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Shedding Light on the Transcriptomic Dark Matter in Biological Psychiatry: Role of Long Noncoding RNAs in D-cycloserine-Induced Fear Extinction in Posttraumatic Stress Disorder

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Abstract

Biological psychiatry scholarship on posttraumatic stress disorder (PTSD) is making strides with new omics technologies. In this context, there is growing recognition that noncoding RNAs are vital for the regulation of gene and protein expression. Long noncoding RNAs (lncRNAs) can modulate splicing, influence RNA editing, messenger RNA (mRNA) stability, translation activation, and microRNA–mRNA interactions, are highly abundant in the brain, and have been implicated in neurodevelopmental disorders. The largest subclass of lncRNAs is long intergenic noncoding RNAs (lincRNAs). We report on lincRNAs and their predicted mRNA targets associated with fear extinction induced by co-administration of D-cycloserine and behavioral fear extinction in a PTSD animal model. Forty-three differentially expressed lincRNAs and 190 differentially expressed mRNAs were found to be associated with fear extinction. Eight lincRNAs were predicted to interact with and regulate 108 of these mRNAs, while seven lincRNAs were predicted to interact with 22 of their pre-mRNA transcripts. Based on the functions of their target mRNAs, we inferred that these lincRNAs bind to nucleotides, ribonucleotides, and proteins; subsequently influence nervous system development, morphology, and immune system functioning; and could be associated with nervous system and mental health disorders. We found the quantitative trait loci that overlapped with fear extinction-related lincRNAs included traits such as serum corticosterone level, neuroinflammation, anxiety, stress, and despair-related responses. To the best of our knowledge, this is the first study to identify lincRNAs and their RNA targets with a putative role in transcriptional regulation during fear extinction in the context of an animal model of PTSD.

Keywords: biological psychiatry, posttraumatic stress disorder, long noncoding RNAs, fear extinction, animal model, transcriptomics

Introduction

Posttraumatic stress disorder (PTSD) is a severe and debilitating psychiatric disorder that is highly prevalent in individuals who experience one or more traumatic events (American Psychiatric Association, 2013). Dysfunctional fear extinction plays an integral role in the disorder (Garfinkel et al., 2014; Milad et al., 2009), as the development of PTSD involves a fear-conditioning process, during which fear and anxiety responses are exaggerated and/or are resistant to extinction (Amstadter et al., 2009; Caddell, 1984; Cohen et al., 2006).

During classical fear conditioning, a neutral (conditioned) stimulus is paired with an aversive (unconditioned) stimulus. Following adequate pairing, the conditioned stimulus will eventually result in the same response as the unconditioned stimulus which is referred to as the conditioned response. The conditioned stimulus subsequently elicits a conditioned fear response, which can be triggered upon encountering a harmless stimulus that becomes paired with the trauma (Estes and Skinner, 1941). In PTSD, the trauma is considered to be the unconditioned stimulus and the conditioned fear response experienced by PTSD patients, even in the presence of seemingly harmless stimuli, is the conditioned response (Foa et al., 1989; Grillon et al., 1998).

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To relieve the anxiety and fear associated with the conditioned stimulus deconditioning/desensitization to the learned fears, thus fear extinction, has to occur (Wolpe, 1968). Systematic desensitization to the conditioned stimulus relies on extinction and counter-conditioning, two processes that involve learning. Exposure therapy is dependent on extinction learning to reduce the conditioned response (Bouton et al., 2001; Rothbaum and Davis, 2003). Recent research indicates that fear extinction involves the formation of a new memories that compete with, and inhibit, the fear response, rather than deleting the original (traumatic) memory (Milad and Quirk, 2012; Rescorla and Heth, 1975).

Treatment options for PTSD include exposure-based cognitive behavioral therapies (CBT; which are based on the theories discussed above) and pharmacological treatments, such as selective serotonin reuptake inhibitors (Stein et al., 2006) and serotonin-norepinephrine reuptake inhibitors (Davidson et al., 2006). Despite the relative efficacy of these treatments, a large number of PTSD patients do not respond optimally and/or relapse over time (Foa et al., 2006; Kessler et al., 1995; Marit Sijbrandij et al., 2007).

D-cycloserine (DCS) is a partial N-methyl-D-aspartate (NMDA) receptor agonist at the glycine site on the NMDAR1 receptor subunit. DCS has been found to be effective in facilitating extinction learning in rats when administered before or immediately after extinction training (Ledgerwood et al., 2003, 2005; Yang and Lu, 2005). The coadministration of DCS and exposure-based CBT has also been proven to be more effective than either of these treatments alone in extinguishing fear in human trials of anxiety disorders (Hofmann et al., 2006; Otto et al., 2010) and PTSD (Difede et al., 2014; de Kleine et al., 2012). DCS administration facilitates generalized extinction of fear (Ledgerwood et al., 2005) and reduces the rate of relapse following successful exposure-based CBT (Ledgerwood et al., 2004).

The majority of studies investigating mechanisms of DCS facilitated fear extinction, have focused on either intra-amygdalar (Mao et al., 2006) or systemic administration (Gabriele and Packard, 2007; Yamamoto et al., 2008) and the resultant alterations in gene and/or protein expression (Yamamoto et al., 2008). Memory consolidation, and by extension, fear extinction, require dynamic gene and protein expression regulation; however, few studies have investigated transcriptional and posttranscriptional regulation during fear conditioning and fear extinction.

Only one-fifth of the human transcriptome is associated with protein-coding genes; noncoding RNAs (ncRNAs) are highly prevalent and outnumber coding genes (Kapranov et al., 2010), contributing significantly to the diversification of eukaryotic transcriptomes and proteomes. There are several types of ncRNAs, including transfer RNAs, ribosomal RNAs, as well as small RNAs such as microRNAs (miRNAs), short-interfering RNAs, PIWI-interacting RNAs, small nucleolar RNAs, small nuclear RNAs, extracellular RNAs, small Cajal body-specific RNAs and long ncRNAs (lncRNAs) (Hombach and Kretz, 2016). The latter are the largest class of noncoding transcripts (Gudenas et al., 2019) and are categorized as sense, antisense, bidirectional, intronic, and intergenic, based on their proximity to protein-coding genes (Mercer et al., 2009; Ponting et al., 2009).

The majority of lncRNA genes are expressed in a cell-type-specific and developmental stage-specific manner (Ramos et al., 2013). lncRNAs are highly abundant in the central nervous system (CNS). A vast number are located adjacent to genes that encode transcriptional regulators and key drivers of neural development, such as neuronal differentiation (Onoguchi et al., 2012), stem cell pluripotency (Ramos et al., 2013), and synaptogenesis (Bernard et al., 2010), implicating these lncRNAs in the regulation of these genes. lncRNAs play a vital role in regulating transcription, and are involved in messenger RNA (mRNA) stability, RNA editing, pre-mRNA splicing, translation activation, or abolition of miRNA-induced repression (Geisler and Coller, 2013).

Long intergenic noncoding RNAs (lincRNAs) are the largest subclass of lncRNAs (Ransohoff et al., 2018). RNA sequencing of postmortem brain samples from schizophrenia and bipolar disorder patients suggests the involvement of lincRNAs in mental disorders (Hu et al., 2016). Significant association with a novel RNA gene, lincRNA AC068718.1, has recently been observed in a genome-wide association study (GWAS) of PTSD in African American women (Guffanti et al., 2013). The authors hypothesized that this lincRNA, with predicted functions for telomere maintenance and immune function, may be a risk factor for PTSD in women.

These studies add to emerging evidence that lncRNAs play a critical role in psychiatric conditions, including stress-related disorders (Cui et al., 2017; Ren et al., 2015). However, the modes of action and functions of most lncRNAs in disease remain to be elucidated. lncRNAs have a rapid turnover rate (median half-life of 3.5 hours), enabling them to swiftly mediate genomic responses to external stimuli (Clark et al., 2012). CNS lncRNAs could, therefore, be involved in rapid cellular and molecular responses, such as those required for memory consolidation or extinction, making them attractive regulators to investigate in pathologies, in which memory processes are affected. Although several different classes of ncRNAs exist, we focused on the largest subgroup of the lncRNAs, namely lincRNAs.

The aims of the present study were (1) to identify lincRNAs associated with fear extinction as facilitated by the coadministration of behavioral fear extinction and intrahippocampal DCS administration, in an animal model that simulated the core PTSD phenotypes and (2) to predict the role of these lincRNAs in regulating the transcriptome during fear extinction.

Materials and Methods

Animal model

All applicable international, national, and institutional guidelines for the care and use of animals were followed. All animal-related procedures were conducted in accordance with the ethical standards of Stellenbosch University's Research Ethics Committee: Animal Care and Use (REC:ACU) (Ref: ACU/2010/006(A1)). An adapted version of the PTSD animal model was utilized as described earlier (Siegmund and Wotjak, 2007). Please refer to Malan-Müller et al. (2016) for a full description.

In brief, 120 adult male Sprague-Dawley rats were grouped into four experimental groups (30 rats per group) based on an associated fear-conditioning paradigm using electric foot shocks. The groups received intrahippocampal administration of either DCS or saline:

1. fear-conditioned + intrahippocampal saline administration (FS),
2. fear-conditioned + intrahippocampal DCS administration (FD),
3. control + intrahippocampal saline administration (CS), and
4. control + intrahippocampal DCS administration (CD)

A series of 10 single electric footshocks (postnatal day [PND] 61) were used to induce fear in the rats. Freezing behavior was captured during the first exposure, second re-exposure, and following the presentation of a neutral tone to assess fear conditioning.

The fear extinction paradigm consisted of coadministration of behavioral fear extinction and intrahippocampal DCS. DCS (10 μg solution) (Walker et al., 2002) and saline were administered intrahippocampally (left dorsal hippocampus [LDH]) 30 min before each fear extinction protocol (PND 62–67). For the behavioral fear extinction protocol, animals were reexposed to the shock chamber for 3 min without shock application; conditioned or associative fear was also measured during this time (PND 62–67) (Supplementary Fig. S1).

