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Amygdala DCX and blood Cdk14 are implicated as cross-species indicators of individual differences in fear, extinction, and resilience to trauma exposure

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Abstract

Doublecortin (DCX) has long been implicated in, and employed as a marker for, neurogenesis, yet little is known about its function in non-neurogenic brain regions, including the amygdala. This study sought first to explore, in rodents, whether fear learning and extinction modulate amygdala DCX expression and, second, to assess the utility of peripheral DCX correlates as predictive biomarkers of trauma response in rodents and humans. Pavlovian conditioning was found to alter DCX protein levels in mice 24 hours later, resulting in higher DCX expression associated with enhanced learning in paradigms examining both the acquisition and extinction of fear ($p < 0.001$). This, in turn, is associated with differences in freezing on subsequent fear expression tests, and the same relationship between DCX and fear extinction was replicated in rats ($p < 0.001$), with higher amygdala DCX levels associated with more rapid extinction of fear. RNAseq of amygdala and blood from mice identified 388 amygdala genes that correlated with DCX ($q < 0.001$) and which gene ontology analyses revealed were significantly over-represented for neurodevelopmental processes. In blood, DCX-correlated genes included the Wnt signaling molecule Cdk14 which was found to predict freezing during both fear acquisition ($p < 0.05$) and brief extinction protocols ($p < 0.001$). High Cdk14 measured in blood immediately after testing was also associated with less freezing during fear expression testing ($p < 0.01$). Finally, in humans, Cdk14 expression in blood taken shortly after trauma was found to predict resilience in males for up to a year post-trauma ($p < 0.0001$). These data implicate amygdala DCX in fear learning and suggest that Cdk14 may serve as a predictive biomarker of trauma response.

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INTRODUCTION

Doublecortin (DCX) is a broadly-conserved microtubule-associated protein whose function in neuronal migration was identified due to its role in X-linked lissencephaly and subcortical band heterotopia (double cortex syndrome) [1]. DCX's contribution to neuronal development and motility derive from its regulation of microtubule bundling and polymerization, and it has been shown to be a critical regulator of nuclear translocation, dendritic extension, and growth cone formation [2–5]. Given its involvement in nervous system development and its robust expression in migrating neuroblasts, DCX rapidly gained prominence in the field of adult neurogenesis as a marker of immature neurons within neurogenic niches. However, over the past two decades a growing volume of work has reported DCX expression in non-prototypically neurogenic regions of the adult brain, including the amygdala and adjacent structures [6–8].

Although relatively little work has been published on the specific contributions of DCX to the function of non-mitotic neurons, DCX has been proposed to play a role in synaptic stabilization at the neuromuscular junction [9]. A second study exploring the role of microRNA-129–5p, of which DCX is a target, likewise found that microRNA-129–5p's capacity to downscale excitatory synapses was impaired when DCX expression was maintained [10]. Existing evidence suggests that DCX expression in non-newly-generated neurons is variable and modulated in response to stress [8] [11]. Notably, two recent publications have provided support for the hypothesis that DCX function may be important for affective regulation and development in humans. Work by Alvarez-Buylla and colleagues confirmed past reports of DCX-expressing cells in the human amygdala and paralaminar nucleus and extended these findings with evidence of ongoing changes in DCX expression throughout adolescence that differed among individuals with autism spectrum disorder [12]. In their recent genome-wide association study of 11,492 U.S. soldiers, Stein and colleagues reported that a locus upstream of doublecortin-like kinase 2 (DCLK2) was significantly associated, at a genome-wide level, with resilience following deployment [13]. Like DCX, DCLK2 is involved in dendritic remodeling and growth cone dynamics and, in rodents, has been shown to partially compensate for the loss of DCX [14] [15]. Still, in the absence of any experimental characterization of DCX's relationship to behavior, its potential relevance to non-neurogenesis-related processing remains unclear.

The amygdala occupies a central role in mediating the affective repercussions of trauma exposure, and in particular the persistent over-expression of fear that typifies Post-Traumatic Stress Disorder (PTSD). Its contributions to fear learning and expression rely upon convergent inputs from sensory cortex and thalamus to the lateral nucleus, as well as modulatory afferents into the basal nucleus from limbic and precortical regions. Plasticity in the form of long-term potentiation at thalamocortical-basolateral amygdala synapses is required for both the acquisition and expression of conditioned fear, and distinct subpopulations of basolateral neurons seem to either facilitate (“fear on” neurons) or inhibit (“fear off” neurons) fear responding via excitation of distinct intra- and extra-amygdala targets [16] [17]. Although recent work has only begun to illuminate the complexity and heterogeneity of the amygdala's cellular composition [17], our relatively detailed understanding of the gross circuits involved in classical fear conditioning, and its

well-documented involvement in fear-related disorders, make this region ideal terrain for examinations of molecular contributions to fear learning.

The present series of studies sought first to elucidate how DCX expression within post-mitotic neurons of the amygdala, a brain region crucial to affective processing and learning, may relate to fear learning and expression. Subsequent work focused on identifying a peripheral marker with comparable dynamics that might serve as a clinically-useful predictor of traumatic learning in humans.

METHODS

Mice & Mouse Behavior

Male, 7 week old c57BL/6 mice were acquired from Jackson Labs (Bar Harbor, ME, USA). Fear conditioning consisted of 5 pairings of a 30 second single-frequency tone at 85db with a co-terminating 0.5 second, 0.6 mA foot-shock delivered via electrified grid flooring. 6 kHz tones were employed for most fear conditioning experiments, however, for studies exploring fear expression to non-conditioned auditory stimuli, conditioned and non-conditioned (testing) tones ranged from 2.2 and 12 kHz. Fear extinction was completed in a second, novel context 1–14 days after conditioning and consisted of 15–45 CS tone presentations. All subsequent fear expression, generalization, or re-acquisition tests were performed in a third novel context, and freezing behavior was captured and analysed using FreezeFrame 3 software (Actimetrics, Wilmette, IL, USA). Sample sizes were chosen based on behavioral effect sizes from other published studies from our laboratories [17–19, 25, 40–41]. For all animal studies the investigator was blinded to condition at time of behavioral analysis. See Supplementary Materials for additional details.

Rats & Rat Behavior

Adult male Sprague-Dawley rats were either purchased from the Animal Resources Centre Western Australia or bred at The University of New South Wales. Rat housing, behavioral studies, and analyses were completed as previously described [18]. At the time of testing, the male rats were 60–120 days of age and weighed between 300–575g. Auditory fear conditioning, extinction, and extinction retention testing of were completed on consecutive, days 24 hours apart. Conditioning employed 5 CS-shock pairings consisting of 10 seconds of white noise co-terminating with a 1s 0.4mA shock. Fear extinction training was completed in a novel context, and presentation blocks of 3 CSs employed for manual freezing analyses.

