TITLE: Gas5 regulates early life stress-induced anxiety and spatial memory

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ABSTRACT

The early life stress (ES) of maternal separation (MS) evokes anxiety and memory deficits. Emerging studies have shown that MS-induced gene expression in the hippocampus is operated at the level of transcription. However, the involvement of non-coding RNAs in MS-induced behavioural deficits is poorly understood. Here, we have explored the role of synapse-enriched long non-coding RNAs (lncRNAs) in anxiety and memory upon MS. We observed that MS led to an enhancement of expression of the lncRNA Gas5 (Growth Arrest Specific-5) in the hippocampus; accompanied by increased levels of anxiety and deficits in spatial memory. The knockdown of Gas5 in early life could reduce anxiety and partially rescue the spatial memory deficit of maternally separated adult mice following MS. Gene Ontology analysis revealed that Gas5 exerts its function by regulating RNA metabolism and translation. Our study highlights the importance of MS-regulated lncRNA in anxiety and spatial memory.

SIGNIFICANCE STATEMENT

The mechanism of early-life stress-induced gene expression and its implications in associated behavioural deficits are poorly resolved. The study fills this void by identifying lncRNA as a regulatory factor linked with early life stress of maternal separation. This study demonstrates that the reversal of Gas5 expression, a synapse-enriched lncRNA, enables the partial rescue of anxiety and memory deficits. Our study highlights an RNA-based approach to alleviate the adverse effects of early life stress.

INTRODUCTION

Maternal separation, a well-established model of early-life stress, has been extensively studied to investigate the long-term consequences of adverse early life events during this critical period of brain function and behaviour (Taylor et al., 2010; Korosi et al., 2012). Stress due to maternal separation (MS) in rodents leads to anxiety-like behaviours in adulthood as characterized by heightened fear, avoidance, and stress sensitivity (Banqueri et al., 2017; Lundberg et al., 2020; Balouek *et al.*, 2023). The ventral hippocampus, a key brain area affected by MS (Dimatelis et al., 2014; Marais et al., 2009), has been shown to regulate emotions (Jimenez et al., 2018;

H. Li et al., 2022; McNaughton, 1997). MS-induced anxiety can lead to changes in the total volume of the hippocampus (Kalisch et al., 2006) and alterations in the expression of glutamatergic receptors (Pickering et al., 2006), both NMDA (Barkus et al., 2010) and AMPA (Katsouli et al., 2014); GABAergic receptors (Sterley et al., 2013), and serotonergic as well as cholinergic receptors (File et al., 2000). Moreover, early-life stress can impact spatial memory that involves the dorsal hippocampus (Morris *et al.*, 2006; Blum *et al.*, 1999). Such behavioural deficits are linked with structural and functional modifications in the hippocampus (Izquierdo and Medina, 1997; Revest *et al.*, 2009; Kremmyda *et al.*, 2016). These observations indicate that the enduring effects of maternal separation on anxiety and memory involve alterations in synaptic plasticity (Monroy et al., 2010; Bannerman *et al.*, 2014) and neurotransmitters, for example, noradrenaline and serotonin (Daniels et al., 2004).

More recently, transcriptomics analysis following MS identified a distinct set of miRNAs in specific brain areas including the hippocampus (Mckibben and Dwivedi, 2021). Some of these miRNAs are localized at the synapse and have been shown to regulate synaptic plasticity and memory (Mckibben and Dwivedi, 2021). A recent study from our laboratory has identified a set of synapse-enriched lncRNAs from the hippocampus. Among these transcripts, a specific lncRNA has been shown to regulate hippocampus-dependent memory (Samaddar et al., 2023). LncRNA functions as scaffolds, decoys, guides, or enhancers to modulate chromatin remodelling, transcription, mRNA stability, and RNA-protein interactions (Mattick et al., 2023; Mercer et al., 2009). Recent attention has focused on lncRNA activity at synapses that govern synaptic plasticity, neuronal development, and neurological disorders (Zhang et al., 2017) (Shi et al., 2017) (Wang et al., 2017). The regulatory function of lncRNA has also been attributed to anxiety (Spadaro et al., 2015) and memory (Soutschek et al. and Schratt et al., 2023; Cui et al., 2022).

Prompted by these observations, we have investigated the impact of MS on the expression of lncRNAs and their involvement in MS-induced anxiety and memory. Our study identified that MS enhanced the expression of the lncRNA Gas5. The knockdown of Gas5 partially alleviates the enduring consequences of early-life stress as detected by the reduction in anxiety and the occlusion of memory deficits following MS.

MATERIAL AND METHODS

Animal ethics, acquisition, and maintenance

All animals were issued from the National Brain Research Centre (NBRC) animal house facility from the protocol numbers NBRC/IAEC/2015/171, NBRC/IAEC/2015/172 and NBRC/IAEC/2015/178 approved by IAEC (Institutional Animal Ethics Committee) of NBRC registered with the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) (Registration number: 464/GO/ReBi-S/Re-L/01/CPCSEA).

Animals were housed in standard housing facilities in cages with food water *ad libitum*. A temperature of around 24± 0.5 °C and humidity of 40 ± 5% was maintained along with a 12-hour light/dark cycle for all animals.

Maternal Separation

A chronic early-life stress paradigm was used on C57BL/6 mice pups (Sood et al., 2018). A litter size of 5-7 pups was selected for the experiment. Postnatal day 2 (P2) pups were separated from their mother for a period of 3 hours (0900 to 1200) till P14. The mother was first moved into a different fresh cage and kept in a separate room. The pups were then placed in a beaker with soft bedding and protective paper lining and the temperature was maintained at 37℃ throughout the period of separation. After 3 hours, first, the pups were kept back in the original cage followed by the mother. No separation was done from P15 till weaning on P21. Handling of the pups was done very gently. Cage changes were done every three days starting from P4. For the control group, designated as homecaged (HC), all litters and mothers were kept undisturbed from P0 to P21 except for regular maintenance and cage changes every three days. Male pups were sacrificed at P21, and hippocampi were isolated for further biochemical processes.

Screening of Lnc RNAs

P21 male pups were sacrificed and the whole hippocampi were harvested in Trizol reagent (Invitrogen) for total RNA isolation. SuperScript III First Strand Synthesis System (Life Technologies, Invitrogen) kit was used for synthesizing cDNA following the manufacturer's protocol. Further, qPCR of the cDNA prepared was done using SyberGreen (Applied Biosystems). See Table 1 for qPCR primer details.

TABLE 1.

No	LncRNA	Forward Primer (5' – 3')	Reverse Primer (5' – 3')
1	Tubulinβ-1	GCGCCTTTGGACACCTATTCA	CCGCGCCCTCCGTATAGTGC
2	GAPDH	CGTATTGGGCGCCTGGTCAC	CGGCCTCACCCCATTTGATG
3	Hprt		
4	Gas5	AGGAGGATGAAGGCTTACGA	AACTGTCTATCCAGCCTCAA
5	Pvt1	TTGCCTGGAGATGTTACAAG	AACATTCTCATTGAGTCAAG
6	SynLAMP (2410006H16Rik)	AAGAGTGGAAGATTGCCAAC	GATTACCAAATGTGCCCACC
7	2900052NO1 Rik	ACAATCCAAATCTACCCACGAG	TGGGATTGTAGATTGTGCTGTC
	1700020IRik	TGACTGCATCTCCGAGGAAT	GGCCTGCTACTATTGCTTACA
9	Zfas1	CGCCGCCAAGGTCTATATAA	CAGGTCAACCATCTGCAAGT
10	Pantr1	ATTTCCTGAGTCAACATGGC	AGAGTATGCCACATGGATGATG
11	Snhg8	CAGTGGAGGAAGTGTTAAGGT	CCACTACAGTACCTGCAAGAA
12	Snhg12	TCTCCGGATGATTCGTGAGA	TCTGCTGTATCGTTGTCCAT
13	Rmst1	CATGAATGTTGTCGACACTGT	GAGCTTGGCAGATCCTTCAA

Cloning and Virus preparation

EF1-α promoter from pLVTHM (Addgene # 12247) was used to replace the CMV promoter in pAAV.U6.shRLuc.CMV.ZsGreen.SV40 (Penn Core Vector) plasmid by excising out the CMV promoter using the EcoRI and NHE-I (NEB #R3131) cut sites. Infusion cloning (Takara-Bio In-Fusion HD cloning Kit; 639650) was used to clone in EF1-alpha promoter generating a pAAV.U6.shRLuc.EF1-α.ZsGreen.SV40 plasmid and express ZsGreen under EF1-α promoter (Table 2). Universal Control (UC) and

Gas5 shRNAs (shRNA # 1= G5C1 and shRNA # 2= G5C2) were cloned by replacing the existing Luciferase shRNA (shRLuc) under a U6 promoter using BamHI (NEB #R0136L) and EcoRI (NEB #R3101) restriction sites (Table 3). The final vector was coined as pAAV.U6.shRNA(UC).EF1-α.ZsGreen.SV40, pAAV.U6.shRNA(G5C1).EF1-α.ZsGreen.SV40 and pAAV.U6. shRNA(G5C2).EF1-α.ZsGreen.SV40 respectively. All clones were verified by Sanger sequencing.