Typical phenotypes associated with PTSD were assessed in this model (Ritov et al., 2016): anxious/fearful behavior by using the light/dark (L/D) avoidance test (PND 62) (Serchov et al., 2016) and open-field test (PND 64) (Cryan and Sweeney, 2011) and anhedonia by using the forced swim test (PND 66) (Strekalova et al., 2004). On these days (PND 62, 64 and 66), the fear extinction protocol was performed 3 h before the behavioral tests. Rats were sacrificed on PND 67 by decapitation. The left hippocampal tissue was divided into left ventral and dorsal hippocampus (LDH) sections, respectively; the dorsal region was used for subsequent analyses to determine the effect of DCS at the infusion site.

The L/D avoidance test was used to differentiate maladapted (MA; animals that displayed anxiety-like behavior) from well-adapted (WA; animals that did not display anxiety-like behavior) subgroups. Six animals that spent the most time in the light compartment of the L/D box were selected as the WA (less anxious) subgroup and six animals that spent the longest amount of time in the dark compartment were selected as MA (anxious) subgroup in both the Fear + Saline and Fear + DCS groups (resulting in 6 Fear + Saline WA [FSW], 6 Fear + Saline MA [FSM], 6 Fear + DCS WA [FDW], 6 Fear + DCS MA [FDM]).

The following subgroups were also of interest in the present study: (1) control animals that received intrahippocampal saline (CS, modeling a human control group); (2) fear-conditioned animals that received intrahippocampal saline (FS) and were MA (FSM, thus modeling a PTSD-like group), fear-conditioned animals that received intrahippocampal DCS (FD) and were WA (FDW, modeling a patient group exhibiting effective fear extinction due to treatment).

We focused on two subgroup comparisons, namely the FSM versus CS (modeling the fear-conditioning process by comparing a PTSD-like group to controls) and FDW versus FSM (modeling the fear extinction process by comparing a group that exhibit effective treatment-induced fear extinction to a PTSD-like group), and honed in on differentially expressed transcripts that were regulated in opposite directions in the two comparison groups. We, therefore, aimed to identify lincRNA and mRNA transcripts that were upregulated in response to fear conditioning in the FSM versus CS group, but downregulated during fear extinction in the FDW versus FSM

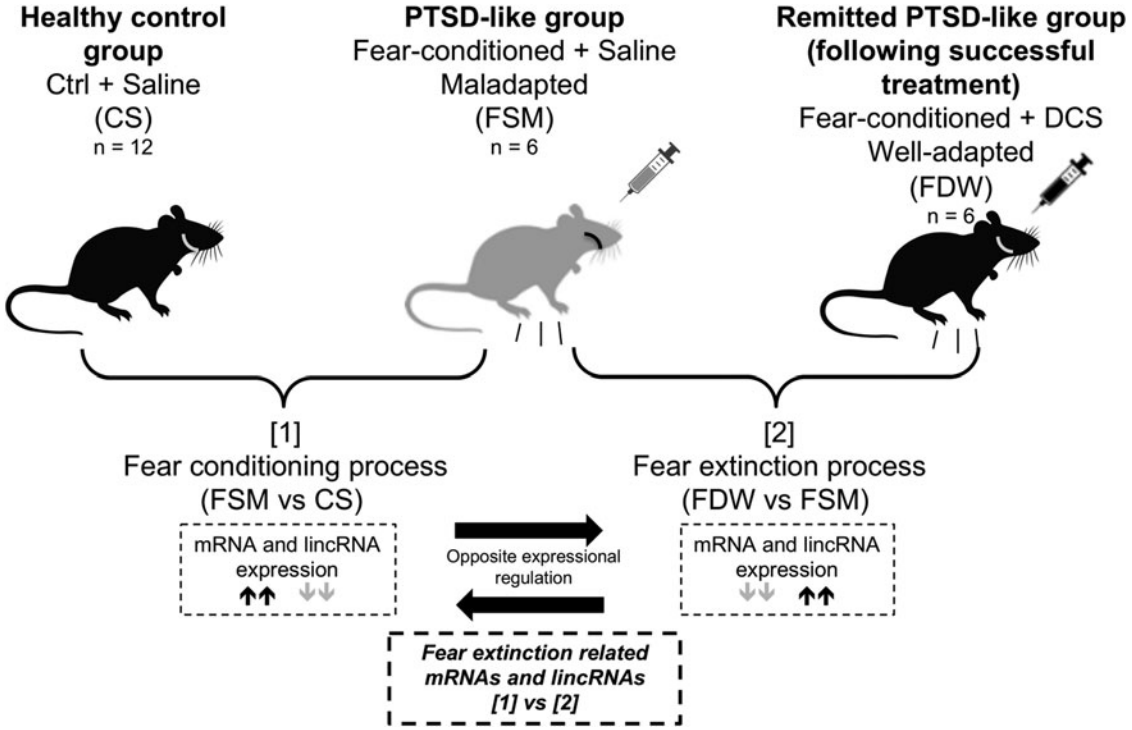


FIG. 1. Animal subgroup comparisons utilized to identify fear extinction-related mRNAs and lincRNAs following the coadministration of DCS and behavioral fear extinction. CS, Control + Saline; DCS, D-cycloserine; FDW, 6 Fear + DCS WA; FSM, 6 Fear + Saline MA; lincRNA, long intergenic noncoding RNA; mRNAs, messenger RNAs; PTSD, post-traumatic stress disorder.

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group, and *vice versa*, to identify lincRNAs and mRNAs specifically associated with the process of fear extinction induced by the coadministration of DCS and behavioral fear extinction (Fig. 1). These sets of opposite, differentially expressed lincRNAs and mRNAs will henceforth be referred to in this article as the fear extinction-related lincRNAs and fear extinction-related mRNAs.

RNA extraction and sequencing

RNA was extracted from the LDH regions of 30 rats (six rats each per FSM, FDW, FSM subgroups, and 12 rats in the CS subgroup) using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA extraction, quantification, and sequencing were performed on the 30 LDH RNA samples as described by Malan-Müller et al. (2016).

Bioinformatics and statistical analyses

FASTQC was used for quality assessment of RNA sequencing data. To identify differentially expressed transcripts for the subgroup comparisons FSM versus CS and FDW versus FSM, expression was quantified using *Salmon*

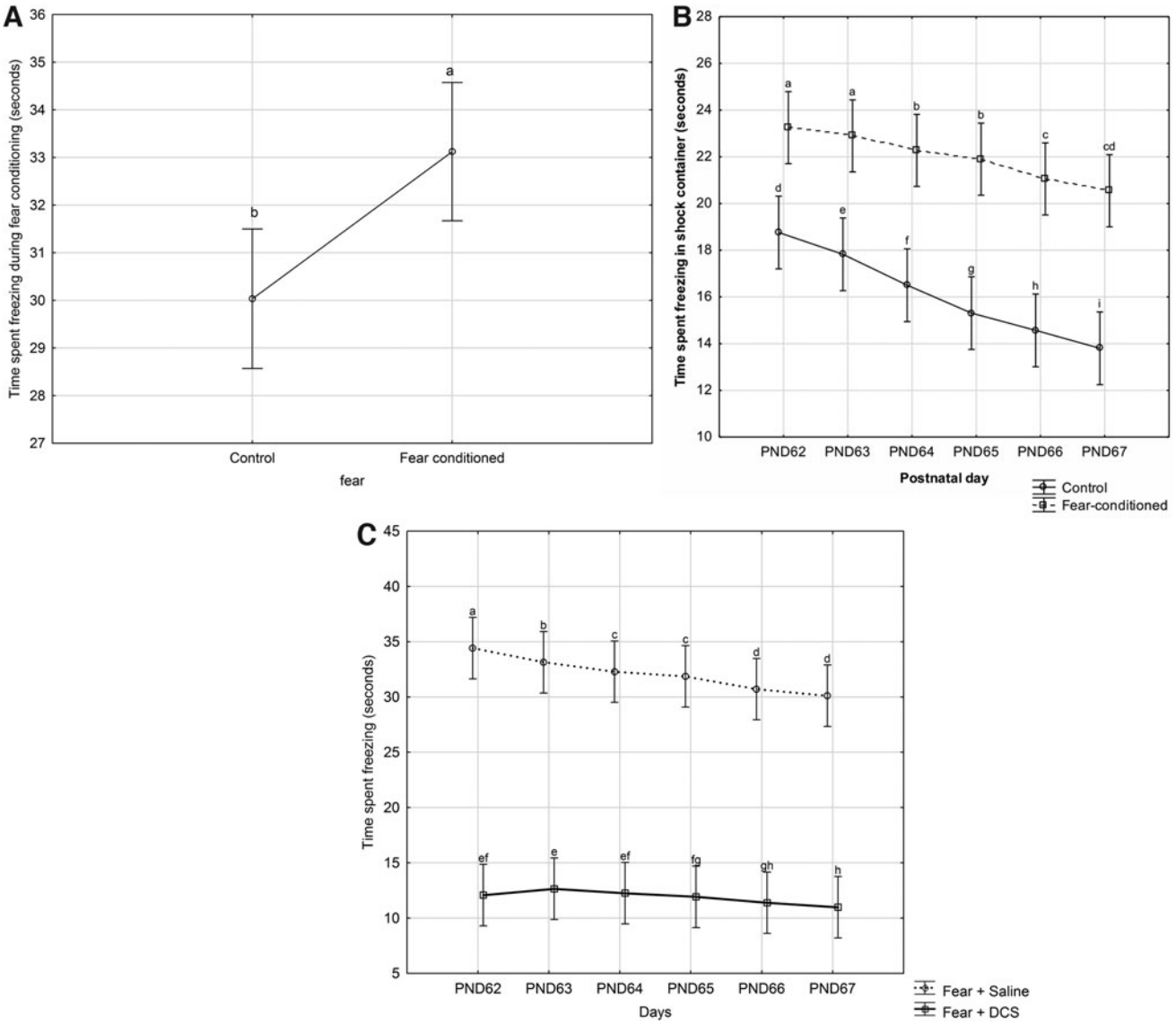


FIG. 2. **(A)** There was a significant difference in mean freezing times (first exposure and second exposure) for control and fear-conditioned animals during the fear-conditioning paradigm, with higher levels of freezing in the fear-conditioned animals (one-way ANOVA). **(B)** Freezing times for fear-conditioned and control animals measured over the 6-day fear extinction paradigm. A reduction in freezing times was noted from PND 62 – PND 67 for both groups, however the fear conditioned animals spent significantly more time freezing compared to controls (mixed model, repeated measures ANOVA). **(C)** Freezing times for Fear + Saline and Fear + DCS groups measured over the 6-day fear extinction paradigm. Saline-treated animals spent significantly more time freezing compared to DCS-treated animals (mixed model, repeated measures ANOVA). *Whiskers* denote confidence intervals. *Letters* on the graphs indicate significance ($p < 0.05$); *common letters* between two groups indicate that there is no significant difference; *different letters* between groups indicate a significant difference. ANOVA, analysis of variance; PND, postnatal day.