Protein isolation & Immunoblotting

10ug of total protein isolated from fresh-frozen mouse and rat tissue punches centered on the amygdala was resolved using SDS polyacrylamide gel electrophoresis then transferred to PVDF membranes. Immunoblots were probed for DCX (Cell Signaling Technologies, Danvers, MA, USA; 1:2000) and GAPDH conjugated to horseradish peroxidase (Cell Signaling Technologies, Danvers, MA, USA; 1: 2000). Bands were visualized using a SuperSignal West Dura Extended Duration chemiluminescent kit (Thermo Scientific,

Rockford, IL, USA) and DCX expression was normalized to endogenous GAPDH expression (Supplementary Figure 1). See Supplementary Materials for additional details.

Mouse RNAseq

RNA extraction, QC, library preparation and sequencing were conducted by the Yerkes Non-Human Primate Genomics Core (Atlanta, GA) as previously described [19]. Quality scores and read characteristics of sequencing data were assessed with FastQC, and Cutadapt 1.4.2 was employed to trim low quality base calls and remove adapter sequences [20] [21]. Alignment to the mm10 UCSC Mouse Assembly (GRCm38) was performed using STAR (version 2.5.3), genomic alignment completed with STAR, and the cufflinks suite (version 2.2.1) was used to calculate differential gene expression [22] [23]. Initial RNA seq data is presented in GEO from prior analyses [19]: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE116630>. See Supplementary Materials for additional details.

qRT-PCR

qRT-PCR samples were run in duplicate, triplicate, or quadruplicate 3–10 ng reactions using custom SYBR probes (ThermoFisher Scientific, Waltham, MA, USA) (see Supplementary Table 1 for primer sequences) on the ABI 7900HT Fast Real-Time PCR System. qRT-PCR data were analyzed using the 2^{-Ct} method on Sequence Detection System software (Applied Biosystems, Waltham MA, USA). See Supplementary Materials for additional details.

Human Participants

Participants were patients enrolled from the emergency department (ED) of Grady Memorial Hospital in Atlanta (GA) who had experienced a traumatic event within the past 24 h. Participants were included if they spoke English, were 18–65 years of age, endorsed a criterion A trauma as defined by the DSM-IV-TR, and provided contact information for follow-up visits. Exclusion criteria included previous hospitalization for mental health reasons, current suicidal ideation, attempted suicide in the past 3 months, current intoxication, or altered mental status during the ED visit. Informed consent was obtained from all research subjects, and all procedures were approved by the Institutional Review Board of Emory University School of Medicine and the Grady Health Systems Research Oversight Committee. Note that more detail is provided related to the enrollment and participants from two recent additional analyses from this study. [47–48]

Questionnaires

Total scores on the modified PTSD Symptom Scale (PSS), Beck Depression Inventory (BDI), and Connor-Davidson Resilience Scale (CD-RISC) were evaluated with regards to blood Cdk14 expression. The CD-RISC is a 25-item self-report scale designed to assess resilience-related characteristics including self-esteem, stress-coping, action-orientation, and adaptability, among other factors. Scores on the CD-RISC correlate negatively with measures of stress vulnerability, and are lower among individuals with PTSD and anxiety disorders than non-traumatized, psychiatrically-healthy populations [26].

RNAseq of Human Samples

RNAseq and questionnaire data were collected from males recruited as part of the Grady Trauma Project, a larger study investigating genetic and environmental risk factors for post-traumatic stress disorder (PTSD) as previously described above. Blood samples were collected following admission to the emergency department after trauma exposure, and questionnaires completed during follow-up assessments at 1, 3, 6, and 12 months post-trauma. Venous blood was collected in Tempus Tubes (RNA, Applied Biosystems) in the ED in the immediate aftermath of trauma exposure. After RNA extraction, mRNA libraries were created using the TrueSeq preparation kit and sequenced on the Illumina HiSeq 2000. Reads were aligned to GRCh37 and the Limma package was used for normalization, converting raw counts to log-CPM, adjusting for library sizes, and reducing heteroscedasticity.

Statistical Analyses

All statistical analyses for behavioral, protein, qRT-PCR, and questionnaire data were completed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and SPSS version 19.0 (IBM, Armonk, NY, USA). For all comparisons between “High” and “Low” expressor groups, animals were segregated using a simple median-split approach. In the case of oddly-numbered groups of animals, the median value was excluded from analyses. Note that a median split procedure used for mice and human samples, but in some cases, as outlined in results, a slightly different procedure used for the rat samples. One-, Two-, or Three-Way ANOVAs were employed for all multigroup analyses, and Tukey’s tests employed for posthoc comparisons where appropriate. Pearson’s correlations were employed to assess bivariate correlations. Analyses of the relationship between self-report questionnaire scores and gene expression data from human participants were likewise completed using bivariate correlations and 2-way ANOVA. For RNAseq correlation analyses of amygdala gene expression and inclusion in subsequent gene ontology analyses, the false discovery rate (FDR) was set at 0.1% ($q < 0.001$). To identify candidate DCX correlates in blood, we employed more permissive criteria and selected 6 candidate genes from among the 10 most significant hits ($q = 0.0078$ – 0.1018) for subsequent experimental validation.

RESULTS

Amygdala DCX protein is associated with individual differences in fear learning in mice

Evaluation of DCX protein levels in the amygdala 2, 4, 6, and 24 hrs after fear conditioning, and 24 hrs after extinction training or retention testing did not reveal any group differences in expression ($F = 1.033$, $p = 0.4126$) (Figure 1a), nor did DCX expression differ between hemispheres ($F = 0.3334$, $p = 0.5693$) (Figure 1b). Both mice exposed to paired (FC group; 95% CI 31.35–21.38, $p < 0.0001$) and unpaired shocks and tones (SHK group; 95% CI 31.61–21.64, $p < 0.0001$), displayed significantly more freezing than did unshocked tone-exposed controls (TEX) (Figure 1c). Assessed as a whole, the data presented in Figure 1 suggest that fear expression, per se, may not be associated with DCX levels in the amygdala at the group level.

Of note, despite the absence of group difference in DCX protein expression, amygdala DCX levels were significantly associated with freezing within groups when cue conditioned (FC)

and context conditioned (SHK only) mice were subdivided via median-split into high vs. low DCX expressors, suggesting robust individual differences within each behavioral group. We found that mice with higher DCX levels in the amygdala 24 hrs after training were found to have displayed more freezing during fear acquisition ($F = 211.2$, $p < 0.0001$) (Figure 1d). DCX protein 24hrs after training correlated positively with total freezing during acquisition in both FC ($R^2 = 0.7042$, $p = 0.0092$) and SHK groups ($R^2 = 0.5454$, $p = 0.0364$) (Figure 1e–f). No such relationship was observed among TEX control animals at 24 hrs after tone exposure ($R^2 = 0.0937$, $p = 0.5167$, nor among mice sacrificed within 6 hrs of training ($R^2 = 0.0008$, $p = 0.8788$) (Figure 1g–h). These findings suggest that individual differences in fear acquisition result in differential modulation of amygdala DCX protein expression and that this process requires more than 6 hrs to occur. Thus, although learning does not alter the mean amygdala DCX protein expression of fear conditioned animals as a group, learning does modulate individuals' DCX expression so that an individual's position within the distribution may change.