TABLE 2.

No.	Primers for replacing CMV with EF1-α in AAV backbone	Infusion primer
1.	Forward primer 5'-3'	TTTTACGCGTGAATTCGGCTCCGGTGCCCGTCAG
2.	Reverse primer 5'-3'	GACCGGTAGCGCTAGCTCACGACACCTGAAATGGAAG

TABLE 3.

No.	Lnc-RNA	shRNA Antisense Sequence (5' – 3')
1.	UC shRNA	ATCTCGCTTGGGCGAGAGTAAG
2.	G5C1 shRNA	GAGGCTGGATAGACAGTTTGA
3.	G5C2 shRNA	GGTCCTTCATTCTGAATTTCA

The expression vector was propagated using the *E.coli* Stbl3 strain and the AAV packaging plasmids pAdDeltaF6 and pAAV2.9 (Penn vector Core) were propagated using the DH5α strain. Endotoxin-free Maxiprep kit (Qiagen) was used to prepare purified plasmid for HEK293T cell transfection. Calcium phosphate transfection was done using 10μg of the above-mentioned transfer vector, 10μg pAAV2/9n and 20μg of pAdDeltaF6 into HEK293T cells of density $2X10^6$ cells. The culture was maintained at 37℃, 5% CO_2 in low-glucose DMEM (Gibco) with 10% foetal bovine serum with one-time media change after 8 hours. Post 96 hours of infection, NaCl (final concentration: 500 mM) was added to the plates for 3 hours to lyse the cells. The supernatant was collected and stored at 4℃. The remaining cells on the plates were scraped and collected separately. The cells went through three freeze-thaw cycles where they were frozen at -80℃ and thawed at 37℃ for complete dissociation of virus particles from the cell membrane. The filtrate was added to the supernatant collected before;

together they were filtered through a 100μ and a 0.45μ filter successively. The final filtrate, containing the virus, was then centrifuged using Amicron-Ultra-15 (Merk Millipore #UFC910024) filters to concentrate the virus and determine the titre by fluorescence-based cell counter. The multiplicity of infection (MOI) was calculated by infecting 80K HEK293T cells with serially diluted AAV solutions. An MOI of 1-2 was used for further experiments.

Primary Neuronal Culture

Hippocampal neurons were cultured following the previous protocol by Kaech & Banker, 2006. Briefly, hippocampi from embryonic day 16 (E16) CD1 mice were dissected and collected in dissection media (Gibco). It was then digested with the addition of 2.5% trypsin to the dissection media at 37℃ for 10 mins. Trypsin-treated tissue was washed with dissection media and collected in Glial Minimum Essential Media (GMEM; Gibco). Further, trituration was done with a fire-polished Pasteur pipette to obtain a single-cell suspension which was plated on poly-L-lysine coated 60mm dishes (1 mg/mL, Sigma) at a concentration of 200-250 cells/mm^2. The culture was maintained at 37℃, 5% CO_2 in Neurobasal Media (Gibco) with B27 supplement (Gibco) throughout the experiment. Cells were infected with AAV at DIV 7 and harvested at DIV 21 for further experiments.

Stereotaxic injection

Surgery was done in P0 pups 6-8 hours post-birth only after the appearance of the milk spot (Kim et al., 2014). Half of the litter was taken at a time and kept on a heating pad at 37˚C. For anaesthesia, the pup was kept on a cold metal plate kept on the ice for 2-3 mins till there was no movement and the body colour turned whitish pink from reddish pink. It was then lightly taped to the platform with its head erect. The platform was kept at 7-8℃ to maintain the anaesthesia. In case the pup's body colour started to show a bluish tinge, the temperature was raised to 12-15℃. Stereotaxic arms were moved to (X, Y) = (0.8, 1.5) mm coordinates and the needle was lowered till at Z= -1.8 mm (Christensen et al., 2010). Each hippocampus was injected with 0.5 μl of AAV with MOI=1. A volume of 0.1 μl was injected per minute with a gap of 10 mins. On completion of 0.5 μl virus injection, an additional gap of 10 mins was given before the needle was pulled out very slowly to prevent any back suction. We proceeded to inject into the second hippocampus after 15 minutes during which the first wound site was

allowed to close. The pup was then kept on a 37℃ heating pad and warmed till movement and colour were regained. They were returned to the mother together to minimise disturbances. The cage was left completely undisturbed for the next day.

Light/dark box assay

The apparatus consists of a smaller dark box with a light intensity of 2 Lux (one-third of the total area) and a bigger light box with a light intensity of 390 Lux (two-thirds of the total area) separated by a door (Bourin and Hascoët, 2003) (Takao and Miyakawa, 2006). P42 mice were habituated for 2 minutes to handling and to a transport vessel in dim light (2-5 Lux) for 1 week in the experimental room. Animals were housed in standard cages and a 12-hour light/dark cycle was maintained as usual. On test day, the mouse was kept in the dark box with a closed door. The door was opened 10 seconds later and the mouse was left free to explore the light box for 10 minutes. The behaviour assays and habituations were performed between 0900 hours to 1800 hours. Arenas were cleaned using 70% alcohol between tests to prevent any odour-related bias (Takao and Miyakawa, 2006). The video was recorded using a Logitech HD webcam C270 overhead-mounted camera and analysed using online free software (Toxtrac) (Rodriguez et al., 2018), which was also blind tested manually. The total distance covered, time spent in the light box, and the number of transitions made between the two boxes were considered.

Spatial object recognition assay

All animals were housed in single cages a week before the experiment. P50 male C57Bl/6 mice were habituated for 2 minutes to handling and a transport vessel for 1 week followed by spatial object recognition assay which comprised a total of 5 sessions, each 10 minutes long. The process is similar to the classical object recognition assay with a few variations (Wimmer et al., 2012). A light intensity of around 15 Lux was maintained at the centre of the arena (Leger et al., 2013). Session 1 involved introducing the animal to the empty arena (12 cm X 15 cm with spatial cues) for short habituation to the open field. In sessions 2 to 4, the animal was trained to explore two objects of different shapes kept in their fixed coordinates, with a 5 cm gap between wall and object, through all the sessions. The animal was returned to its original cage for 5 minutes to relieve stress and the arena and objects were wiped with 70% alcohol between each session. The test session (5th session) was carried out

after 24 hours of the training sessions when only one object was moved to a new coordinate with leu to the spatial cues and the animal was allowed to inspect for 10 minutes. All recordings were done using a Logitech HD webcam C270 overhead-mounted camera and a MATLAB-based autotyping software was used to calculate the number of bouts on each object (Patel et al., 2014), which was also blind tested manually. A bout was considered when the animal moved its nose within a space 2 cm radially from the object. Any attempt to climb or jump on the object was not considered a bout. The Discrimination Index (DI), which is the ratio of the difference in time spent with either object to the total time spent with both objects during the test session, was calculated to verify the ability of the animal to successfully recognize the displaced object (Denninger et al., 2018).