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(version 0.8.2) (Patro et al., 2017) with the Ensembl release-87 catalog (coding and noncoding transcripts) (Kersey et al., 2018). The *tximport* (version 1.12.0) (Soneson et al., 2015) pipeline was used to import transcript counts into R v3.5.1 (R Core Team, 2013), and the *edgeR* package (Robinson et al., 2010) was used to identify differentially expressed lincRNAs and mRNAs. The robust generalized linear model approach, as described by Zhou et al. (2014), was used to estimate the dispersion parameter and make inferences for changes in expression.

Raw RNA-sequencing data are available on ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-8647.

lincRNA-mediated gene expression regulation during fear extinction

The LncTar tool (Li et al., 2015b) was used to identify potential lincRNA–mRNA and lincRNA-pre-mRNA interactions within the sets of fear extinction-related lincRNAs and mRNAs. LncTar uses base pairing and determines the minimum free energy joint structure of the two RNA molecules (Li et al., 2015b). The FASTA sequences of fear extinction-related lincRNA, mRNA, and pre-mRNA transcripts were sourced from ensemble.org and used as the input files. The normalized free energy (ndG) cutoff, which indicates the relative stability of internal base pairs in the paired

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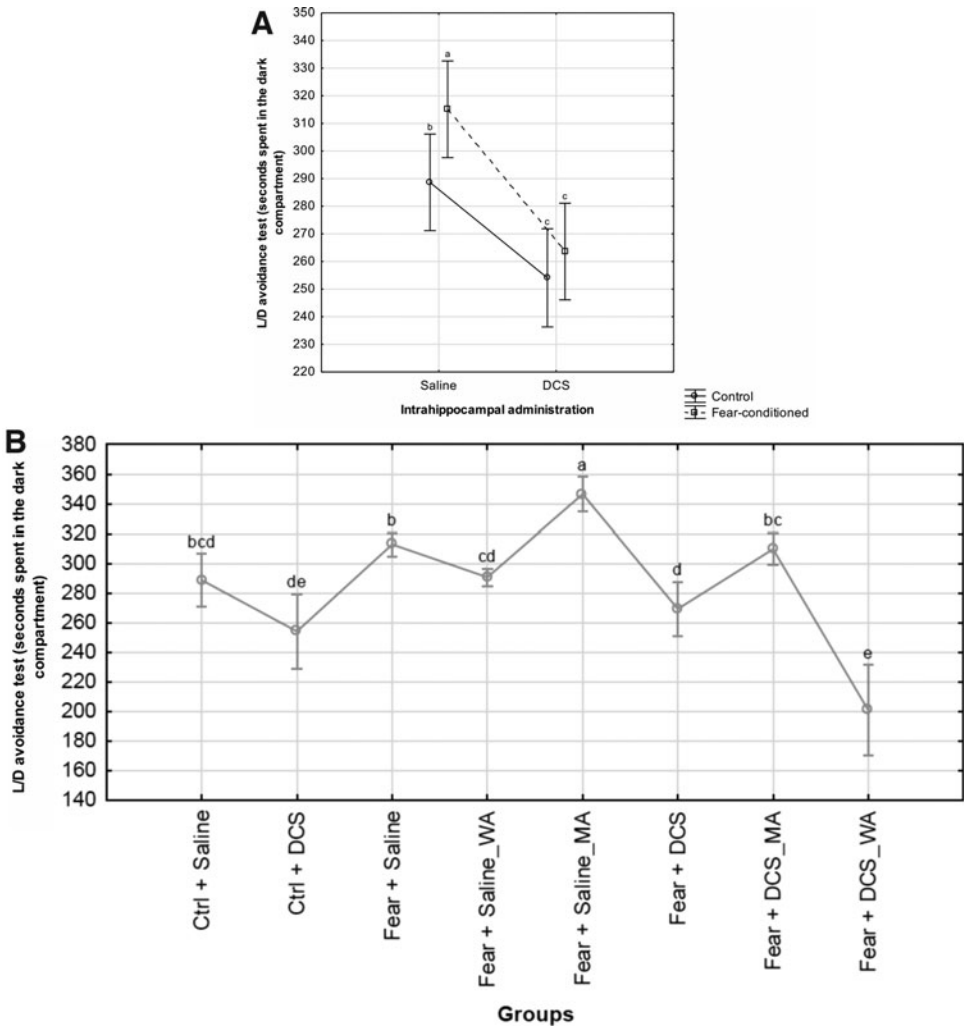


FIG. 3. **(A)** Fear conditioning significantly affected exploratory/anxiety-related behavior in the L/D avoidance test (two-way ANOVA). Fear + Saline animals spent more time in the dark compartment compared to Control + Saline animals. DCS also significantly influenced exploratory behavior—DCS administered animals spent less time in the dark compartment compared to saline-treated animals. **(B)** Time spent in the dark compartment of the L/D avoidance test for fear-conditioned and control animals that were treated with either saline or DCS, with the different MA and WA subgroups. The MA and WA subgroups exhibited the extreme behaviors of their respective groups (either Fear + Saline or Fear + DCS) (two-way ANOVA with Games Howell *post hoc* test). The FSM spent significantly more time in the dark compartment compared to the CS, indicating fear conditioning in the FSM subgroup and the FDW animals spent significantly less time in the dark compartment compared to the FSM animals, indicating successful fear extinction in the FDW subgroup. *Letters* on the graphs indicate significance ($p < 0.05$); *common letters* between two groups indicate that there is no significant difference; *different letters* between groups indicate a significant difference. *Whiskers* denote confidence intervals. Ctrl, control; DCS, D-cycloserine; L/D, light/dark; MA, maladapted; WA, well-adapted.

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RNAs (Dimitrov and Zuker, 2004; Markham and Zuker, 2008; Rehmsmeier et al., 2004), was set to the second-highest stringency of –0.15, to only report interactions with a high probability.

Putative functions of fear extinction-related lincRNAs

The functions of lincRNAs can be deduced from their genomic location or the functions of their targets. Gene set enrichment analyses were used to group transcripts together based on their functional similarity (Cline et al., 2007). We investigated the biological processes, molecular functions, pathways (using Comparative Toxicogenomics Database [CTD]; http://ctdbase.org/tools/analyzer.go) (Davis et al., 2019), and diseases (Rat Genome Database [RGD]; https://rgd.mcw.edu/rgdweb/enrichment/start.html) (Twigger et al., 2002) associated with the predicted interacting fear extinction-related mRNAs. Biological processes and molecular function categories were considered overrepresented if the Bonferroni-corrected p-value was <0.01 and for pathways when Bonferroni-corrected p-value was <0.05.

lincRNA quantitative trait loci overlap

The genomic locations of the fear extinction related-lincRNAs were inspected to determine proximity to quantitative trait loci [QTLs; genes or genomic loci that contribute significantly to the variation in phenotypes/traits (Zeng, 1994)], which could suggest putative functions of these lincRNAs (Cabili et al., 2011; Kutter et al., 2012; Li et al., 2015a). RGD (Twigger et al., 2002) was used to identify corresponding RGD names of the fear extinction-related lincRNAs, which were subsequently used to identify QTLs that overlap with the location of these lincRNAs.

Results

Animal behavioral results

The fear-conditioning protocol induced fearful behavior in the fear-conditioned animals (Fig. 2A, B). The fear extinction paradigm was successful in significantly reducing freezing behavior in the fear-conditioned animals and the coadministration of behavioral fear extinction and DCS was more effective in extinguishing fear compared with coadministered saline (Fig. 2C).

The L/D avoidance test was the most sensitive behavioral test to detect the effects of coadministered behavioral fear extinction and DCS and was subsequently used to differentiate MA (animals that displayed anxiety-like behavior) from WA (animals that did not display anxiety-like behavior) subgroups (Malan-Müller et al., 2016). In the L/D avoidance test, fear-conditioned animals showed more anxious behavior compared with the control animals and DCS elicited an anxiolytic effect—as noted in the exploratory behavior (Fig. 3A). MA animals spent significantly more time in the dark compartment of the L/D test arena compared with the WA animals and DCS-administered animals spent significantly less time in the dark compartment compared with saline-treated animals (Fig. 3B).

The FSM subgroup spent significantly more time in the dark compartment compared to the CS subgroup (Fig. 3B), justifying the use of these subgroups to investigate fear conditioning. The FDW subgroup spent significantly less time in the dark compartment compared to the FSM subgroup (Fig. 3B)—justifying the use of these subgroups to investigate fear extinction (induced by co-administration of DCS and behavioral fear extinction).

RNA sequencing and differential expression analysis

RNAs with false discovery rate <0.05 and absolute log-fold-change (logFC) ≥1 are illustrated as red points on the minus-add plot of logFC versus log-counts-per-millions (Fig. 4A). The overlap of detected differentially expressed features were calculated and plotted using the *UpSet* (Lex et al., 2014) package (Fig. 4B).