In order to more specifically assess the relationship between amygdala DCX regulation and learning, mice were exposed to fear acquisition, expression, and extinction training, followed 1 week later by fear generalization testing immediately prior to sacrifice (Fig 1i). Interestingly, animals with high DCX expression 1 week after extinction (and 2 hours after generalization) displayed less freezing during their extinction training ($F = 30.1$, $p < 0.0001$) (Figure 1j). These data suggest that the DCX levels may be most associated with the level of plasticity or learning potential within the most recent behavioral paradigm, as opposed to directly representing valence (e.g., fear or inhibition of fear) itself.

If the levels of amygdala DCX protein represent something akin to “learning potential”, defined here as one's individual capacity to form or modify associations between a cue and outcome, then greater susceptibility to subsequent fear re-training to a reinforced cue after extinction may also be associated with increased DCX. Consistent with this hypothesis, we found that freezing during subsequent exposure to a previously- conditioned stimulus (CS), as well as to non-conditioned stimuli (NCS) that differed in frequency from the CS, was also associated with increased levels of DCX expression ($F = 13.93$, $p < 0.001$) (Figure 1k), similar to the initial fear acquisition result. Importantly, since DCX levels were assessed 1 week following extinction training but within 2 hrs of generalization testing, these results suggest that post-training amygdala DCX expression might influence future fear behavior. Overall, these initial data in Figure 1 suggested that, rather than reflecting the strength of fear acquisition itself, the DCX protein levels in amygdala may best represent an animal's learning potential for amygdala-dependent processes.

Fast- and slow-extinguishing rats display differences in amygdala DCX expression

Having established a relationship between learning and amygdala DCX regulation in mice, we next sought to validate and expand our findings in a second rodent model: rats. Work by King and colleagues had previously found that fast and slow extinguishers differ in their propensity for fear renewal, reinstatement, and spontaneous recovery following mild, but not strong, relapse protocols [18]. We hypothesized that DCX may also be a marker for rate or efficacy of extinction in this rat model as it was in mice (Figure 1j, above).

In examining the rat samples and behavior, a consistent threshold was set for fast and slow extinguishers across several experiments, including those from which the behavior and brains were analyzed. Examining amygdala DCX levels in rats revealed that, as in mice, DCX expression was significantly higher among fast extinguishers who were sacrificed 24 hrs ($F = 21.25$, $p < 0.0001$), but not 2 hrs after extinction training ($F = 0.2449$, $p = 0.6217$) (Figure 2a–b). Chi-square analyses further confirmed that rats characterized as fast extinguishers (rats requiring 13 blocks of 3 CS presentations) were significantly more likely to fall within the high-DCX group than were rats characterized as slow extinguishers (rats requiring 16 blocks of 3 CS presentations to reach extinction criterion) ($X^2 = 10.94$; $p = 0.0121$) (Figure 2c). Extinguished rats subjected to a single CS 24 hrs after extinction and sacrificed 2 hrs later (26 hrs after extinction) again showed a trend towards less freezing in response to the CS when amygdala DCX expression was high than when DCX levels were low, supporting the hypothesis that higher DCX is associated with more robust extinction ($F = 3.454$, $p = 0.0665$) (Figure 2d).

Given the previously-reported association between protocol strength and sensitivity to fear relapse among fast- and slow-extinguishers, we next employed correlational analyses to explore whether the robustness of extinction protocols might mediate the relationship between freezing and amygdala DCX protein levels in these same animals. Among rats trained with a shorter extinction protocol, the onset of extinction—defined as the last instance of complete immobility during CS presentation—was significantly correlated with amygdala DCX levels 24 hrs later ($R^2 = 0.1214$, $p = 0.0402$). Longer extinction protocols, however, resulted in no correlation between freezing and amygdala DCX levels ($R^2 = 0.01336$, $p = 0.4604$) (Figure 2e–f). These data suggest that DCX as a marker of plasticity or learning potential may be most associated with learning in a sub-optimal paradigm, whereas with more prolonged learning there may be a ceiling effect such that the level of DCX is less important for amygdala-dependent learning.

A large number of plasticity-related genes are regulated in tandem with DCX following fear learning

We next examined whether DCX is a marker of a set of genes related to learning potential, that together may further our understanding of which neurons may be incorporated into a new memory engram, as well as to potentially provide molecular markers of neural plasticity. In order to determine what genes might be co-regulated with amygdala DCX, we employed correlational analyses of RNAseq data from control, fear conditioned, and extinguished mouse amygdala punches taken 2 hrs after training. DCX mRNA expression in the amygdala differed from unmanipulated controls 2 hrs after fear learning, suggesting at least transient modulation of DCX (Supplementary Figure 2). RNAseq revealed 388 genes whose expression in amygdala correlated significantly with DCX ($q < 0.001$). Of these, over 90% were positively associated with DCX expression (Figure 3a), therefore gene ontology analyses were performed twice: once using all DCX-correlated genes, and once using only positively-correlated genes. The results of both analyses overlapped substantially (Figure 3b); we present here the analyses for all genes correlated positively with DCX (see Supplementary Table 2 for overall analyses). Genes were significantly over-represented among GO biological processes, most notably including “nervous system development”

($p = 8.46E^{-13}$), “generation of neurons” ($p = 5.76E^{-11}$), “regulation of localization” ($p = 2.95E^{-11}$), and “neurogenesis” ($p = 4.52E^{-11}$), all of which displayed greater than 2-fold gene enrichment (Figure 3b, Table 1). Of the significant process clusters identified during these analyses, 25 were associated generally with nervous system development, and a further 6 with regulation of microtubule polymerization. Thus, there is considerable over-representation of developmental plasticity-related genes in amygdala whose regulation remains tightly linked with DCX during adult fear learning in the absence of mitosis, and presumably relate to structural and other changes that occur with dendritic and axonal arborization and synapse development.

Cdk14, a Wnt signaling molecule, was one of the neuroplasticity-related genes in the amygdala that was found to correlate most positively with DCX ($q = 0.031$) and which would emerge again in our next series of experiments designed to identify peripheral markers of amygdala DCX expression and/or learning. As predicted by our RNAseq data, validation experiments confirmed that high amygdala Cdk14 mRNA was associated with faster extinction ($F = 6.773$, $p = 0.01$) (Figure 3c). Because of these blood – brain correlations between blood Cdk14 and DCX, we chose to further examine Cdk14 as a potential predictive biomarker.