DI= [time spent with object displaced- time spent with not displaced]/ [time spent with displaced+ time spent with not displaced]

Cryo-sectioning and imaging

Following behaviour assessments, animals underwent sacrifice at P60. Mice were sedated with a heavy dose of Ketamine (60mg/kg) and Xylazine (10mg/kg) cocktail. They were perfused intracardially with a solution of 1XPBS (phosphate buffer saline) followed by 4% paraformaldehyde, for maintaining tissue integrity. To ensure thorough fixation, brains were extracted and immersed in 4% paraformaldehyde for an additional 5 days post-perfusion. Subsequently, the fixed brains were incubated in 40% sucrose solution for a week till they settled to the bottom, enabling optimal cryo-sectioning. Brains were flash-frozen in dry ice and coronal sections of 50µ thickness were obtained and mounted on slides pre-coated with gelatin. The tissue sections were washed 3 times with autoclaved Mili-Q water and mounted with Vectasheild DAPI to stain the nucleus and visualized under the microscope.

Coronal sections of the mouse brain were visualized using Zeiss Apotome3 (Figure S2). All imaging parameters were kept uniform across experiments. Images were stitched post-acquisition using Fiji image analysis software (MosaicJ plugin).

Western blot

The hippocampal tissue was obtained from C57BL/6 male P21 pups, previously subjected to injections of UC, G5C1, or G5C2 shRNAs at P0. The tissue was lysed in

RIPA buffer (composed of 150mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4) and subsequently centrifuged at 4°C for 10 minutes at 4000 g to remove debris. Protein quantification was performed using the Pierce BCA Protein Assay Kit (Thermofisher) with the resulting clear supernatant.

For experimental purposes, specific sample lysates were mixed with Laemmli Buffer and heated to 95°C. An amount of 5 µg of each sample was utilized for housekeeping gene tubulin-β1 (Tuj-1) blot, while 30 µg of each sample was employed for pan-Ago blot. The samples were resolved by electrophoresis on a 10% polyacrylamide gel and then transferred onto a nitrocellulose membrane. Subsequently, the membrane was blocked with 5% BSA and incubated with primary antibodies against Tuj-1 (dilution 1:10,000, Sigma, Catalog # T8578) and pan-Ago (dilution 1:1200, MerkMillipore, Catalog # MABE56) overnight at 4°C. Following the primary antibody incubation, the membrane was exposed to secondary antibodies (dilution 1:10,000 for Tuj-1 and 1:5000 for pan-Ago, Goat Anti-Mouse, Thermofisher Scientific, Catalog # A11030). Luminescence on the blot was detected using the ECL chemiluminescence kit (Millipore). Band intensities and the expression of pan-Ago were quantified using ImageJ software, with normalization against tubulin-β1.

Statistics

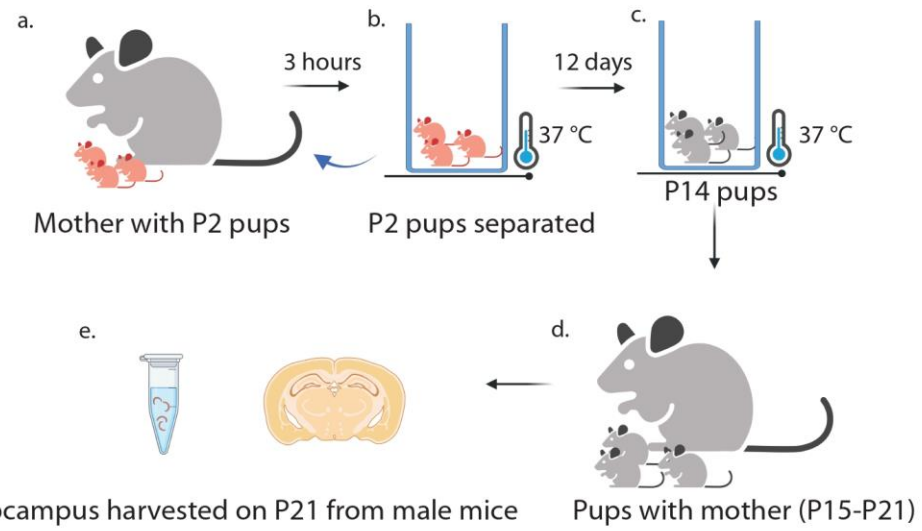
Statistical analyses were performed for all experiments. Data shown are represented as mean±SEM. The number of independent experiments is represented by 'n.' qRT-PCR data for lncRNA screening and Argonaute immunoblots were analyzed using unpaired Student's t-test with Welch's correction for unequal variance. qRT-PCR data for Gas5 knockdown efficiency were analyzed using paired Student's t-test. One-way ANOVA with Fisher's LSD test was used for analyzing light/dark and SOR data analysis. Immunoblot band intensity analysis and image stitching were done using ImageJ software. Gene Ontology plots were computed using R. Data with a p-value less than 0.05% were considered significant. All data were analysed using GraphPad Prism 9.

RESULTS

Maternal separation alters the differential expression of synapse-enriched lncRNAs

To investigate the impact of early life stress, this study used the maternal separation (MS) paradigm (Figure 1A) and the differential expression of a subset of synapse-enriched lncRNAs was analyzed by qPCR. We observed a significant upregulation in the expression of specific lncRNAs, such as Pantr1 (146.3 ± 56.78% increase, p=0.041) and Gas5 (79.6 ± 19.14% increase, p=0.006) and without any change in expression of 1700020I14Rik (8.30 ± 13.14% increase, p=0.5522) upon MS. Although, we observed an increase in expression of 2410006H16Rik (105.5 ± 75.28% increase, p=0.210), 2900052NO1Rik (100.3 ± 70.88% increase, p=0.206), Pvt1 (82.9 ± 46.95% increase, p=0.127), RMST_1 (49.9 ± 51.05% increase, p=0.366), Snhg6 (95.4 ± 81.12% increase, p=0.284), Snhg8 (80.5 ± 73.63% increase, p=0.316), Snhg12 (86.3 ± 48.60% increase, p=0.126) and Zfas1 (37.4 ± 23.57% increase, p=0.163); these enhancement in expression are not statistically significant (Figure 1B). Gas5 was selected as the target lncRNA and two shRNAs (G5C1 and G5C2) against Gas5 were cloned into an AAV vector (Figure S1A). Knockdown efficacy was assessed in primary hippocampal neuronal culture and successful knockdown was evaluated for both the constructs (82.82 ± 9.33 % decrease, p= 0.003 for G5C1 and 84.93 ± 13.68 % decrease, p= 0.25 for G5C2 w.r.t GAPDH; 81.3 ± 8.46 % decrease, p=0.0024 for G5C1 and 83.71 ± 6.9 % decrease, p=0.0067 for G5C2 w.r.t Tubulin-β1) (Figure S1C-D).

A.


a.
3 hours
b.
12 days
c.
37 °C
37 °C
Mother with P2 pups
P2 pups separated
P14 pups
e.
d.
Hippocampus harvested on P21 from male mice
Pups with mother (P15-P21)

B.

