pre-mRNA showing known alternative splice sites, coding exons (solid gray), non-coding exons (striped gray). Letters correspond to *Stat92E* transcripts, exon numbers labeled, transcript-distinguishing major (thick line) and minor (dashed line) splice junctions. (B and C) Relative fold change of primer pair combinations in adult whole *10XSTAT-GFP* reporter flies following mock or pre-exposure treatment (n = 4, 4). Fold change was normalized to *RpL32* (B) or *Stat92E* P4 (C) and relativized to mock control. Two-tailed student's *t*-test statistics (*$P < .05$).

(Figure 3A). Primers were tested and correct amplicons were confirmed via PCR with a DNA template (Supplemental Figure 3A). Next, RNA was extracted, reverse-transcribed into cDNA, and used to verify expected amplicons after RNA processing (Supplemental Figure 3B). We confirmed the specificity of our RNA isolation method, because this analysis included intron-spanning primer sets (P4 and P8) as well as negative controls that should not produce an amplicon from processed RNA transcripts or from short PCR amplification time (P1/P1a and P2/P4).

To quantify isoform changes in response to pre-exposure, RNA was isolated from reporter flies subjected to mock- or pre-exposure and used in One-Step-RT-qPCR with fold changes determined using the $2^{-\Delta\Delta Ct}$ method (Figure 3B and C). Upon normalization to *Rpl32*, no statistical differences were identified as a result of pre-exposure to ethanol, likely due to extensive variability across replicates. There was, however, a tendency toward increased usage of exon 1a-containing transcripts in response to pre-exposure. To better consider transcript usage given the total amount of *Stat92E* transcripts, results were re-analyzed using the ubiquitous P4 amplicon for normalization (Figure 3C). This approach further supported the possibility that pre-exposure to ethanol increased the use of exon 1a ($T = -2.94$, $P = .047$*). There was also a tendency, although not statistically different, in reduction of the *10XSTAT-GFP* reporter following pre-exposure. Together, these findings suggest that pre-exposure to ethanol can alter *Stat92E* mRNA transcript usage by increasing the use of exon 1a and possibly reducing STAT signaling in the whole fly.

Discussion

In this work we investigated the role of *Drosophila* Stat92E in ethanol-induced locomotion and the impact that previous ethanol exposure has on *Stat92E* mRNA processing and downstream signaling activity. We found that ubiquitous expression of *Stat92E-RNAi*, particularly in animals previously exposed to ethanol, significantly increased ethanol-induced locomotion. Furthermore, when decreasing or increasing Stat92E in MB neurons, flies showed a bidirectional tendency to increase or decrease ethanol-induced locomotion, respectively. Previous studies have demonstrated that dialing up or down either innate immune or dopaminergic signaling bidirectionally modifies naïve sedation response[41] and ethanol-induced hyperlocomotion,[42] respectively. It is unclear how these signaling pathways intersect and change upon repeated ethanol exposure, but insights may be gained by considering dopamine as an immune transmitter.[43] Considering the major impact of ubiquitous rather than cell-type specific modulation of Stat92E, there is likely a role for Stat92E in non-neural tissues, such as the fat body, in affecting ethanol-induced behavior. Another possibility is that aberrant immune regulation ultimately triggers a host of

blood brain barrier and neurodegeneration issues that reduces plasticity and recovery.[44] In mammals, previous exposure to addictive drugs can produce hyperactivity when subsequently challenged with the same drug or an associated cue, a characteristic known as behavioral sensitization.[45] Our findings suggest that Stat92E signaling may contribute to this behavioral state and could thereby influence predisposition toward addiction.

Our immunohistochemical analysis of STAT reporter animals did not suggest any changes in STAT activity or localization following pre-exposure to ethanol (Figure 2). Conclusions from this finding are complicated by the developmental contribution STAT signaling and different cell-specific turnover rates of GFP. Destabilized GFP (DGFP) produces signal for up to 8 h,[46] and the *10XSTAT-DGFP* reporter[27] line showed little to no signal in the adult brain, nor did DGFP reporter levels change after our ethanol pre-exposure treatment (data not shown). A newer strategy using dual destabilized and stable fluorescent reporters for transcriptional reporting[47] may help future experiments delineate the spatiotemporal dynamics of STAT signaling following repeated ethanol exposure.

To our knowledge, we are the first to report STAT activity during the overexpression of dominant active Stat92E$^{\Delta N \Delta C}$. STAT activity was increased upon either pan-neuronal or pan-glial overexpression, suggesting that the pathway is inducible in both adult cell types. Interestingly, though, only subsets of cells showed consistent STAT sensitivity. The alpha/beta, but not gamma or alpha'/beta', lobes of the MB consistently had STAT activity upon STAT overactivation in all neurons. Previous work has demonstrated that Stat92E in these neurons is required for long-term memory.[25] Together this information supports a model where repeated ethanol exposure modifies a vulnerable JAK/STAT pathway that ultimately contributes to lasting addiction-associated behavior.[26,48] Translationally, this model is similar to recent work by Lasek and colleagues, showing mammalian STAT3 activation in hippocampal and prefrontal cortex regions during a state of alcohol withdrawal, although their findings place STAT activity in astrocytes.[7,8] There is also an intriguing link in *Drosophila* between Jun amino-terminal kinase (JNK) immune signaling and self-medication of alcohol in response to parasitoid wasps.[49,50] This suggests a possible coevolution of host-parasite immune challenges[51] and ethanol-seeking behavior. Further work could directly assess how pharmacologic approaches to inhibiting either STAT or other innate immune pathways influence the prevention, progression, and treatment of alcohol addiction-like behavior in animal models.

To determine *Stat92E* transcript usage following pre-exposure to ethanol, we performed RT-qPCR with whole fly tissue (Figure 3). There was a significant increase in detection of exon 1a following ethanol pre-exposure. The alternative transcriptional start site of *Stat92E* has been previously reported,[22,23] and was even detected from RNA-seq of MB neurons in the context of ethanol exposure.[6] A possible method to explain *Stat92E* promoter control is via self-regulation, as a search with JASPAR[52] for possible Stat92E binding sites (ID: MA0532.1) predicts 4 high confidence matches: 1 site within exon 1, 2 sites between exon 1 and 1a, and 1 site just downstream of exon 1a. These loci provide interesting candidates in which to explore the differential impact of ethanol pre-exposure via ChIP-PCR or ChIP-seq. Perhaps the long-lasting neuromolecular changes induced by ethanol are the result of chronically altered transcriptional feedback signals. It is also important to consider cell-specific chromatin landscapes and the epigenomic changes that ethanol exposure can elicit on sensitive circuitry.[53,54] Lastly, the molecular impact of STAT signaling and its inhibition or promotion of downstream targets can provide a more comprehensive view of how repeated ethanol exposure alters neurobiological processes. Using "big data" from *Drosophila* models of AUD is an undeniably powerful approach for identifying novel, or characterizing previously implicated, ethanol-induced transcriptional effects.[55]

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Supplemental Material

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