Blood Cdk14 correlates with amygdala DCX expression and predicts future fear in mice

Given the inherent limitation of not being able to readily assess amygdala DCX levels in living animals in advance of behavioral testing, as well as our own RNA sequencing data demonstrating DCX’s extremely low expression in blood, we wondered if there may be peripheral markers that correlate with DCX RNA in the early aftermath of fear learning that would also serve as biomarkers of learning ability. Thus, blood RNAseq was employed in order to identify a dependable peripheral marker that might serve as a proxy for amygdala DCX expression in future studies. Multiple candidate genes were identified whose expression was correlated with amygdala DCX expression, albeit less robustly than our within-amygdala analyses ($p = 4.538E^{-5} - 3.3296E^{-7}$, $q = 0.0078 - 0.1019$). Correlations between amygdala DCX and the top 6 candidates in blood ranged from an R^2 of 0.79–0.91, and their potential utility as peripheral retrospective and predictive biomarkers of fear-related learning were subsequently validated using qRT-PCR in an independent sample (see Figure 4a for experimental details and Supplementary Table 1 for PCR primers).

In all, high vs. low expression of 4 of our 6 candidates in blood was significantly associated with differences in behavior during fear acquisition (Supplementary Figure 3), fear extinction (Supplementary Figure 4), or both. Notably, many of these genes showed a negative relationship with amygdala DCX levels, with high peripheral expression of these genes being associated with low amygdala DCX. Of these, blood Cdk14 displayed the greatest range of predictive utility (Fig 3c). Baseline Cdk14 mRNA levels measured in blood 24hrs prior to conditioning predicted freezing during fear acquisition, with low Cdk14 mRNA expression being associated with more rapid acquisition than high Cdk14 ($F = 6.645$, $p = 0.0113$) (Figure 4b). Low Cdk14 mRNA in blood 2 hrs after fear acquisition likewise predicted more rapid extinction learning 24 hrs later ($F = 14.97$, $p < 0.001$) (Figure 4c).

To validate and extend our previous finding that extinction protocol length appears to mediate the strength of the relationship between DCX and past learning, we next examined amygdala DCX mRNA and blood Cdk14 mRNA in mice extinguished using either 15 or 45 non-reinforced CS presentations. High amygdala DCX mRNA levels 2 hrs after extinction were associated with less freezing only in mice extinguished using the shorter protocol ($F = 7.2, p < 0.01$) (Figure 4d). Likewise, low blood Cdk14 expression 24 hrs prior to extinction predicted less freezing during extinction training only in animals extinguished with 15 CSs, and not in animals exposed to 45 CSs ($F = 10.07, p < 0.01$) (Figure 4e). These data suggest that not only is DCX sensitive to extinction protocol length, but so too is the blood biomarker its expression helped to identify.

Finally, in order to assess the relationship between blood Cdk14 levels and extinction retention, a second cohort of mice were exposed to a single CS presentation 1 week after a 15 CS extinction protocol; blood was collected immediately following removal of mice from fear expression testing chambers (Figure 4f). No relationship was observed between blood taken 1 week after fear extinction and freezing during extinction training itself (Figure 4g). However, high blood Cdk14 expression at time of the subsequent fear expression test was associated with less freezing in response to the tone ($F = 7.979, p = 0.0062$) (Figure 4h).

Blood Cdk14 expression predicts resilience in a traumatized human population

In order to assess whether blood Cdk14 expression might have utility as a predictive marker of traumatic learning in a human population sample, Cdk14 mRNA expression was examined in blood collected from 100 adult males shortly after trauma exposure (Figure 5a, Table 2). Among other measures, these subjects had been assessed for level of resilience using the Connor-Davidson Resilience Scale (CD-RISC) to assess resilience-related characteristics including self-esteem, stress-coping, action-orientation, and adaptability.[26] Correlational analyses of Cdk14 levels at time of emergency department admission and total scores on trauma-related questionnaires taken 1 month following trauma did not reveal any significant associations. However, Cdk14 expression taken immediately after trauma correlated significantly with CD-RISC scores at 3 months ($R^2 = 0.08104, p = 0.0047$), 6 months ($R^2 = 0.0654, p = 0.0181$), and 12 months post-trauma ($R^2 = 0.0961, p = 0.0085$) (Figure 5b–d).

2-way ANOVA confirmed that high Cdk14 expression was associated with differences in resilience following trauma ($F = 8.11, p < 0.0001$) (Figure 5e), and this remained significant following sequential Bonferroni corrections controlling for the number of questionnaires assessed. Individuals in the top 2 quartiles of blood Cdk14 expression had significantly higher CD-RISC scores than did individuals in the lowest 2 quartiles (Tukey's MCT: 1st Q vs. 3rd Q $p = 0.0193$; 1st Q vs. 4th Q $p = 0.0001$; 2nd Q vs. 3rd Q $p = 0.0296$; 2nd Q vs. 4th Q $p = 0.0003$). Assessment of CD-RISC scores among participants who had completed this questionnaire at 1, 3, 6, and 12 months suggested that the above group differences were better explained by greater resilience across time (Main effect of Group: $F = 11.44, p < 0.001$) than by differences in recovery or score trajectory (Main effect of Month: $F = 0.6995, p = 0.5539$) (Figure 5f). Cdk14 levels did not correlate significantly with total scores on either the PTSD Symptom Scale or Beck Depression Inventory, however. Together, these

findings suggest that higher post-trauma resilience associates with higher peripheral blood Cdk14 mRNA levels near the time of trauma exposure, possibly consistent with the data in Figure 4f, in which higher blood cdk14 mRNA in mice was indicative of lower fear expression.

DISCUSSION

The goal of the present series of studies was to determine whether dynamic adult amygdala expression of doublecortin (DCX) expression was modulated by, or associated with, fear learning. Our findings suggest that this is indeed the case. Although fear learning itself did not result in global differences in amygdala DCX protein levels between groups, individual differences in fear learning and expression were associated with differential DCX expression. Rapid acquisition and extinction of fear were both associated with higher amygdala DCX protein 24 hrs later, suggesting that amygdala DCX expression *reflects learning potential, or plasticity*, rather than greater/lesser fear. Importantly, in both rats and mice, the association between fear extinction and DCX expression emerged only following shorter extinction protocols, implying that the importance of DCX recruitment during fear learning may vary depending upon the number of presented trials and, presumably, how many of those trials are required to robustly encode and/or consolidate that memory.

Differential recruitment of cellular subpopulations and neuroplasticity has been reported during spatial and perceptual learning paradigms in which task difficulty was modulated [27] [28], and a large volume of work acknowledges that repeated exposure to stress results in different patterns of gene expression and changes in neuronal morphology compared to acute stress [29] [30]. Such chronic stress exposure is most often executed over the course of days and weeks, whereas acute stressors are generally operationally defined as a single exposure occurring within whatever discreet period of time the experimenter has chosen to use. Although the fear conditioning and extinction protocols employed herein occurred during a single/acute session, each individual CS presentation within this session might also be conceptualized as an individual trial. Indeed, behavioral measures of extinction learning are more sensitive to the number of CS presentations than to the absolute amount of time over which CSs were presented [31]. As with chronic versus acute stress, then, there may be a minimum threshold of CS presentations required to induce the changes in DCX and Cdk14 expression reported here, as well as a ceiling beyond which these genes no longer contribute to, reflect, or predict the learning that takes place. Further work employing more fine-tuned variation in the number of CS presentations given during both conditioning and extinction would help clarify whether this is the case.