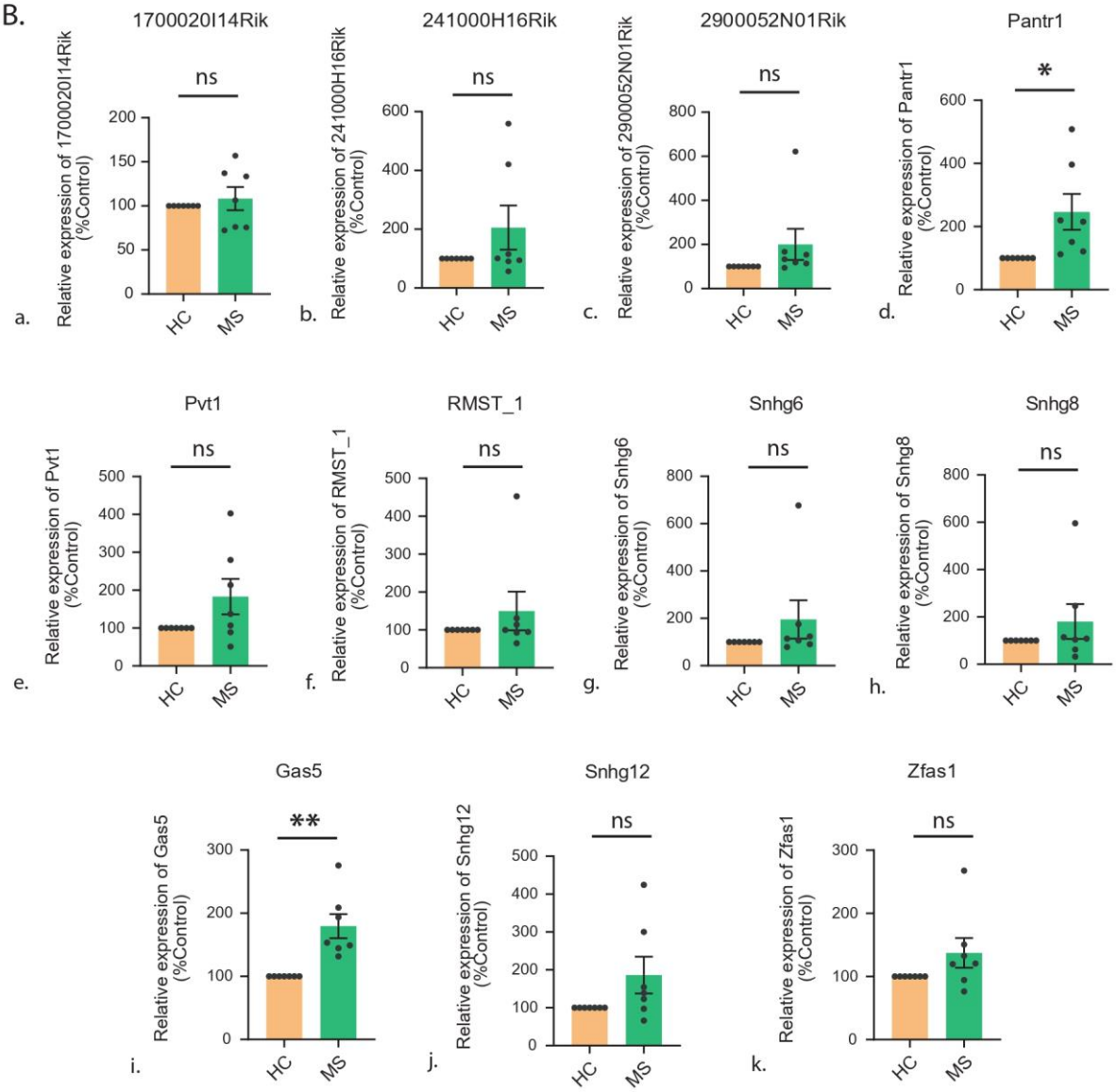

1700020I14Rik
241000H16Rik
2900052N01Rik
Pantr1
Pvt1
RMST_1
Snhg6
Snhg8
Gas5
Snhg12
Zfas1
ns
*
**
Relative expression of 1700020I14Rik (%Control)
Relative expression of 241000H16Rik (%Control)
Relative expression of 2900052N01Rik (%Control)
Relative expression of Pantr1 (%Control)
Relative expression of Pvt1 (%Control)
Relative expression of RMST_1 (%Control)
Relative expression of Snhg6 (%Control)
Relative expression of Snhg8 (%Control)
Relative expression of Gas5 (%Control)
Relative expression of Snhg12 (%Control)
Relative expression of Zfas1 (%Control)
HC
MS
a.
b.
c.
d.
e.
f.
g.
h.
i.
j.
k.

Figure 1. Maternal separation affects the expression of synapse-enriched lncRNAs. (**A**). Maternal separation paradigm followed: P2 pups separated from their mother for 3 hours during P2 – P14 and following MS pups were kept till P21. (**B).** Differential expression of synapse-enriched lnc-RNAs (a) 1700020I14Rik, (b) 2410006H16Rik, (c) 2900052NO1Rik, (d) Pantr1, (e) Pvt1, (f) RMST_1, (g) Snhg6, (h) Snhg8, (i) Gas5, (j) Snhg12, (k) Zfas1 as obtained through qPCR, *p < 0.05, **p < 0.01, ns = not significant. n=7, Data shown as mean ± SEM. Unpaired Student's t-test with Welch's correction.

Gas5 knockdown partially alleviates MS-induced anxiety

We have analysed open-space anxiety of mice that are injected with AAV expressing shRNAs against Gas5 or non-targeting control. Mice were bilaterally injected with AAV expressing either G5C1 or G5C2 in the hippocampus at P0. These mouse pups were maternally separated or homecaged (P2 – P14); following which they were kept with their respective mother till P21 (day of weaning). Young-adult male mice (P48) were subjected to the light/dark box assay (Figure 2A) (Wang et al., 2022). Maternally separated mice exhibited avoidance of brightly-lit open areas as detected by subsequent reduction in time spent (102.2 ± 24.36 second (s) decrease, p<0.0001); distance covered (17.02 ± 2.924 meters (m) decrease, p<0.0001), and fewer transitions (13.24 ± 3.527 decrease, p= 0.0003) into the brightly lit arena as compared to the homecaged control. However, the maternally separated Gas5 RNAi mice showed a partial rescue in anxiety level as measured by time spent (65.57 ± 27.04 s increase, p= 0.0178 for G5C1 and 66.62 ± 25.21 s increase, p<0.0101 for G5C2), distance covered (16.64 ± 3.246 m increase, p <0.0001 for G5C1 and 13.25 ± 3.027 m increase, p<0.0001 for G5C2) and number of transitions (11.11 ± 3.915 increase, p= 0.0059 for G5C1 and 8.308 ± 3.651 increase, p=0.0258 for G5C2) into the brightly lit arena with respect to maternally separated mouse expressing non-targeting control shRNA (Figure 2C-E). We have also observed that homecaged mice expressing Gas5 shRNA did not show any change in time spent, distance covered and the number of transitions into the brightly lit arena as compared to homecaged mice expressing non-targeting shRNA (Figure 2C-E). These observations indicate that Gas5 RNAi does not alter any physiological phenotype of the animal.