Our data revealed that lincRNAs made up the majority of the lncRNA class; only one antisense and one sense intronic RNA were expressed in opposite directions in the two comparison groups of interest, and we, therefore, focused on lincRNA and mRNA transcripts. A total of three transcripts

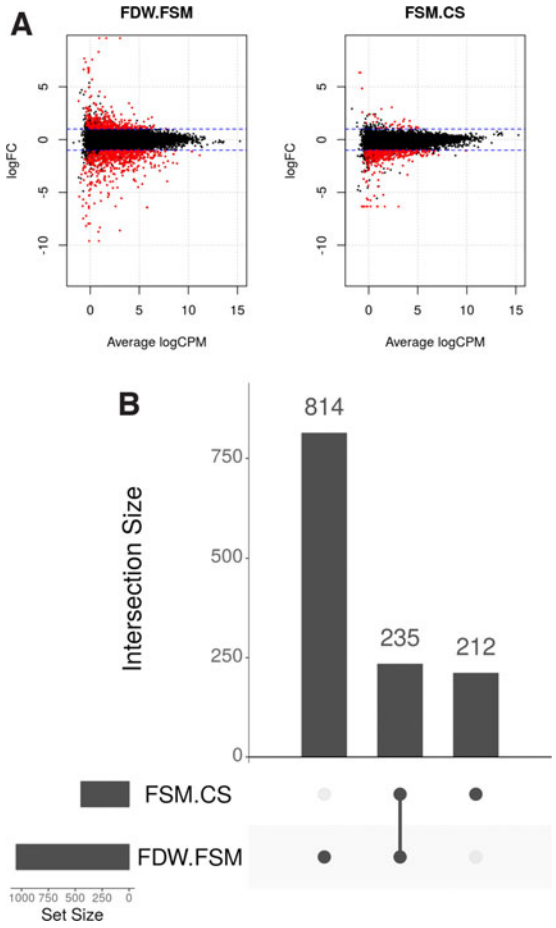


FIG. 4. **(A)** Minus-add plots of logFC versus average logCPM/average abundance of lincRNA and mRNA transcripts. The *blue dotted lines* indicate logFC cut off values of >1 or <–1. *Red points* are significantly differentially expressed transcripts (including lincRNAs and mRNAs) at a false discovery rate of 5%. **(B)** An UpSet plot illustrating unique and shared differentially expressed lincRNA and mRNA transcripts between the two subgroup comparisons, FSM versus CS and FDW versus FSM. An UpSet plot is a visualization approach for the quantitative analysis of sets, their intersections and aggregates of intersections, and serves as an alternative to Venn diagrams. logCPM, log-counts-per-millions; logFC, log-fold-change.

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were upregulated in the FSM versus CS and downregulated in FDW versus FSM, and a total of 230 transcripts were downregulated in FSM versus CS and upregulated in FDW versus FSM (including mRNA and lincRNA transcripts) (Supplementary Tables S1–S5). One transcript was downregulated in both these subgroup comparisons and another transcript was upregulated in both subgroup comparisons, resulting in a final sum of 235 overlapping transcripts (Fig. 4B and Supplementary Figs. S2 and S3 show all differentially expressed mRNA and lincRNA transcripts for the two comparison groups).

A total of 190 fear extinction-related mRNA transcripts were regulated in opposite directions between the two subgroup comparisons of interest ([1] FSM vs. CS and [2] FDW vs. FSM), with three transcripts upregulated and 187 transcripts downregulated in the fear-conditioning comparison group [1] relative to the fear extinction comparison group [2] (Fig. 5a and Supplementary Table S6) and 43 lincRNA transcripts were downregulated in the fear-conditioning comparison group [1] relative to the fear extinction comparison group [2] (Fig. 5b and Supplementary Table S7). A breakdown of the differentially expressed lincRNA and mRNA transcripts is provided in Table 1 (Supplementary Figs. S2 and S3, and Supplementary Tables S2–S7).

lincRNA-mediated gene expression regulation during fear extinction

LncTar predicted 119 lincRNA–mRNA interactions (Supplementary Table S8), from the 43 fear extinction-related lincRNA transcripts, eight lincRNAs were predicted to interact with 108 fear extinction-related mRNAs (Fig. 6). Nine mRNA transcripts were targeted by more than one lincRNA and the lincRNA ENSRNOT00000076905 had the highest number of predicted mRNA interactions, 89 in total.

LncTar predicted 30 interactions between differentially expressed lincRNAs and pre-mRNAs, with seven lincRNAs predicted to interact with 22 pre-mRNA transcripts (Fig. 7, Supplementary Table S9). There were six pre-mRNA transcripts, for which there was no corresponding interaction between the lincRNA and its mature mRNA transcript (Table 2, indicated with asterisks in Fig. 7).

Gene ontology, disease, and pathway enrichment analyses to predict functions of fear extinction-related lincRNAs

A total of 81 lincRNA-interacting fear extinction-related mRNAs were used in the enrichment analyses, 27 transcripts were clone-based transcripts with unknown functions and were excluded by CTD and RGD databases. Only the higher-order parental or ancestral terms for enriched diseases and higher GO levels for biological processes (levels 4–6) and molecular functions (levels 3–5) will be reported to simplify results and highlight key findings. Figure 8 shows the most enriched disease (top 25; Fig. 8a), biological process

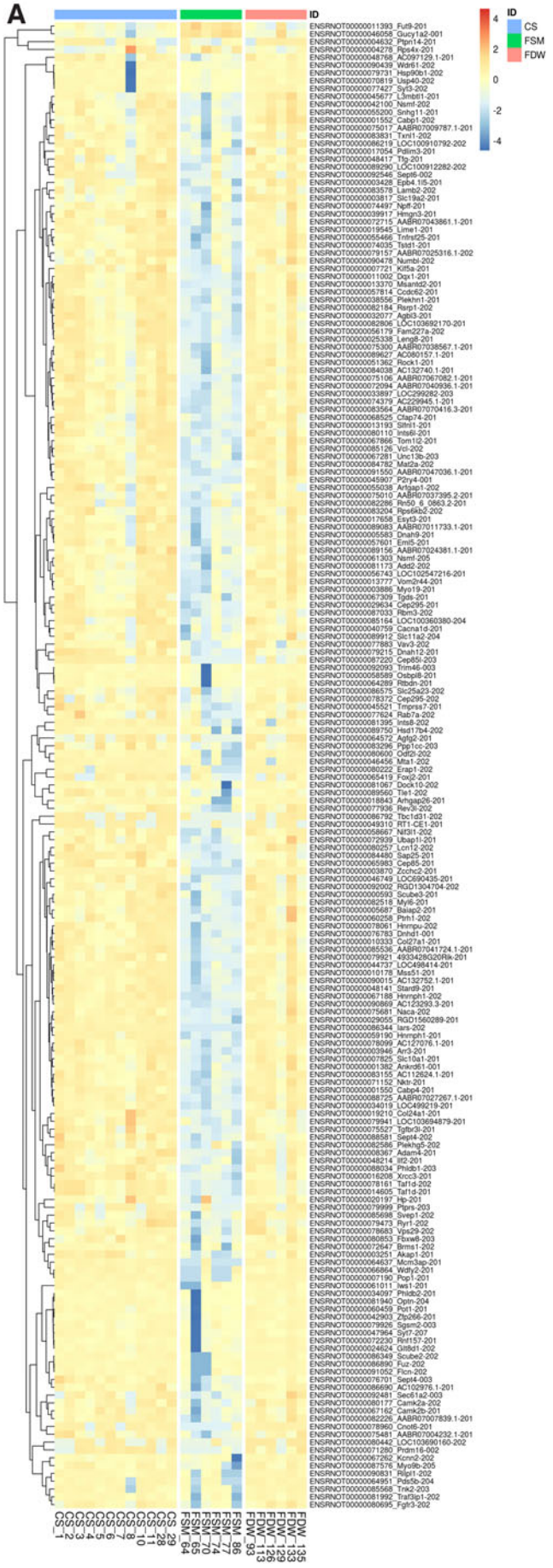


FIG. 5. **(A)** Heatmap of the 190 fear extinction-related mRNA transcripts that were differentially expressed between [1] FSM versus CS and [2] FDW versus FSM. **(B)** Heatmap of the 43 fear extinction-related lincRNA transcripts that were differentially expressed between [1] FSM versus CS and [2] FDW versus FSM.

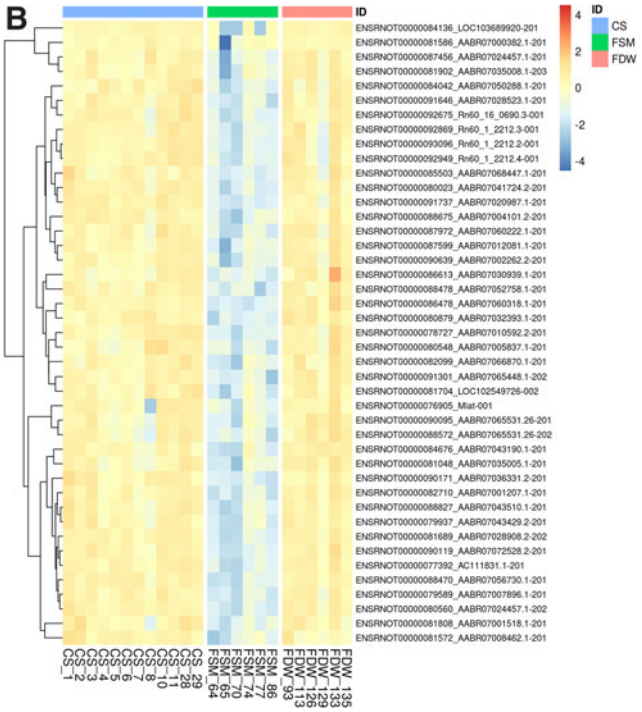


FIG. 5. (Continued).