Although we chose to focus our initial work on the relationship between amygdala DCX and behavior during fear and extinction learning, our RNAseq analyses implicate a host of other neuroplasticity-related genes alongside it. Within the amygdala, 388 genes were found to correlate robustly with regional DCX expression, and many of these are involved in early neurodevelopmental processes, despite the postmitotic nature of these neurons. These findings complement recent RNAseq data from DCX-expressing cells in the human paralamina nucleus, which likewise revealed over-representation of genes associated with neurogenesis and neuronal development [12]. It therefore seems likely that, rather than

being uniquely reflective of individual differences in learning, DCX is but one member of a larger group of neuroplasticity-related genes that, together, respond to and regulate amygdala-dependent plasticity, and thus fear learning, en masse. The extent to which they are recruited, in turn, likely underlies some of the individual variation we observed.

The intrinsic excitability of individual neurons has previously been demonstrated to underlie the selective recruitment of mature neurons into fear memory traces [32]. Enhancement or knock-down of CREB in amygdala and hippocampal neurons exerts bidirectional control of memory trace allocation and expression [33] [34]. Notably, increasing the excitability of a small subset of lateral amygdala neurons 2 days before conditioning was sufficient to enhance fear learning, and subsequent reactivation of neurons transfected with excitability-enhancing genes appeared to function as a memory retrieval cue [33]. These alterations in excitability are associated, in turn, with morphological changes, including the presence of more dendritic spines [35]. If, as our results suggest, DCX expression reflects general amygdala plasticity, then our results tend to support the notion that greater amygdala excitability in the hours prior to training is associated with enhanced amygdala-dependent learning. The potential utility of blood Cdk14 as a marker of amygdala excitability prior to fear acquisition may provide a unique means of predicting susceptibility to enhanced fear learning in humans. Longitudinal tracking of Cdk14 expression may additionally provide important insight about the extent to which amygdala excitability remains stable over time and following trauma exposure.

Among the blood markers correlated with amygdala DCX expression, Cdk14 mRNA levels were found to be robustly predictive of subsequent fear acquisition and extinction. Notably, Cdk14 levels measured at time of fear expression testing were higher among those mice that froze less in response to the CS, paralleling our human RNAseq data showing that high blood Cdk14 levels are associated with greater resilience over the following year among males exposed to trauma. Although no such association was observed between Cdk14 and the PTSD symptom scale (PSS), multiple lines of evidence have established the association between greater levels of neural plasticity and resilience to stress. Thus, the association between resilience, neural plasticity, and improved learning may underlie our findings. For instance, greater amygdala plasticity in the period following exposure to trauma may facilitate extinction of acquired fear memories, resulting in improved long-term outcomes and enhanced resilience to trauma.

Investigating the specific nature of the association between blood Cdk14 and amygdala DCX expression was beyond the scope of this project; however, peripheral expression of centrally-acting genes and non-coding RNAs have been found to associate with affective symptoms across multiple studies and cohorts [36–39]. Such blood-brain correlations are often suggested to be the result of transport across the blood-brain barrier or common regulatory mechanisms operating in tandem centrally and peripherally [38–39]. While there are no obvious connections in the literature linking Cdk14 and DCX-signaling, there are multiple sources of evidence linking Cdk14 signaling to Wnt regulation [49–51] and additional studies linking Wnt regulation and DCX signaling [52–54]. Thus these pathways are likely connected and our findings may provide the first evidence associating these pathways together and with brain-blood markers of neural plasticity. Nonetheless, until

further research is completed on the Wnt-signaling molecule Cdk14 itself, any explanation for the observed correlation between blood-based Cdk14 and amygdala DCX will remain speculative.

It is notable, although not entirely unexpected, that amygdala expression of Cdk14 was likewise associated with differences in fear learning in mice. Past work from our lab has found that Wnt signaling is necessary for synaptic de-stabilization/re-stabilization during fear learning [40] [41]. Wnt signaling through the LRP6 receptor, in turn, relies upon a 2-step process involving receptor priming via phosphorylation of cyclin y/Cdk14, followed by Wnt-mediated phosphorylation upon binding [42]. This dual-phosphorylation also facilitates the sequestration of beta-catenin required during mitosis although, to the best of our knowledge, Cdk14's role in mediating beta-catenin signaling in post-mitotic cells remains unexplored [42]. Still, that another gene family crucial to both synaptic stabilization and neural development served as a peripheral marker for fear learning is consistent with our findings that DCX correlates in the amygdala tend to be genes involved in neurodevelopmental processes, as well as the idea that such genes are re-employed to facilitate plasticity in the adult [43].

The association between blood Cdk14 expression at time of emergency department admission following trauma and later resilience as measured by the CD-RISC tentatively suggests that DCX-related mechanisms of learning and plasticity may be conserved in humans as well as between rodent species. Although the identification of a significantly predictive biomarker of resilience in humans based upon our correlative analyses of RNAseq data from mice is hopeful, our data regarding the sensitivity of Cdk14 to variations in extinction protocols calls for careful consideration. We were not able to assess the extent to which the participants included in this study sought or adhered to post-traumatic care but, if Cdk14 expression patterns in humans do indeed recapitulate those seen in rodents, we would anticipate that the utility of this gene as a biomarker would vary as a function of treatment and/or the extent to which patients were successful in extinguishing responding to fear-related cues. This possibility would be prudent to bear in mind when designing future studies involving measures of Cdk14 expression. It is also notable that the human participants that took part in this study did not display particularly low scores on the CD-RISC; in fact, mean scores were comparable to those reported for healthy populations [26]. Of note, while the human subjects cohort in this study had experienced a recent trauma (mostly motor vehicle crashes), they were not treatment-seeking for psychiatric symptoms. Rather they were recruited from the general emergency department due to their trauma exposure, and only a subset of them went on to meet depression or PTSD criteria. In fact, one could argue that given the level of trauma exposure, but lack of severe PTSD / Depression symptoms, that they are indeed a resilient cohort. Also note that a correlation was found between blood Cdk14 levels and a measure of resilience, which is in line with the data garnered from rodents: the associations between amygdala DCX/Cdk14 and fear learning are present in the absence of clear pathology, and thus likely represent a fundamental mechanism of fear learning. Future studies examining Cdk14 expression in blood prior to trauma exposure may help elucidate whether it constitutes a stable biomarker of trait resilience. Finally, future work must extend these findings by examining whether the reported associations hold for both female rodents and women.