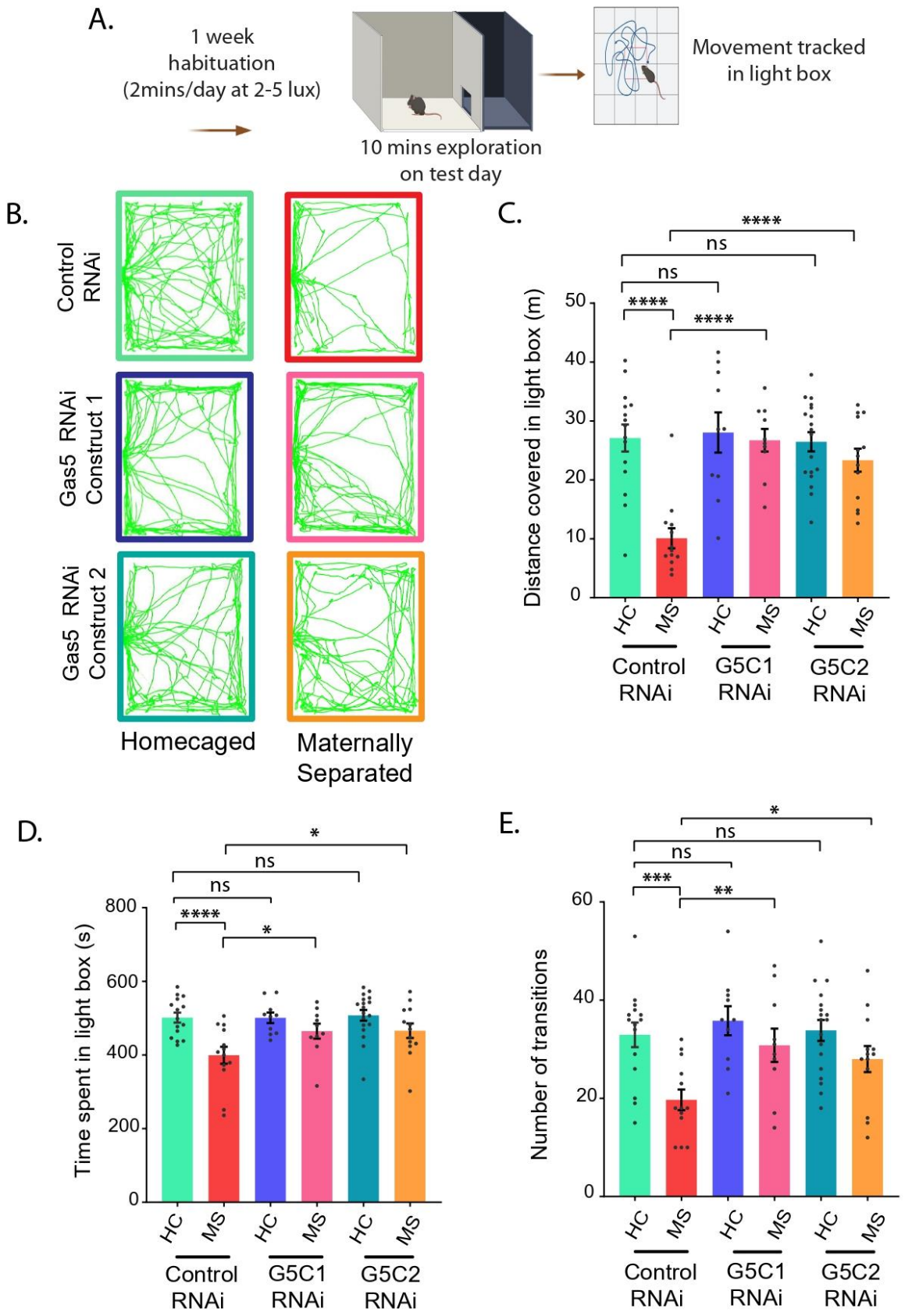

A.
1 week
habituation
(2mins/day at 2-5 lux)
10 mins exploration
on test day
Movement tracked
in light box
B.
Control
RNAi
Gas5 RNAi
Construct 1
Gas5 RNAi
Construct 2
Homecaged
Maternally
Separated
C.
Distance covered in light box (m)
HC
MS
Control
RNAi
G5C1
RNAi
G5C2
RNAi
D.
Time spent in light box (s)
E.
Number of transitions

Figure 2. Gas5 knockdown reduces anxiety following maternal separation. A. Paradigm depicting light/dark box assay to measure anxiety. **B**. Representative trace showing the movement of the mouse in the bright-lit arena in the light/dark box. **C.** HC = Homecaged, MS = Maternally Separated, G5C1= shRNA # 1, G5C2 = shRNA # 2. Distance covered in the light box (in meters). **D.** Time spent in the light box (in seconds). **E.** Total number of transitions made between light and dark box. Data shown as mean±SEM, n=10 - 18, *p<0.05 **p< 0.01, ***p<0.005, ****p<0.0001, ns not significant. One-way ANOVA with Fisher's LSD test.

Gas5 RNAi rescues MS-induced spatial memory deficit

We have analysed the impact of early-life stress on memory using spatial object recognition (SOR) test and evaluated the role of Gas5 following its knockdown (Figure S1A). Mice were injected (P0) with AAV expressing shRNA against Gas5 or non-targeting control. These mice were maternally separated or homecaged (P2 – P14) and subjected to SOR (P56). Mice were allowed to explore two distinct objects placed in two specific locations within an arena during the training session (Figure 3A, S3). On the test day, 24 hours after training, one of the objects was displaced within the same arena and the exploration time of non-displaced and displaced objects was analysed (Figure 3A-3B). We observed that the maternally separated mice expressing non-targeting shRNA displayed reduced recognition of the displaced object during the test session as determined by the discrimination index (DI) (0.3916 ± 0.104 decrease, p=0.0004) when compared to the homecaged control (Figure 3D). Next, we examined the effects of maternal separation on memory following the knockdown of Gas5. We observed that the maternally separated mice having Gas5 RNAi could partially enhance DI (0.3222 ± 0.104 increase, p=0.0031 for G5C1 and 0.2757 ± 0.107 increase, p=0.0127 for G5C2) as compared to the maternally separated mice injected with non-targeting control shRNA. Also, we have detected comparable DI of homecaged mice injected with AAV expressing either Gas5 shRNAs or non-targeting control shRNA (Figure 3D). Furthermore, the exploration of objects that were not displaced within a separate arena remains unaffected following Gas5 RNAi and maternal separation as we did not observe any change in DI across experimental conditions (Figure 3C). These observations indicate that the reduction of Gas5 expression could partially rescue deficits in spatial memory following maternal separation.

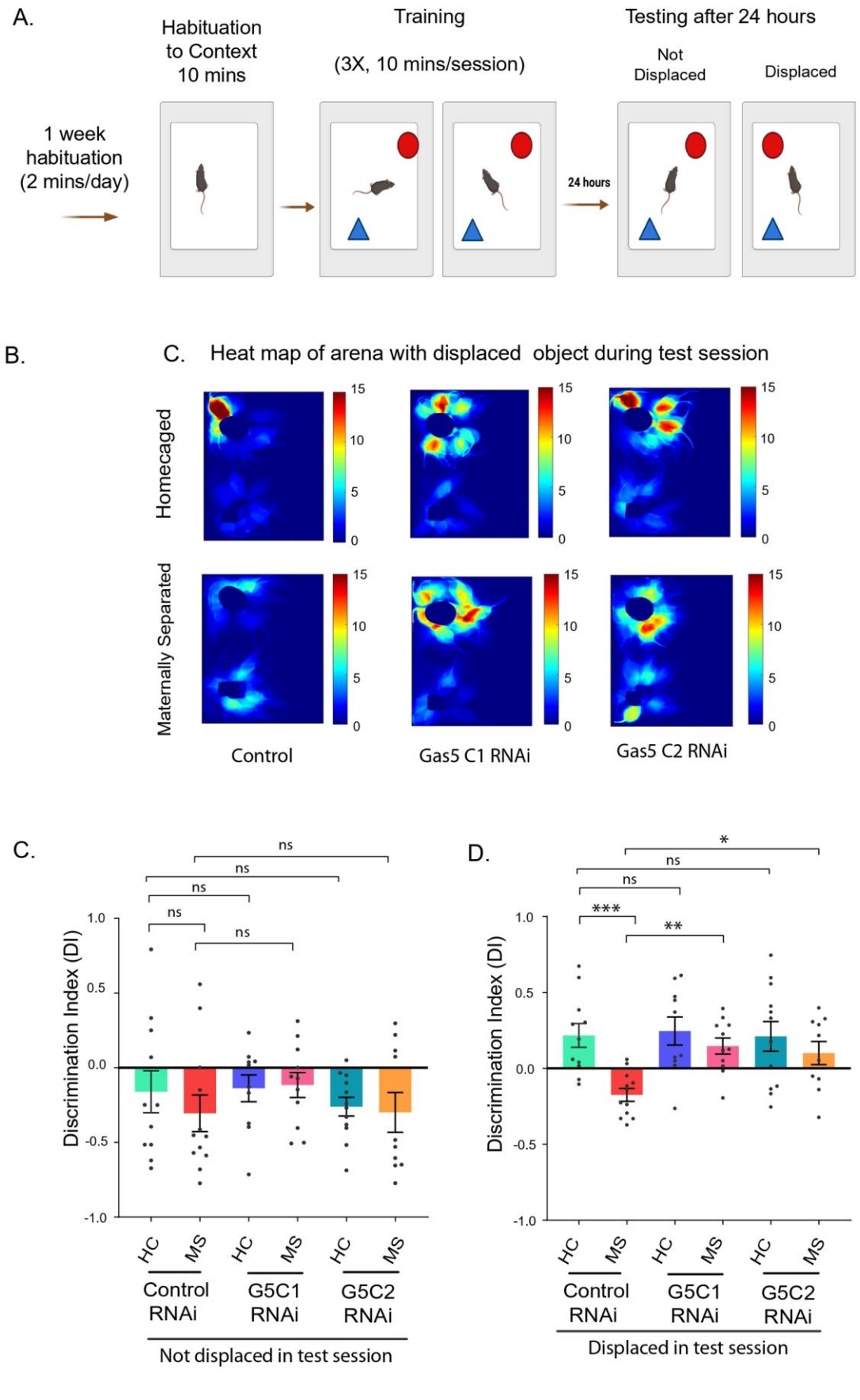

A.
Habituation to Context 10 mins
Training
(3X, 10 mins/session)
Testing after 24 hours
Not Displaced
Displaced
1 week habituation (2 mins/day)
24 hours
B.
C. Heat map of arena with displaced object during test session
Homecaged
Maternally Separated
Control
Gas5 C1 RNAi
Gas5 C2 RNAi
C.
Discrimination Index (DI)
HC
MS
Control RNAi
G5C1 RNAi
G5C2 RNAi
Not displaced in test session
D.
Displaced in test session