(Fig. 8b), and molecular function terms (Fig. 8c), based on the Bonferroni-corrected *p*-values and the number of annotated genes for each term (Supplementary Tables S10–S12 contain exact *p*-values and all mRNA transcripts associated with each term).

A variety of disease terms were associated with these fear extinction-related lincRNAs (Fig. 8a); *Nervous system disease* was the most significant term and several related terms were also enriched, such as *Brain disease*, *Central nervous system disease*, and *Disease of mental health*. A total of 48 mRNA transcripts were associated with these disease terms, of which the lincRNA ENSRNOT00000076905 was predicted to interact with 45 mRNA transcripts. Additional predicted interactions included ENSRNOT00000088470 with *Arhgap26*, ENSRNOT00000080023 with *Rps6kb2*, and ENSRNOT00000092675 with *Serpina3n*.

Of the 13 enriched biological process terms, *Neuron projection morphogenesis* and *Nervous system development* were of particular interest (Fig. 8b). A total of 17 mRNA transcripts were associated with these terms, of which the lincRNA ENSRNOT00000076905 interacted with 15 neurogenesis-associated mRNA transcripts (*Add2*, *Baiap2*, *Camk2B*, *Fbxw8*, *Fuz*, *Lamb2*, *Nif3L1*, *Numbl*, *Prdm16*, *Ptprs*, *Rnf157*, *Rock1*, *Syt3*, *Traf3lp1*, and *Trim46*), ENSRNOT00000088470 interacted with *Arhgap26* and ENSRNOT00000076905 interacted with *Baiap2*.

Eleven molecular function terms were associated with the lincRNA-interacting mRNA transcripts (Fig. 8c) with the main molecular functions encompassed in the broader terms of nucleotide, ribonucleotide, and protein binding. A total of 26 mRNA transcripts were associated with these terms and 24 of these were predicted to interact with ENSRNOT0000 0076905. Furthermore, ENSRNOT00000080023 interacted with *Rps6kb2* and ENSRNOT00000088470 interacted with *Arhgap26* (Fig. 7).

Table 1. Summary of the Number of Differentially Expressed Messenger RNA and Long Intergenic Noncoding RNA Transcripts for CS Versus FSM and FSM Versus FDW Subgroups

	[1] FSM vs. CS (fear conditioning)	*[2] FDW vs. FSM (fear extinction)*
Differentially expressed mRNA transcripts (↑/↓)	392 (40/352)	983 (317/666)
Differentially expressed lincRNA transcripts (↑/↓)	55 (2/53)	66 (59/7)
Fear extinction-related transcripts		
Opposite mRNA transcripts [1] vs. [2] (↑/↓)	190 (3/187)	
Opposite lincRNA transcripts [1] vs. [2] (↑/↓)	43 (0/43)	

↑/↓ refers to numbers of upregulated/downregulated transcripts in the first group relative to the comparison group (thus 40 upregulated and 352 downregulated mRNA transcripts in the FSM group relative to the CS group). Opposite transcripts refer to transcripts that were differentially expressed in the fear conditioning [1] FSM versus CS and fear extinction [2] FDW versus FSM groups, but in opposite directions (e.g., fear conditioning will result in the downregulation of a particular transcript, and fear extinction will result in the upregulation of that same transcript).

CS, control + saline; DCS, D-cycloserine; FDW, 6 Fear + DCS WA; FSM, 6 Fear + Saline MA; lincRNA, long intergenic noncoding RNA; MA, maladapted; mRNA, messenger RNA; WA, well-adapted.

Two pathways, namely *Immune system* and *Signaling by Rho GTPases*, were associated with 15 of the fear extinction-related mRNAs that interacted with the fear extinction-related lincRNAs (Supplementary Table S13). Fourteen of these transcripts were predicted to interact with ENSRNOT00000076905; ENSRNOT00000080023 interacted with *Rps6kb2*; and ENSRNOT00000088470 interacted with *Arhgap26* (Fig. 7). The *Oxytocin signaling pathway* was associated with four of the fear extinction-related mRNAs; however, the association was not statistically significant (adjusted $p = 0.068$).

lincRNA QTL overlap

Four of the 43 fear extinction-related lincRNAs had available corresponding RGD names to use in the RGD QTL overlap analysis. Table 3 summarizes the most significant (logarithm of the odds ≥3, *p*-value <0.01) and relevant (in the context of fear extinction) QTLs.

Discussion

This study reports new findings on lincRNAs that are putatively involved in the molecular mechanisms of DCS-facilitated fear extinction. To the best of our knowledge, this is the first study to identify lincRNAs and their RNA targets with a putative role in transcriptional regulation during fear extinction in the context of a PTSD animal model.

lincRNAs associated with fear conditioning were identified as differentially expressed in FSM versus CS groups. Those associated with fear extinction were differentially

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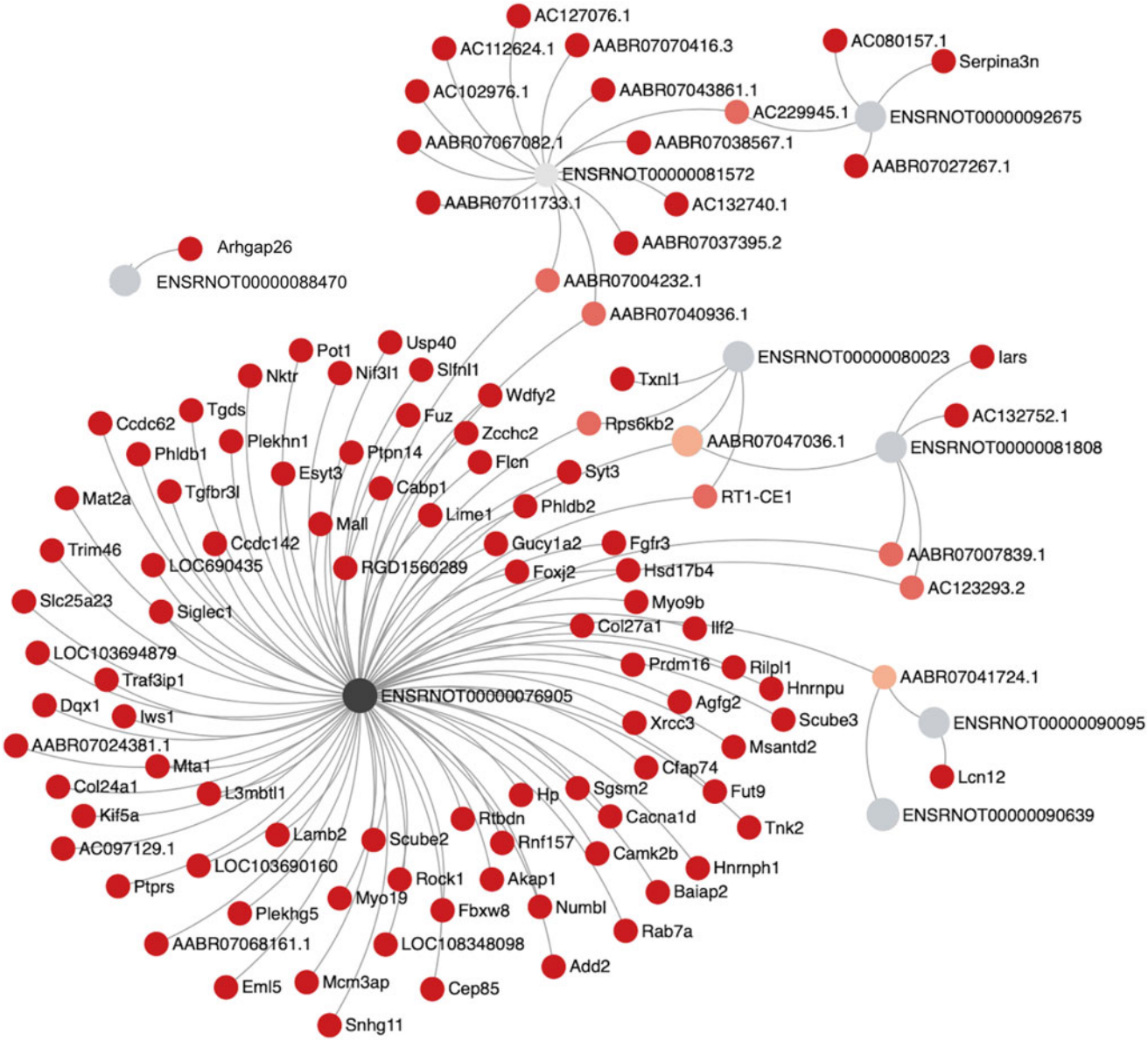


FIG. 6. Fear extinction-related lincRNA-mRNA interactions. LncTar predicted lincRNA-mRNA transcript interactions; *gray circles* represent the eight fear extinction-related lincRNA transcripts predicted to interact with the 119 fear extinction-related mRNA transcripts (*red* and *orange circles*). *Orange circles* represent the nine mRNA transcripts that interacted with more than one lincRNA transcript.

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expressed in FDW versus FSM, and in the opposite direction as in the FSM versus CS group, and were referred to as fear extinction-related lincRNAs.

Our prediction analyses indicated that, based on sequence homology, eight lincRNAs could interact with 108 mature mRNA transcripts. These interactions may have influenced RNA editing, mRNA stability, translation activation, and miRNA-mRNA interactions of genes that are important for fear extinction (see gene ontology discussion). Seven lincRNAs were predicted to interact with 22 pre-mRNA transcripts. Six of these interactions were not predicted for the corresponding mature mRNA transcripts, where the hybridization occurred in intronic regions or within exon-intron boundaries. We hypothesize that these interactions may influence translation and splicing events in those transcripts. Therefore, the differential expression of some of the fear extinction-related mRNAs could be attributed to alternative splicing of their fear extinction-related pre-mRNAs.