The work presented here is the first demonstration that individual differences in DCX expression within non-neurogenic brain regions are associated with measurable differences in learning and behavior. Furthermore, our results demonstrate that correlational analyses of peripheral gene expression with centrally-acting genes of interest may constitute a viable strategy for identifying useful biomarkers even in cases where the primary gene of interest cannot be employed to that end.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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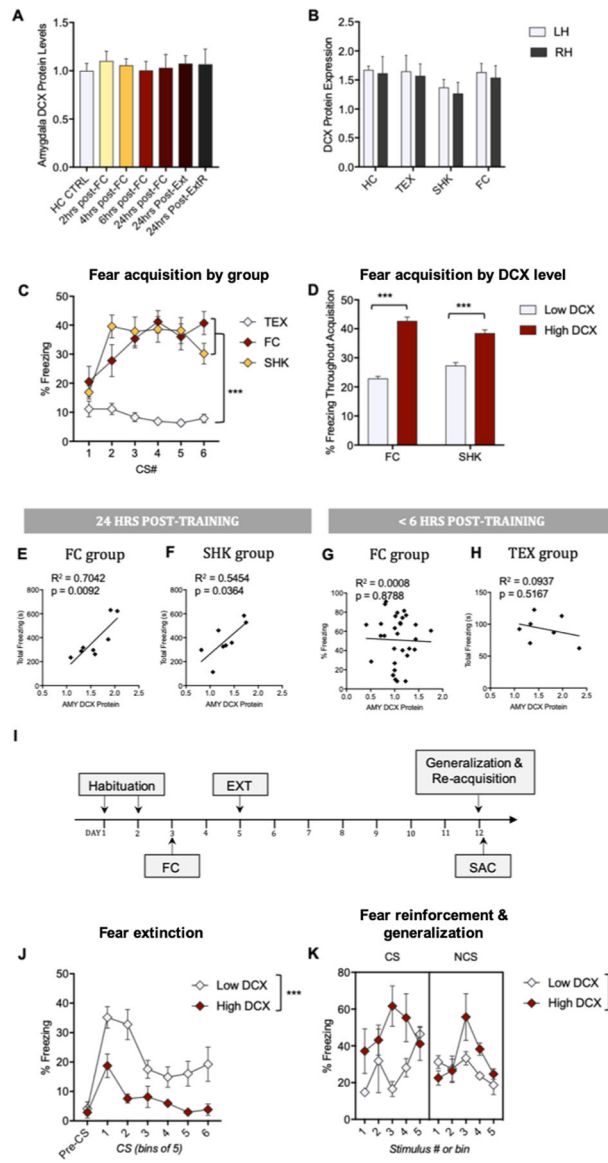


Figure 1. Individual differences in freezing during fear learning are associated with differences in amygdala doublecortin (DCX) expression in mice.

No group differences in amygdala DCX expression are detected 2, 4, 6, or 24 hrs post-fear conditioning (Post-FC), nor 24 hrs post-extinction (Post-Ext) or post-extinction retention testing (Post-ExtR) ($n = 8-17$) (A). DCX protein expression does not differ significantly between right (RH) and left (LH) hemispheres ($n = 3-4$) (B). Classical fear conditioning (FC) and conditioning using unpaired/non-predictive tones and shocks (SHK) both result in significantly increased freezing as compared to controls exposed only to tones (TEX) ($n = 8$) (C). In both FC and SHK groups, mice with higher (top 50%) expression of amygdala DCX 24 hours after fear learning displayed more freezing during acquisition than did mice with lower (bottom 50%) amygdala DCX expression ($n = 4$) (D). In FC and SHK groups, amygdala DCX protein levels 24 hrs after training are significantly correlated with freezing during acquisition (E-F). No correlation is observed between amygdala DCX protein and freezing in FC animals sacrificed within 6 hrs of training (G), nor among unconditioned

controls (H). Timeline of fear conditioning (FC), extinction (EXT), and generalization and re-acquisition experiments, including sacrifice (SAC) (I). Freezing during fear extinction training differs between animals with high (top 50%) versus low (bottom 50%) amygdala DCX levels 24 hrs after learning (n = 4) (J). Amygdala DCX protein levels assessed 2 hrs following exposure to reinforced conditioned stimuli (CS) or non-conditioned tones (NCS) 1 week after extinction training are associated with differences in freezing during re-acquisition and generalization testing (n = 3) (K). * p < 0.05, ** p < 0.01, *** p < 0.001.

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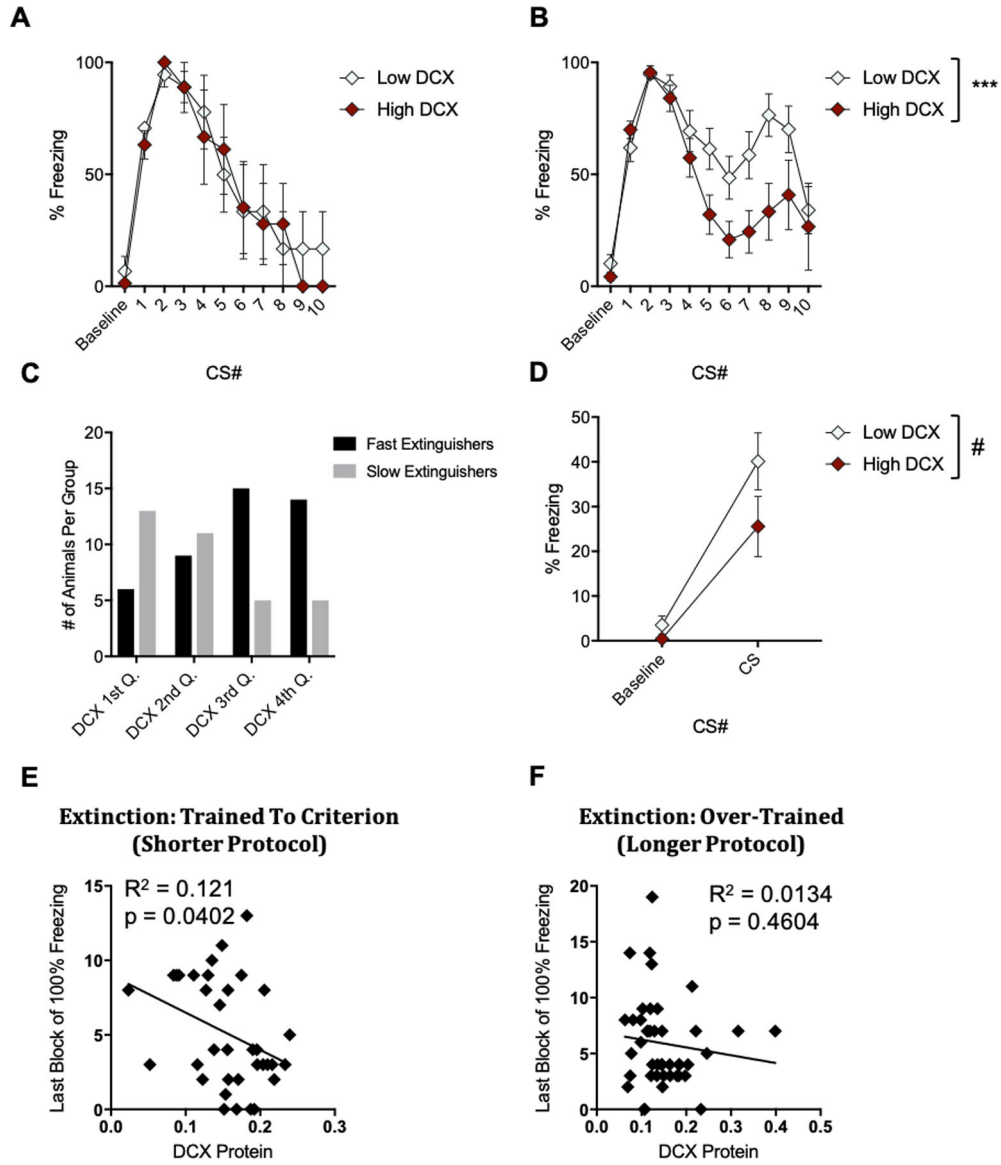