Figure 3. Gas5 reverses the effect of MS-induced deficits in memory. **A.** Schematic showing paradigm for SOR. **B.** Representative heat maps in the arena with the displaced object during the test session, circular (glass) object represents the displaced object. **C.** HC= Homecaged, MS= Maternally Separated, G5C1= shRNA # 1, G5C2= shRNA # 2. Discrimination Index (DI) in the arena with no displaced object during the test session. **D.** DI in the arena with a displaced object during the test session. Data shows mean±SEM. n=10-12, *p< 0.05, **p< 0.01, ***p<0.005, ns = not significant. One-way ANOVA with Fisher's LSD test.

Gas5 is potentially involved in regulating RNA metabolism

To gain a plausible mechanistic insight into the Gas5-mediated regulation of anxiety and memory upon maternal separation, we took an *in silico* approach to identify the expression of transcripts influenced by Gas5. First, we have selected Gas5 interacting coding transcripts from the available CLIP-seq data (POSTAR3 database – http://postar.ncrnalab.org./) (Hu et al., 2017). We then performed a Gene Ontology (GO) analysis of these interacting coding transcripts and focused on significant enrichment (FDR < 0.05) of transcripts in specific GO terms (Figure 4A-C). The cellular processes included enrichment of GO-terms for ribonucleoprotein complex (FRD= 1.05E-31), cytoplasmic stress granule (FDR= 1.41E-17), polysome (FDR= 3.50E-05), somatodendritic compartment (FDR= 0.010067571) and dendrite (FDR= 0.004478929) (Figure 4B) etc. The molecular functions are enriched with GO-terms representing ribonucleoprotein complex binding (FDR= 1.22E-05), mi-RNA binding (FDR= 6.05E-17) and translation regulator activity (FDR= 7.53E-05) (Figure.4C). The biological process displays significant enrichment for GO-terms, such as RNA processing (FDR= 1.58E-43), RNA splicing (FDR= 8.08E-45), regulation of translation (FDR= 2.52E-18) etc. alongside some not significant but enriched terms like dendritic transport of ribonucleopreotein complex (FDR= 0.094670904) and miRNA transport (FDR= 0.063158655) (Figure 4A) (For details see Table 4 in supplementary). Overall, the GO analysis revealed that Gas5 is primarily involved in RNA metabolism including stability and regulation of translation. Prompted by this observation, we focused on the transcript encoding Argonaute (Ago) family of proteins that are involved in translation and RNA stability. As a proof-of-concept, we have analyzed the expression of Ago following Gas5 knockdown. Mice were injected with AAV expressing shRNA against Gas5 or non-targetting control shRNA (P0), and Ago expression in the hippocampus

was analyzed at P21. We observed a significant increase in Ago expression in G5C1 (0.99 ± 0.29 increase, p=0.0123) and G5C2 (0.74 ± 0.28 increase, p=0.0431) injected animals as compared to individuals expressing non-targetting shRNA (Figure.4D-E).

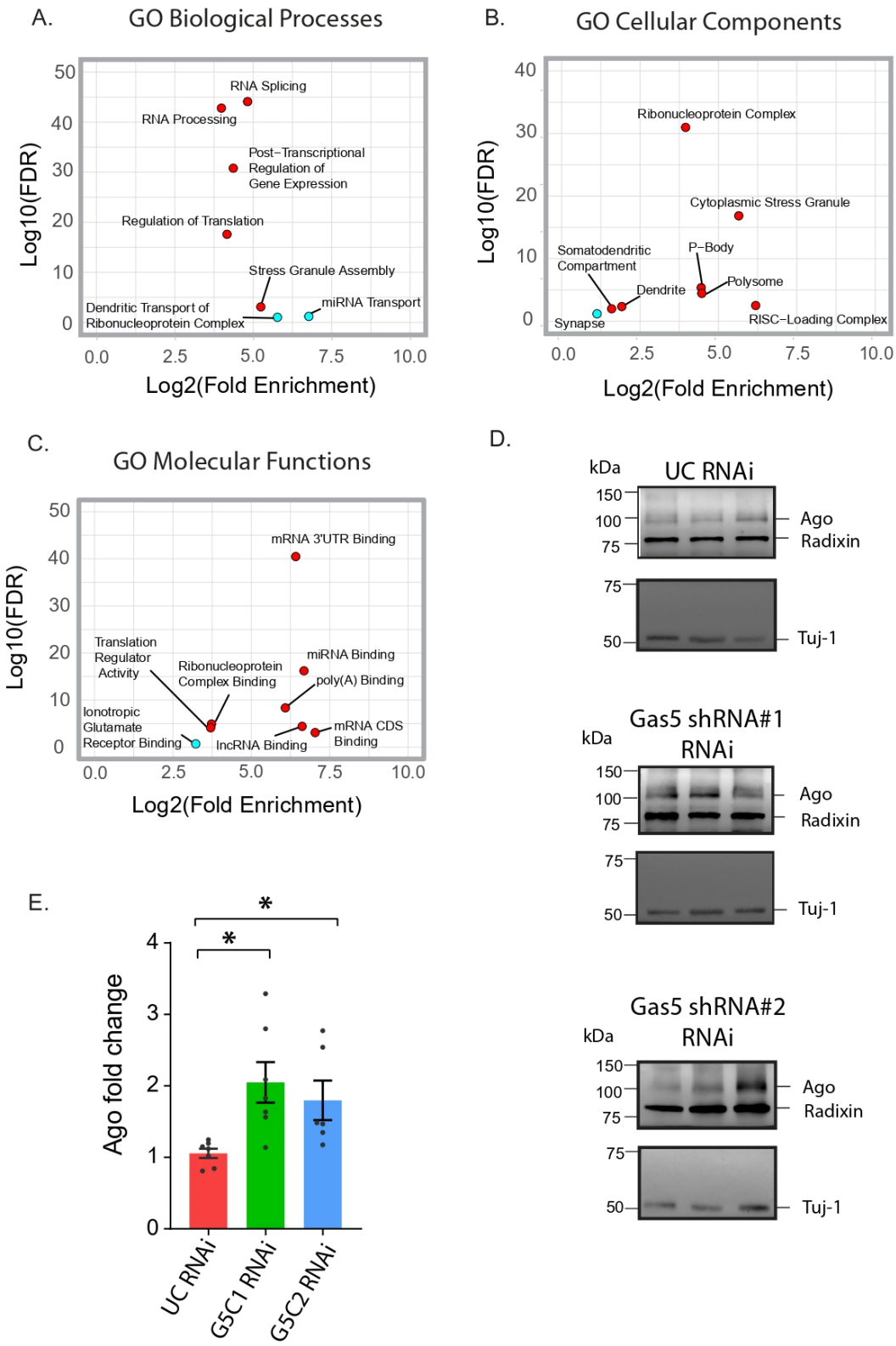


Figure 4. **Involvement of Gas5 in RNA metabolism as revealed by GO-analysis.** Available CLIP-seq data of Gas5 interacting coding transcripts were used to perform gene ontology (GO) analysis showing clusters of transcripts involved in **A**. Biological processes, **B**. Cellular components, and **C**. Molecular functions. Red dots show significant enrichment (FDR< 0.05), blue dots show highly enriched but not significant **D.** Immunoblot of Pan-Ago and Tuj-1 expression at the hippocampus of p21 male animals injected with UC (non-targetting shRNA or Control RNAi), Gas5 C1 (G5C1) or Gas5 C2 (G5C2) shRNA. **E.** Quantification of Pan-Ago with respect to Tuj-1; n=6-7, *p<0.05; Data shows mean±SEM. Unpaired t-test with Welch's correction.