Of particular interest was the interaction between ENSRNOT00000076905 and the pre-mRNA of the NMDA receptor synaptonuclear signaling and neuronal migration factor gene (*Nsmf*), since DCS is a partial NMDAR agonist and binding of DCS to NMDARs facilitates extinction learning (Ledgerwood et al., 2003, 2005; Yang and Lu, 2005). *Nsmf* encodes the protein, Jacob, which is important for cell survival and the stability of synaptic cell contacts and is NMDAR-dependent (Dieterich et al., 2008). A recent *Nsmf* mouse knockout study reported hippocampal dysplasia, impaired BDNF-signaling during dendritogenesis, and phenotypes related to the lack of BDNF-induced nuclear import of

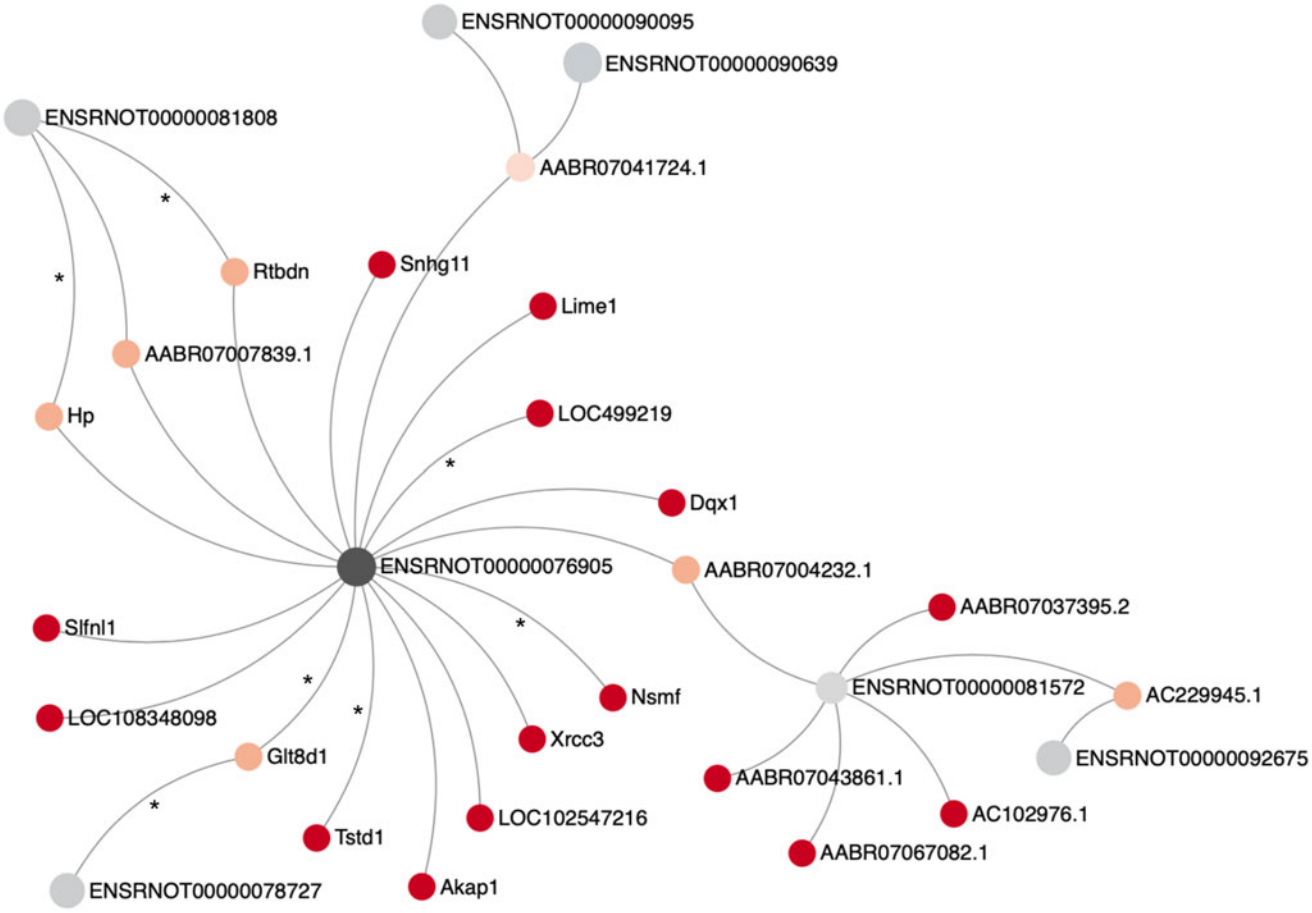


FIG. 7. Fear extinction-related lincRNA-pre-mRNA interactions. LncTar predicted lincRNA-pre-mRNA transcript interactions; *gray circles* represent the seven fear extinction-related lincRNA transcripts predicted to interact with the 22 fear extinction-related pre-mRNA transcripts (*red* and *orange circles*). *Orange circles* represent the nine pre-mRNA transcripts that interacted with more than one lincRNA transcript. *Asterisks* indicate the six pre-mRNA transcripts and seven interactions for which there was no corresponding interaction between the lincRNA and its mature mRNA transcript.

TABLE 2. PREDICTED FEAR EXTINCTION-RELATED LONG INTERGENIC NONCODING RNA AND PREMESSENGER RNA INTERACTIONS THAT MAY AFFECT SPLICING

Pre-mRNA target	*lincRNA*	*Target region*
Glt8d1	ENSRNOT00000076905	intron 2
Glt8d1	ENSRNOT00000078727	intron 2, exon 3, intron 3
Hp	ENSRNOT00000081808	intron 2, exon 3, intron 3
Nsmf	ENSRNOT00000076905	intron 4
LOC499219	ENSRNOT00000076905	intron 1
Rtbdn	ENSRNOT00000081808	intron 1
Tstd1	ENSRNOT00000076905	intron 3 to exon 4

Glt8d1, glycosyltransferase 8 domain containing 1 gene; *Hp*, haptoglobin gene; *LOC499219*, gene transcribing hypothetical protein; NMDA, N-methyl-D-aspartate; *Nsmf*, NMDA receptor synaptonuclear signaling and neuronal migration factor gene; *Rtbdn*, retbindin gene; *Tstd1*, thiosulfate sulfurtransferase-like domain containing 1 gene.

Jacob, implicating the role for the protein in hippocampal dendrite- and synaptogenesis (Spilker et al., 2016).

Our data indicated that *Nsmf* was downregulated during fear conditioning (FSM vs. CS) and upregulated during DCS-facilitated fear extinction (FDW vs. FSM), potentially through the activation of NMDARs by DCS, which facilitates nuclear import of the Jacob protein (Dieterich et al., 2008). This could promote dendrite- and synaptogenesis, and possibly facilitate fear extinction. Furthermore, the *Nsmf* gene undergoes extensive splicing, with more than 20 known splice isoforms (Spilker et al., 2016). The overexpression of one such splice isoform (Δ*ex9-Jacob*) in primary neurons resulted in decreased dendritic complexity and number of synapses (Dieterich et al., 2008), emphasizing the importance of Jacob splice variants in hippocampal synaptogenesis, a process central to learning and memory (Geinisman et al., 2001; Klintsova and Greenough, 1999). Our analysis predicted an interaction between ENSRNOT00000076905 and *Nsmf* pre-mRNA, which may result in alternative splicing of the Jacob protein, with possible implications for hippocampal synaptogenesis and, possibly, fear extinction.

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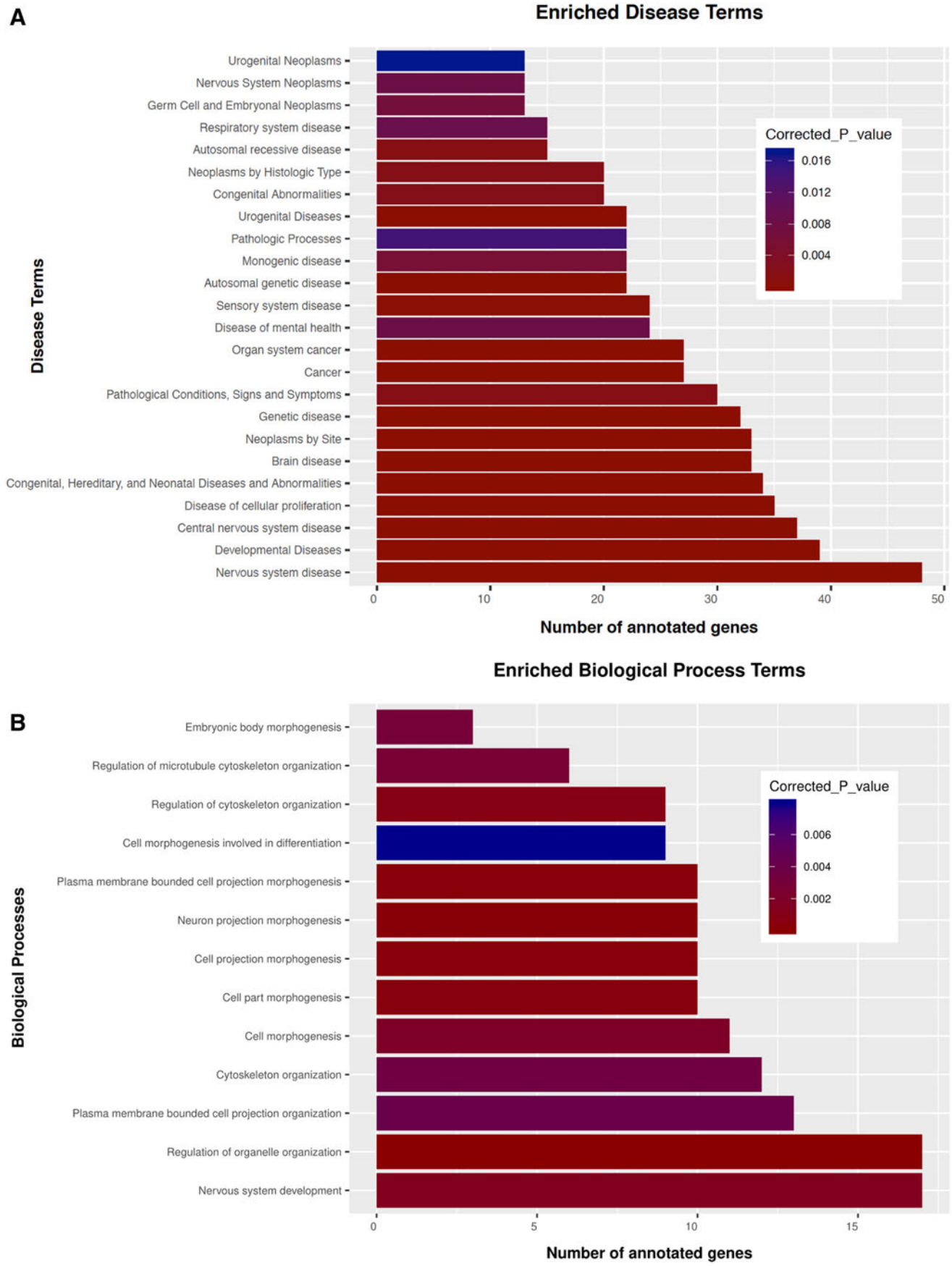