Figure 2. Individual differences in extinction speed are associated with different levels of amygdala DCX expression after learning in rats. Fast Extinguishers show no differences in amygdala DCX protein levels as compared to Slow Extinguishers when sacrificed 2 hrs after extinction training (n = 6) (A), but display significantly higher DCX expression 24 hrs later (n = 25) (B). The distribution of Fast Extinguishers is biased toward the top 2 quartiles of DCX expressors, whereas Slow Extinguishers tend to fall within the bottom 2 quartiles of amygdala DCX protein expression (N = 78) (C). Rats with high amygdala DCX protein levels following extinction show a trend toward reduced freezing during exposure to the extinguished CS (n = 23) (D). Amygdala DCX protein expression correlates with the number of unreinforced CS presentations required to display reductions in freezing during shorter extinction protocols (E), but not longer ones (F). * p < 0.05, ** p < 0.01, *** p < 0.001, # p < 0.1.

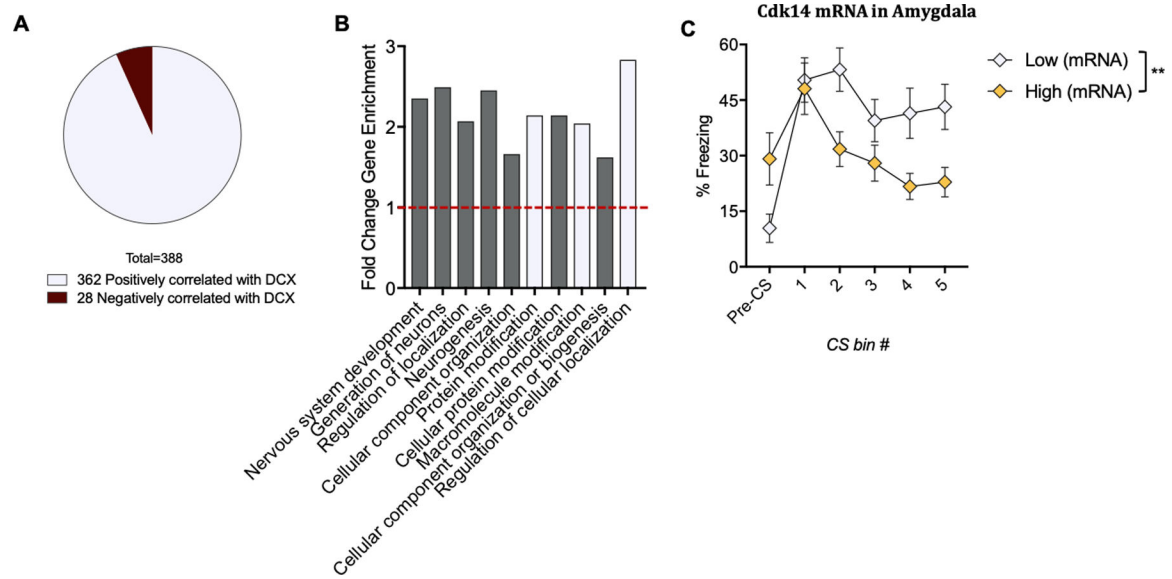


Figure 3. Gene ontology analyses of amygdala correlates of DCX as determined by RNA sequencing.

The majority of the 388 genes that were found to correlate significantly with DCX mRNA expression in the mouse amygdala ($q < 0.001$) display a positive association with DCX expression (A). Gene ontology analyses shows that the top *positively* correlated genes have significant over-representation of processes associated with neuronal development, cellular compartment organization, and protein modification. Dark bars represent gene ontology processes that were likewise among the 10 most significant hits when all DCX-correlates, *both positive and negative*, were included in the analyses (B). qRT-PCR of amygdala tissues taken 2 hrs after extinction confirmed that high amygdala Cdk14 was associated with less freezing during extinction ($n = 6$) (C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

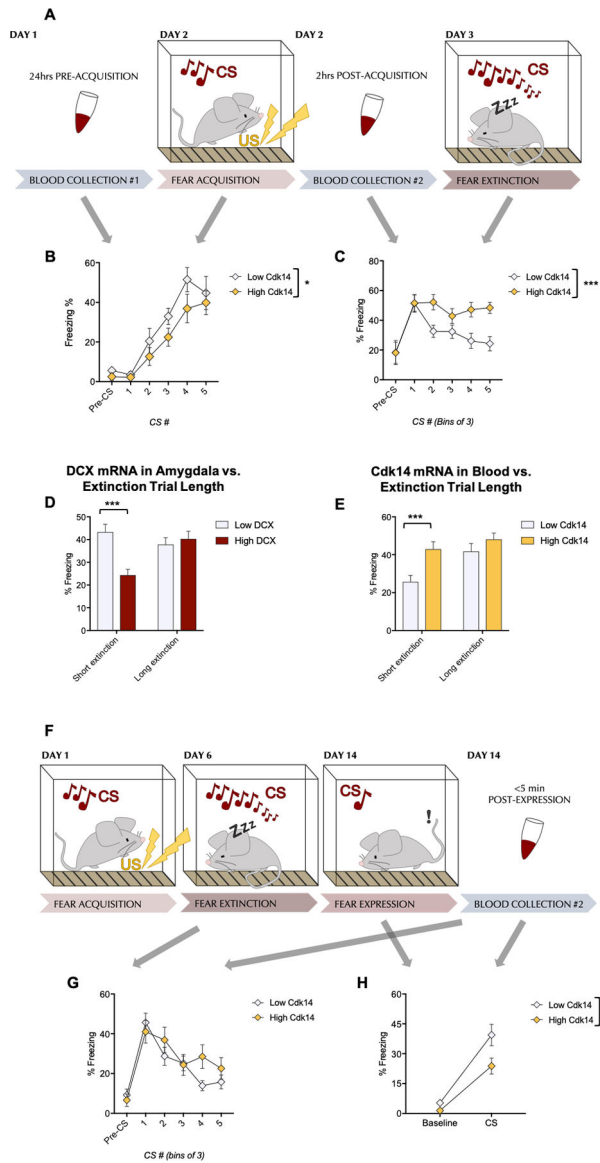


Figure 4. Blood Cdk14 expression is a predictive marker of fear learning and fear expression in mice.