DISCUSSION

LncRNAs emerged as key regulatory RNAs involved in diverse cognitive functions (Mercer et al., 2009) (Samaddar and Banerjee, 2021) (Liau et al., 2021). A recent study from our laboratory identified a set of synapse-enriched lncRNAs from the hippocampus (Samaddar et al., 2023). This study highlighted the importance of one such synapse-enriched lncRNA, Gas5, which in early life stress, evoked behavioural deficits. We observed that early life stress of maternal separation induces the expression of Gas5 in the hippocampus (Figure 1). The reduction of Gas5 expression by RNAi alleviates MS-induced anxiety and rescues memory deficits (Figure 2 – 3). Apart from Gas5, maternal separation also induces the expression of another synapse-enriched lncRNA, Pantr1 (Figure 1). The *in situ* hybridization (Allen Brain Atlas) data showed that Gas5 is expressed in the CA1, CA2, CA3 and DG of both the dorsal and the ventral hippocampus (Patel et al., 2023), whereas, Pantr1 expression is mainly restricted to the CA1 region of the dorsal hippocampus (Goff et al., 2015). Existing literature shows that the dorsal hippocampus is involved in spatial memory formation (Moser and Moser, 1998) and the ventral hippocampus has a prominent role in anxiety (Fanselow and Dong, 2010). Therefore, to probe the lncRNA function in MS-induced anxiety and memory, we have focused on Gas5.

The Gas5 lncRNA has been shown to be involved in aggression (Feldker et al., 2003) and novelty-induced behaviour (Xu et al., 2020). Consistent with these observations, our study demonstrates the involvement of Gas5 in MS-induced anxiety (Figure 2). The maternally separated mouse displays a significant reduction in exploring the displaced object in our spatial object recognition test, indicating deficits in memory upon early life stress (Figure 3). These deficits in memory are partially rescued by

Gas5 knockdown (Figure 3). Our observation of impaired spatial learning following maternal separation is in agreement with previous findings (Huot et al., 2002)(Grace et al., 2009)(Hulshof et al., 2011). However, the impaired cognitive performance with accompanied enhancement in anxiety differs from other studies that demonstrated improved spatial learning along with elevated levels of anxiety following maternal separation (Suri et al., 2013) (McGrath et al., 2008). This discrepancy could possibly arise due to the use of different rodent strains and paradigms to test hippocampus-dependent memory. We speculate that heightened anxiety upon maternal separation could contribute to hyperactivity that may lead to the exploration of both the displaced as well as the non-displaced object in our memory test (Figure 3). Our data shows that there is no bias between home-caged and maternally separated mice in their preference towards any object. Also, no preferential bias for any object is observed between mice expressing Gas5 shRNA or non-targeting control (Figure 3).

It is important to note that the Gas5 RNAi alone does not influence anxiety or memory without any exposure to early life stress (Figure 2 – 3). This observation indicates that the MS-induced Gas5 level above the physiological set point is a prerequisite for anxiety and memory deficits due to early life stress. Furthermore, the knockdown of Gas5 by two distinct shRNAs could reduce anxiety and rescue memory deficits. The use of two distinct shRNAs against Gas5 (Figure S1) eliminates the possibility of an off-target effect and consequent artefactual behavioural rescue. Taken together, our study highlights an RNA-centric approach that could potentially ameliorate the debilitating effect of early life stress.

To gain a plausible mechanistic insight into the Gas5-mediated regulation of anxiety and memory deficit upon maternal separation, we analyzed the functions of Gas5 interacting transcripts identified from the POSTAR3 database. Our GO analysis revealed that Gas5 interacting transcripts are involved in RNA metabolism and synaptic functions. We have noted the regulation of transcripts associated with translation, RISC loading complex, miRNA transport and RNA splicing. The GO analysis also detected regulation of transcripts encoding proteins necessary for dendritic transport of ribonucleoprotein complex, somatodendritic compartment and ionotropic glutamate receptor binding. These observations impinge on the fact that Gas5 could, potentially, regulate synaptic function *via* post-transcriptional regulation of gene expression within subcellular spaces upon exposure to early life stress. Our data

shows that the expression of Argonaute, a component necessary for miRISC loading and miRNA function, is reduced following the knockdown of Gas5. Consistent with previous observations showing the involvement of miRNAs in early life stress-induced gene expression (Allen and Dwivedi, 2020), we speculate that Gas5-mediated regulation of anxiety and memory upon maternal separation involves components of the miRNA pathway. The enhanced expression of Argonaute may shift the miRNA loading between coding transcript and Gas5 *via* promoting molecular decoy activity to alter the translation status (Eulalio et al., 2008) (Li et al., 2020).

Overall, our study highlights the importance of evaluating diverse lncRNA activities during early life stress. To gain mechanistic insight, it would be important to understand how lncRNA activity is modulated by early life stress and its consequence on cognitive performance. We believe that this study will promote further research to probe how early life stress across temporal windows could lead to behavioural diversity *via* non-coding RNA-mediated regulation of gene expression.