FIG. 8. **(A)** The top 25 enriched disease terms associated with the 61 fear extinction-related mRNAs predicted to interact with fear extinction-related lincRNAs. **(B)** Biological processes associated with the 61 fear extinction-related mRNAs predicted to interact with fear extinction-related lincRNAs. **(C)** Molecular functions associated with the 61 fear extinction-related mRNAs predicted to interact with fear extinction-related lincRNAs. Bars are filled according to the significance level of Bonferroni-corrected *p*-values; significance increases from *blue* to *red*.

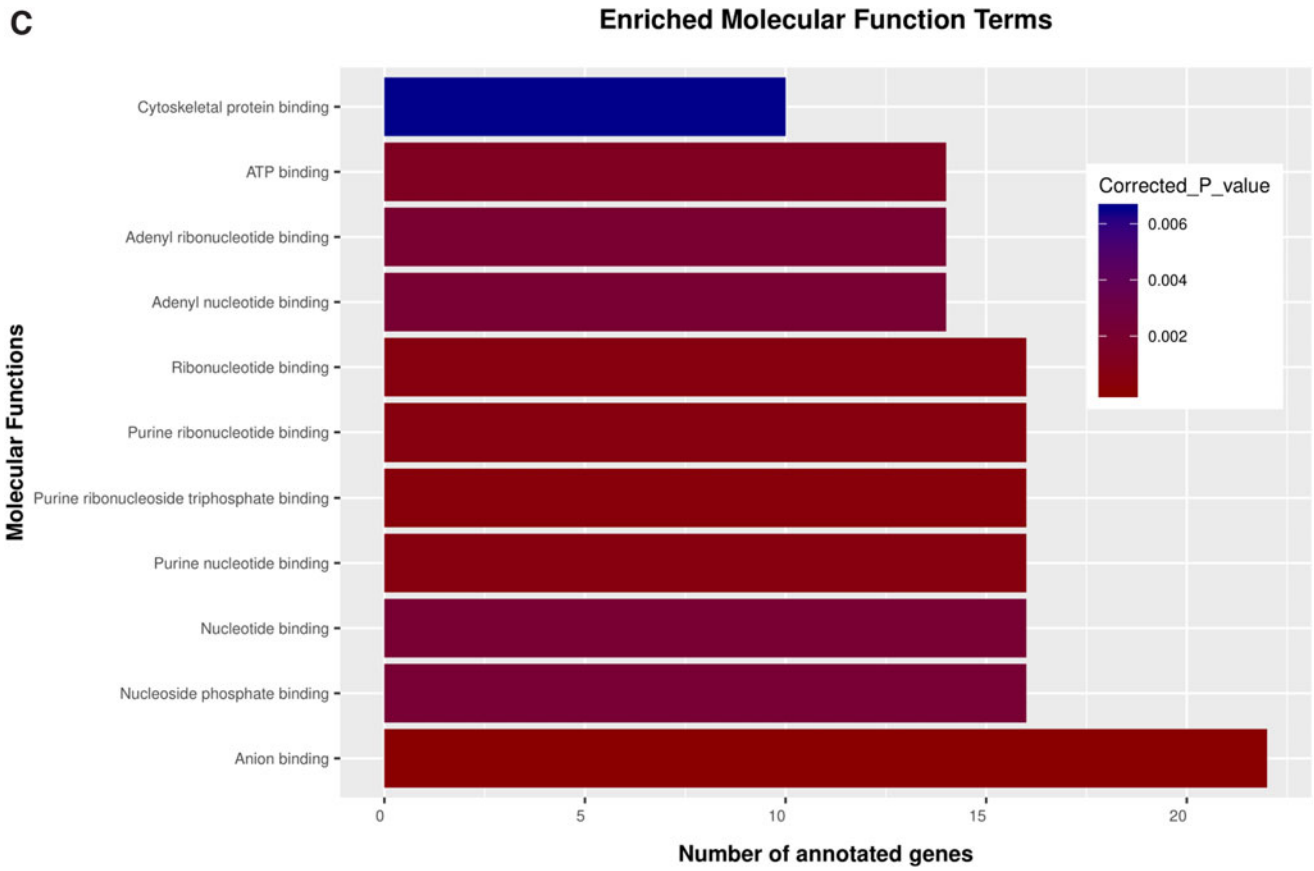


FIG. 8. (Continued).

Disease enrichment analyses on the set of predicted fear extinction related mRNA targets indicated that the most enriched disease term was *Central nervous system disease*, but other synonymous disease terms were also significant, including *Disease of mental health*. The lincRNA ENSRNOT00000076905 was predicted to interact with the majority of mRNAs enriched in these disease terms (Supplementary Table S10). The likely reason for the vast number of predicted interactions of this lincRNA is its short length (140 bp), which increases the likelihood of complementary hybridization to several mRNA transcripts. Additional lincRNAs predicted to interact with mRNAs enriched for CNS disease terms, included ENSRNOT00000088470, ENSRNOT00000080023, and ENSRNOT00000092675. These lincRNAs could, therefore, be involved in diseases that affect the CNS and mental health, by targeting and regulating genes associated with these disease terms.

For biological process enrichment, one lincRNA, ENSRNOT00000076905, was predicted to interact with 15 of the 17 mRNA transcripts involved in *Nervous system development and neuronal projection (neurogenesis)*. We hypothesize that this lincRNA is involved in neurogenesis, neuronal projection, and extension. The fear extinction protocol consisted of reexposure to the shock chamber (without shock application), together with intrahippocampal DCS administration. Our findings, therefore, suggest that DCS facilitated the process of fear extinction by promoting hippocampal neurogenesis. This correlates with earlier findings reporting that hippocampal DCS infusion increased neuronal proliferation and neural plasticity mediated by hippocampal NMDA receptors, thereby promoting the acquisition and retrieval of extinction memory (Ren et al., 2013). These results shed further light on the molecular mechanisms behind DCS-facilitated fear extinction, where the lincRNA ENSRNOT00000076905 may interact and regulate the expression of several mRNA transcripts, to ultimately facilitate fear extinction via neurogenesis.

Other mRNAs that were enriched in the biological process *Neurogenesis* were the brain-specific angiogenesis inhibitor 1-associated protein 2 (*Baiap2*), that was predicted to interact with ENSRNOT00000076905, and Rho GTPase activating protein 26 (GTPase Regulator) (*Arhgap26*), that was predicted to interact with ENSRNOT00000088470. The *Baiap2* gene encodes a synaptic protein whose hippocampal expression is required for learning, memory (Kim et al., 2009), and social competence (Chung et al., 2015). Furthermore, a SNP in *BAIAP2* has been associated with negative modulation of memory strength in humans (Luksys et al., 2014), a process that plays an important role in PTSD (Parsons and Ressler, 2013). A study that investigated early-life programming and related gene x environment interactions in the context of anxiety and depression found that *Baiap2* was downregulated following prenatal stress exposure (Jakob, n.d.). We, therefore, hypothesize that DCS administration reversed the negative effect that fear conditioning had on the expression of *Baiap2*, via a proposed ENSRNOT000000

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TABLE 3. LONG INTERGENIC NONCODING RNAS THAT OVERLAP WITH QUANTITATIVE TRAIT LOCI OF INTEREST

lincRNA	*Symbol*	*QTL name*	*LOD*	p-*Value*	*Trait*	*Chr*
LOC102549726 ENSRNOT00000081704	Despr15	Despair-related QTL 15	NA	0.003	Locomotor behavior trait (VT:0001392)	20
	Scort12	Serum corticosterone level QTL 12	20.46	0.001	Blood corticosterone amount (VT:0005345)	
	Scort15	Serum corticosterone level QTL 15	3.48	0.001	Blood corticosterone amount (VT:0005345)	
LOC103689920 ENSRNOT00000084136	Anxrr19	Anxiety-related response QTL 19	5.07	NA	Body movement coordination trait (VT:0005424)	10
	Neuinf9	Neuroinflammation QTL 9	4.6	NA	Nervous system integrity trait (VT:0010566)	
	Scort13	Serum corticosterone level QTL 13	3.26	0.001	Blood corticosterone amount (VT:0005345)	
	Scort19	Serum corticosterone level QTL 19	6.3	0.001	Blood corticosterone amount (VT:0005345)	
	Stresp21	Stress response QTL 21	3.3	NA	Thymus mass (VT:0004954)	
LOC100912578 ENSRNOT00000088478	Anxrr10	Anxiety-related response QTL 10	3.9	NA	Exploratory behaviour trait (VT:0010471)	3
	Despr11	Despair-related QTL 11		<0.001	Locomotor behavior trait (VT:0001392)	
	Neuinf11	Neuroinflammation QTL 11	3.9	NA	Nervous system integrity trait (VT:0010566)	
	Scort3	Serum corticosterone level QTL 3	23.37	0.001	Blood corticosterone amount (VT:0005345)	
LOC102550455 ENSRNOT00000086613	Neuinf7	Neuroinflammation QTL 7	3.4	NA	Nervous system integrity trait (VT:0010566)	10

Chr, chromosome; LOD, logarithm of the odds; NA, not available (for certain QTLs either the LOD score or *p*-value was unavailable on RGD); QTL, quantitative trait loci; RGD, Rat Genome Database.