Schematic description of experimental timeline for first blood marker validation study (cohort 1) (A). Blood Cdk14 mRNA expression taken 24 hrs before fear acquisition and 24 hrs before fear extinction training predict freezing during fear conditioning (B) and fear extinction (C), respectively (n = 6–10). Both amygdala DCX expression (D) (n = 6–10) and blood Cdk14 (E) (n = 2–3) are sensitive to extinction training robustness; differences in expression were associated with freezing during extinction in animals exposed to 15 non-reinforced CS presentations, but not in those exposed to 45 non-reinforced CSs. Schematic description of experimental timeline for second blood marker validation study (cohort 2) (F). Blood Cdk14 levels assessed immediately following fear expression testing do not reflect past extinction learning directly (G), but are instead associated with freezing during expression testing (H) (n = 6). * p < 0.05, ** p < 0.01, *** p < 0.001.

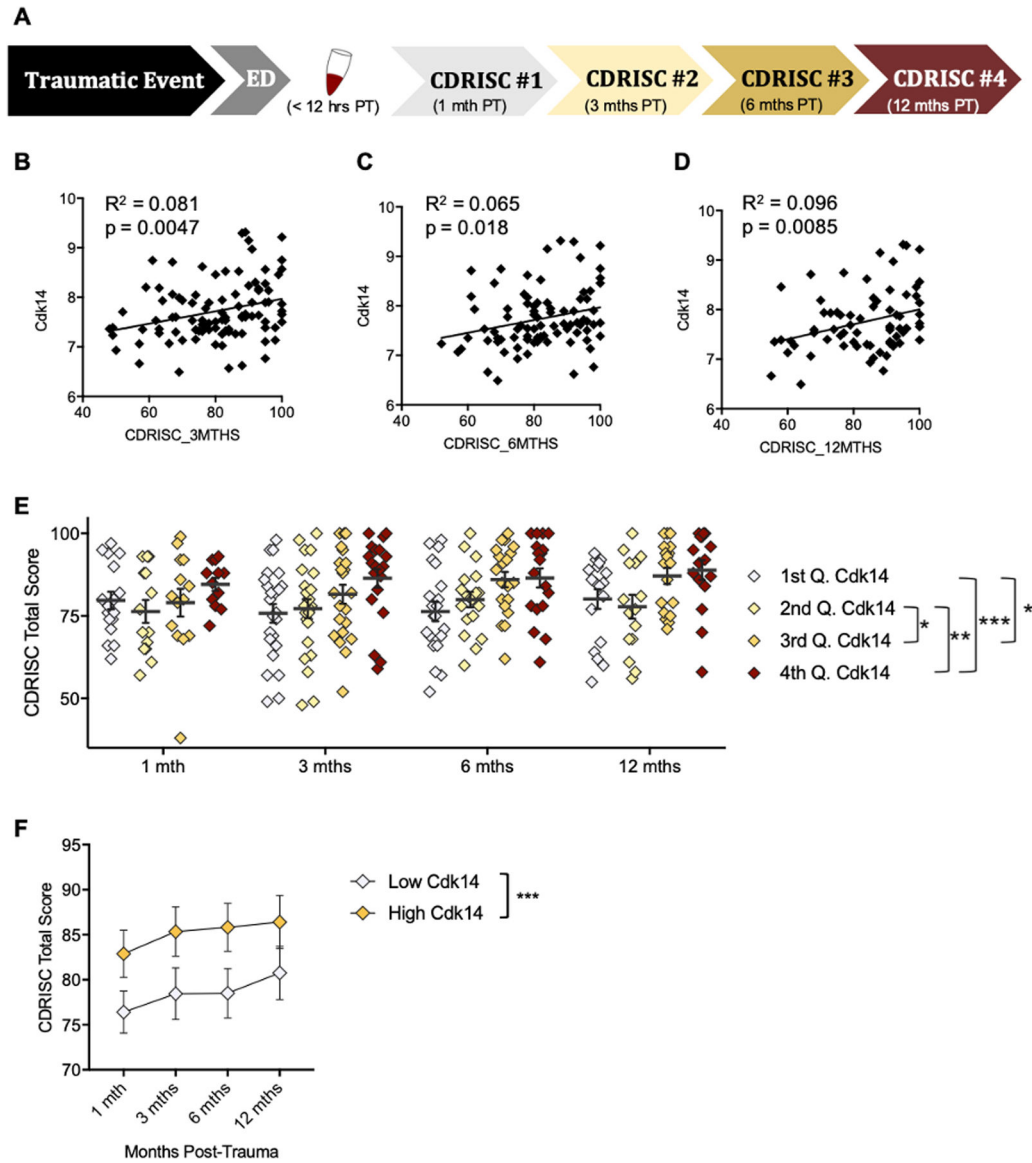


Figure 5. Blood Cdk14 expression measured within hours of trauma predicts resilience in a traumatized human population.

Schematic depiction of experimental timeline (A). Connor-Davidson Resilience Scale (CD-RISC) scores at 3 months (B), 6 months (C), and 12 months (D) post-trauma (PT) correlate significantly with Cdk14 expression in blood taken shortly after trauma-exposure. 2-way ANOVA confirms that blood Cdk14 expression quartile is associated with differences in CD-RISC scores; individuals in the highest 2 quartiles of Cdk14 expression display greater resilience scores than do those in the lowest 2 quartiles (E). Median-split analyses including only participants for whom CD-RISC scores are available at all timepoints (N = 37) reveals that high blood Cdk14 expression is associated with greater resilience overall, rather than a difference in CD-RISC score trajectory post-trauma (F). * p < 0.05, ** p < 0.01, *** p < 0.001.

Table 1.

Gene ontology analyses results for positive correlates of DCX in mouse amygdala that met significance criterion ($q < 0.001$).

	GO BIOLOGICAL PROCESS	# GENES	SIG (p)
1	nervous system development	80	8.46E-13
2	generation of neurons	62	5.76E-11
3	regulation of localization	89	2.95E-11
4	Neurogenesis	65	4.52E-11
5	cellular component organization	131	3.86E-10
6	protein modification process	75	5.6E-10
7	cellular protein modification process	75	5.6E-10
8	macromolecule modification	77	2.12E-09
9	cellular component organization or biogenesis	132	2.12E-09
10	regulation of cellular localization	41	4.57E-09

Gene ontology analyses results for 362 positive correlates of DCX in mouse amygdala. Table shows ten most significant biological processes identified through GO analysis of genes positively-correlated with amygdala DCX.

Table 2.

Participant information (Grady Trauma Project)

Subject gender/sex	Male
Average age (range)	38 (18–62)
# Participants with RNAseq & CD-RISC Scores	100
# Participants with RNAseq & CD-RISC Scores at all timepoints	37
Race (n)	Black (69)
	White (22)
	Asian (2)
	Mixed (4)
	Other (7)
Ethnicity (n)	Hispanic (6)
	Not Hispanic (94)

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