76905-mediated upregulation of *Baiap2*, thereby promoting fear extinction learning.

The *Arhgap26* gene transcribes a protein that is part of the Rho family of GTPases, and interestingly, *Signaling by Rho GTPases* was one of the enriched pathways associated with fear extinction-related lincRNAs. This pathway has been implicated in the regulation of learning and memory (Diana et al., 2007). The rearrangement of synaptic connections in neural networks is one of the proposed mechanisms underlying memory formation. Dendritic spines receive the majority of excitatory synapses (Harris, 1999; Nimchinsky et al., 2002) and undergo dynamic, experience-dependent changes (Trachtenberg et al., 2002). Furthermore, changes in dendritic spine morphology have been observed during long-term potentiation (LTP), a process that models the activity-dependent changes of synaptic efficacy and the cellular basis of learning (Lang et al., 2004; Muller et al., 2000).

Dendritic spine morphology and rearrangement are controlled by the neuronal actin cytoskeleton (Fukazawa et al., 2003; Zito et al., 2004), of which actin assembly, polymerization, and actomyosin contraction are mainly regulated by small GTPases of the Rho family (Edwards et al., 1999; Kimura et al., 1996; Maekawa et al., 1999). LTP induction is associated with actin cytoskeletal reorganization, which is characterized by a sustained increase in F-actin content within dendritic spines. This increased F-actin content is dependent on NMDA receptor activation and involves the inactivation of actin-depolymerizing factor (cofilin) (Fukazawa et al., 2003).

In the present study, therefore, it is possible that DCS activated the NMDA receptor, resulting in increased F-actin content and subsequent alteration in neuronal morphology, such as neuronal projection, mediated by Rho GTPases, ultimately facilitating optimal learning and memory (Luo et al., 1996; Tashiro et al., 2000). We also hypothesize that the lincRNAs ENSRNOT00000076905 and ENSRNOT00000088470 may have been involved in this process by regulating the expression of genes implicated in the Rho GTPase signaling pathway.

Another pathway we found to be associated with the fear extinction-related lincRNAs was the *Immune system*. In recent years, there has been a growing awareness of the importance of the immune system in supporting optimal CNS functioning (Besedovsky and del Rey, 2011) and the detrimental effects that a dysregulated immune system can evoke on neuronal functioning (Molina-Holgado and Molina-Holgado, 2010; Yirmiya and Goshen, 2011) and mental health (Pariante, 2014; Zass et al., 2017). Optimal immune functioning not only supports stress-coping responses but is also essential for learning and memory (Besedovsky and del Rey, 2011; Molina-Holgado and Molina-Holgado, 2010; Yirmiya and Goshen, 2011). In this study, DCS may have facilitated fear extinction by regulating the expression of immune-related genes via lincRNAs such as ENSRNOT00000080023 and ENSRNOT00000076905.

As expected, the main molecular functions associated with fear extinction related lincRNAs were nucleotide, ribonucleotide, and cytoskeletal protein binding, since the main features of lincRNAs are the regulation of gene and protein expression through its interactions with chromatin and RNAs or by recruiting and interacting with transcriptional repressors or enhancers (as reviewed by Dykes and Emanueli, 2017). lncRNAs can even interact with DNA and one mechanism involved in direct RNA–DNA interactions

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involves formation of triple helices. Double-stranded DNA forms triple-helical structures by incorporating a third single-stranded nucleic acid in its major groove, forming Hoogsteen or reverse Hoogsteen hydrogen bonds with a purine-rich strand of DNA (Broitman et al., 1987).

Interestingly, other enriched molecular functions included purine nucleotide-binding and purine ribonucleotide binding. In the nucleus, these triple-helical structures (containing ribosomal DNA [rDNA] and lincRNA) are recognized by DNA methyltransferase DNMT3B, which methylates rDNA promoters and subsequently represses rDNA transcription (Schmitz et al., 2010). Moreover, certain lncRNAs directly interact with DNA in a sequence-specific manner to activate (Mondal et al., 2015; Postepska-Igielska et al., 2015) or repress transcription (Grote et al., 2013; Kalwa et al., 2016) through the recruitment of coactivator or corepressor proteins. Some lncRNAs can also form triple helices in *cis* (autobinding) (Grote et al., 2013; Postepska-Igielska et al., 2015; Zhao et al., 2018), enabling regulation of the exact locations they are transcribed from. Our findings not only highlight the main functions of lincRNAs but also point to directions for future research, namely interrogation of lincRNA-DNA interactions and their subsequent effects on transcriptional and translational regulation.

lncRNAs can regulate the expression of neighboring genes in-*cis* (Ma et al., 2013; Maenner et al., 2010). We, therefore, aimed to identify QTLs that overlapped with the genomic regions of four fear extinction-related lincRNAs, for which there was an RGD ID available (ENSRNOT00000081704, ENSRNOT00000084136, ENSRNOT00000088478 and ENSRNOT00000086613). The selected QTLs of interest were involved in traits such as serum corticosterone level, neuroinflammation as well as anxiety-, stress-, and despair-related responses. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis results in an inability to initiate a normal stress response, which is a key feature of PTSD. The HPA axis is regulated by a negative feedback mechanism where excess cortisol (or corticosterone in rodents) binds to glucocorticoid receptors in the hypothalamus and pituitary and subsequently suppresses the release of corticotropin-releasing hormone and adrenocorticotropin hormone.

The HPA axis also interacts with the immune system to maintain homeostasis (Wong et al., 2002), and there is an intricate relationship among the immune system, brain, and behavior, as discussed earlier. Research has also shown that immune functioning is affected in PTSD patients (Eraly et al., 2014; Heath et al., 2013; Morath et al., 2014). We, therefore, hypothesize that the upregulation of these lincRNAs, following DCS administration, may result in the *cis*-regulation of genes that control cortisone levels and neuroinflammation, which elicited downstream effects on learning and memory to ultimately alleviate anxiety- and stress-related responses and promote successful fear extinction.

Conclusions and Outlook

This study is a first step toward addressing the current knowledge gaps in the role of lincRNAs in fear conditioning and extinction in the context of DCS. We used bioinformatics, *in silico* interaction prediction, and gene set enrichment analyses to identify differentially expressed lincRNAs that putatively target and regulate the expression of mRNAs that are enriched in biological processes, molecular functions and pathways that mediate fear extinction. We also identified lincRNAs with genomic locations in close proximity to QTLs that are associated with fear and anxiety-related traits. Unfortunately, due to insufficient hippocampal tissue, additional qPCR validation of differentially expressed lincRNAs and further investigation of expression levels of mRNA and protein of predicted mRNA targets were not possible.

Future studies could build on the *in silico* results by including functional verification of lincRNA, mRNA, and protein levels. The *in silico* lincRNA-mRNA interaction findings are predictive and serve to guide future functional studies to verify these predicted interactions and splicing events in cell culture and also in knock-out animal models. Furthermore, lincRNAs can regulate gene and protein expression through several mechanisms, however, with the data and material at our disposal, our investigation was limited to the interactions with mRNAs and pre-mRNAs. Future studies could expand this investigation to include other regulatory mechanisms of lincRNAs and the potential role of other ncRNA classes during fear extinction.

While the present findings require further replication in independent studies, this is the first study to identify lincRNAs and their RNA targets that could play a role in transcriptional regulation during fear extinction induced by the coadministration of behavioral fear extinction and DCS. We identified differentially expressed lincRNAs and their predicted mRNA and pre-mRNA targets that could help us decipher the molecular basis of DCS-induced fear extinction.

Notably, four hippocampal lincRNAs, ENSRNOT00000076905, ENSRNOT00000088470, ENSRNOT00000080023, and ENSRNOT00000092675 were predicted to interact with nucleotides, ribonucleotides and proteins, thereby possibly regulating the expression of genes involved in neuronal projection and neurogenesis, a dynamic process required during learning and memory—possibly mediated by the Rho GTPase pathway. Through the regulation of serum corticosterone levels, and subsequently, the HPA-axis, lincRNAs ENSRNOT00000081704, ENSRNOT00000084136, ENSRNOT00000088478, and ENSRNOT00000086613 may also have attenuated anxiety, stress, and despair-related responses through improved neuroimmune functioning. These eight lincRNAs are proposed as potentially important actors in the molecular sequence of events resulting in effective fear extinction observed in this animal model which reflects the core phenotypes of PTSD.

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Author Disclosure Statement

The authors declare they have no conflicting financial interests.

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Supplementary Material

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Abbreviations Used

ANOVA = analysis of variance
CBT = cognitive behavioral therapies
CNS = central nervous system
CR = conditioned response
CS = Control + Saline
CTD = Comparative Toxicogenomics Database
DCS = D-cycloserine
HPA = hypothalamic-pituitary-adrenal
L/D = light/dark
LDH = left dorsal hippocampus
lincRNAs = long intergenic noncoding RNAs
lncRNAs = long noncoding RNAs
logFC = log-fold-change
MA = maladapted
miRNAs = microRNAs
mRNA = messenger RNA
ncRNAs = noncoding RNAs
NMDA = N-methyl-D-aspartate
Nsmf = NMDA receptor synaptonuclear signaling and neuronal migration factor gene
PND = postnatal day
PTSD = posttraumatic stress disorder
QTL = quantitative trait loci
RGD = Rat Genome Database
US = unconditioned stimulus
WA = well-adapted

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