SUPPLEMENTARY FIGURES

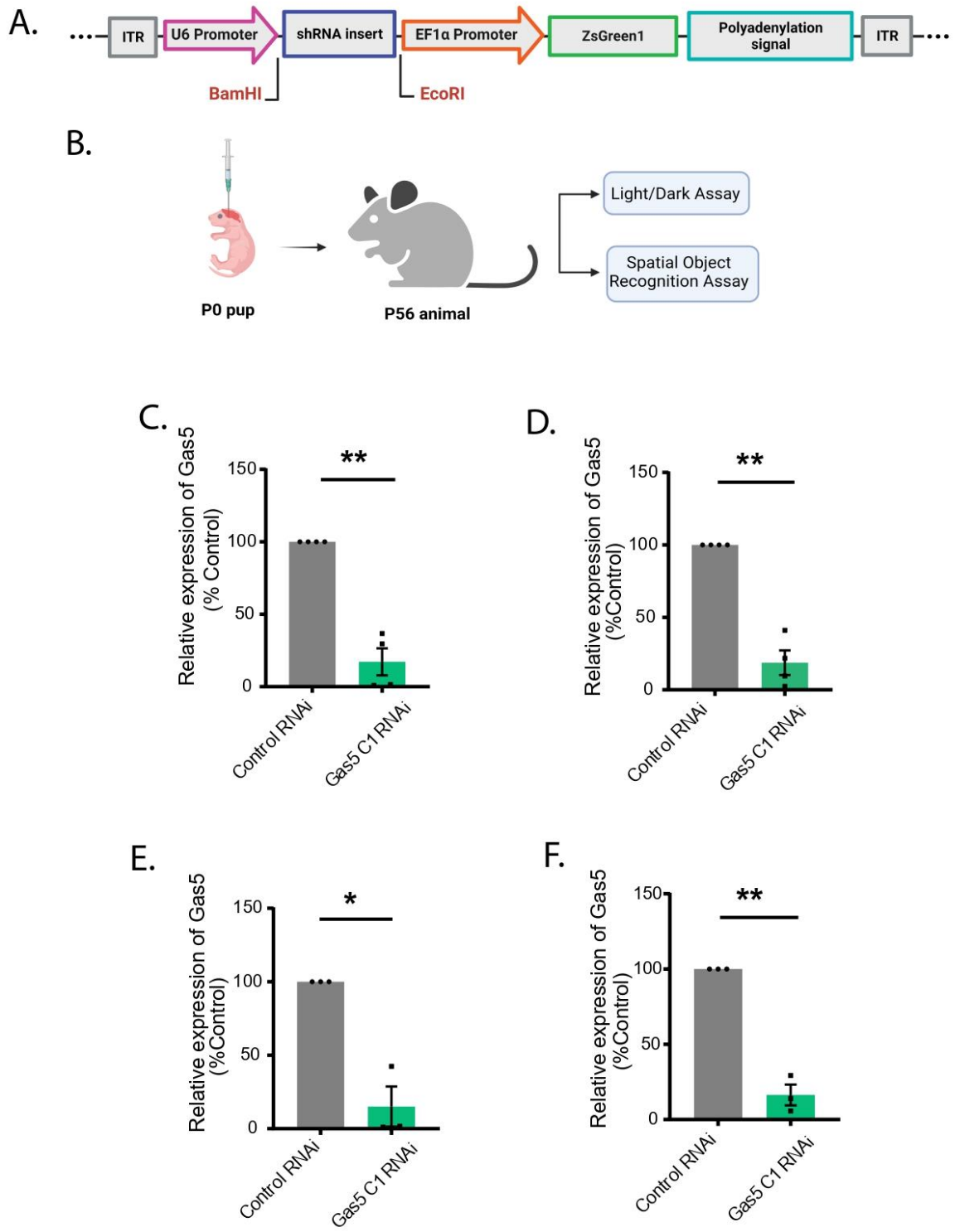


Figure S1. Gas5 knockdown by stereotaxic assay in P0 pups. **A**. Map of vector used for Gas5 knockdown where shRNA is expressed under U6 promoter and Zs-Green under EF1-α; **B**. Stereotaxic injection of AAV was done in P0 pups which were later used for behavioural assays, like light/dark box assay and spatial object recognition assay, at P56. **C**. Gas5 RNAi

tested in primary neuronal culture: G5C1 RNAi with GAPDH as internal control, n=4, **D.** with Tubulin-β1 as internal control, n=4, **E.** G5C2 RNAi with GAPDH as internal control, n=3, **F.** with Tubulin-β1 as internal control, n=3. *p< 0.05, **p< 0.01. Data shown as mean±SEM. Paired student's t-test.

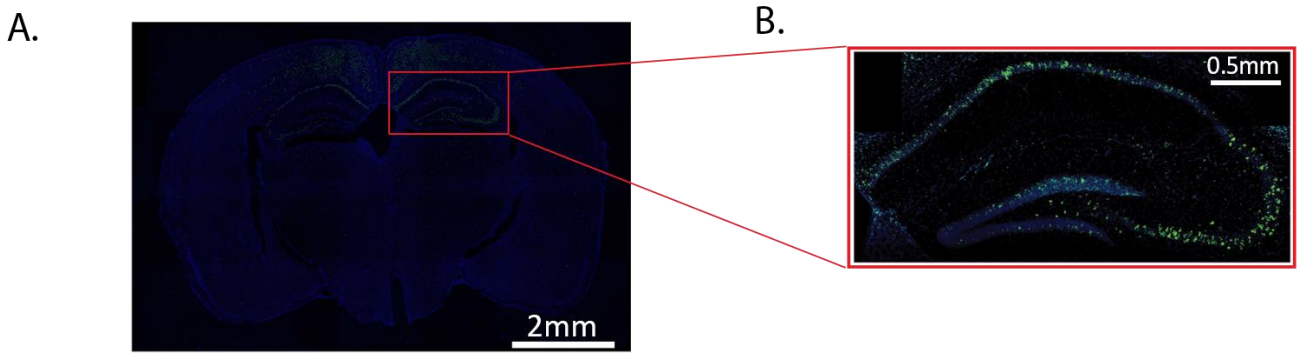


Figure S2. Brain section of P58 mouse showing expression of AAV inducing Gas5 knockdown in hippocampi. A. whole brain section and **B**. hippocampus magnified.

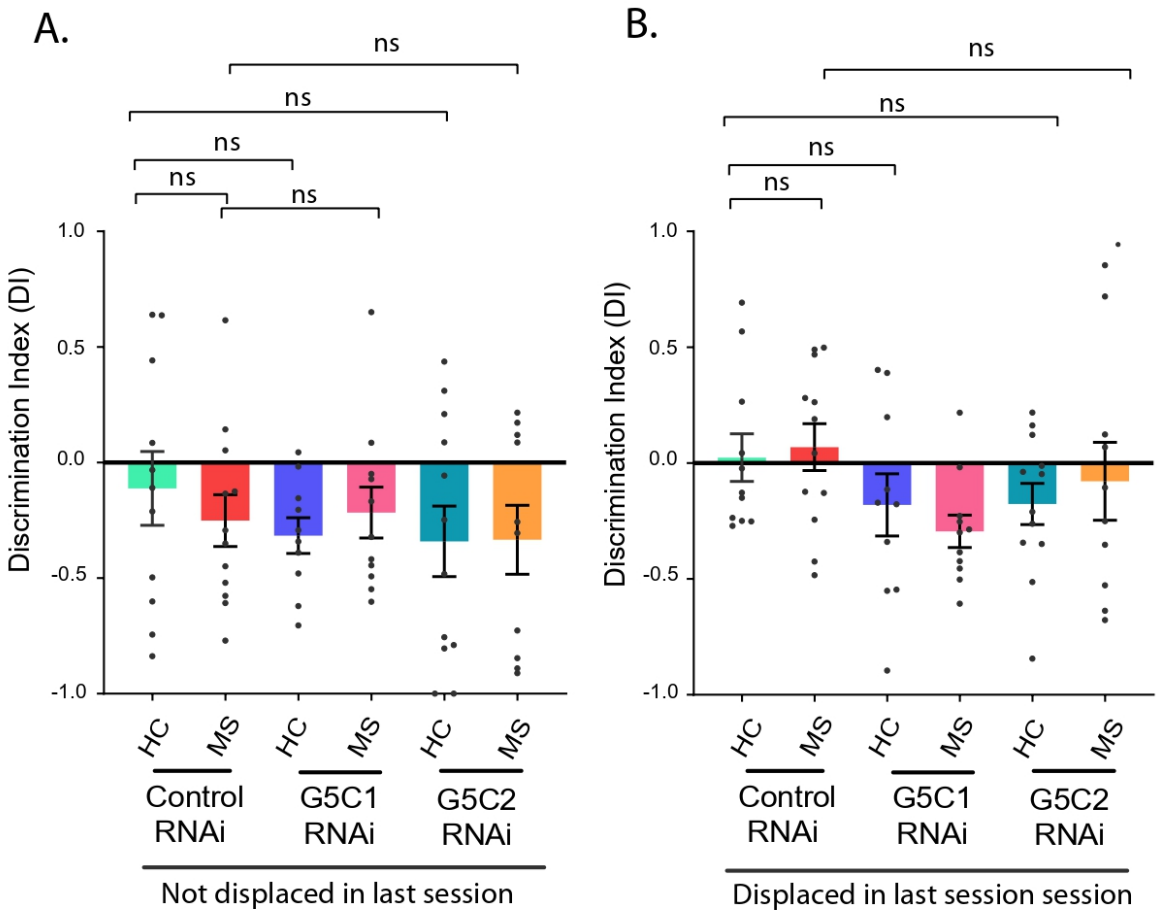


Figure S3. Discrimination index of animals during training. A. Discrimination Index from the last session in the arena where no object was displaced during the test session, **B**. Discrimination Index from the last session in the arena where an object was displaced during the test session. Data shows mean±SEM. One-way ANOVA with Fisher's LSD, n=10-12, ns = not significant.

TABLE 4. Gene Ontology analysis details

BIOLOGICAL PROCESS					
No.	Enrichment FDR	Fold Enrichment	Pathway	Log (2) Fold enrichment	Log (10) FDR
1.	8.08E-45	28.25532203	GO:0008380 RNA splicing	4.820450727	4.41E+01
2.	1.58E-43	15.832586	GO:0006396 RNA processing	3.984825011	4.28E+01
3.	1.64E-31	20.57737628	GO:0010608 post-transcriptional regulation of gene expression	4.362987138	3.08E+01

4.	2.52E-18	17.9186037	GO:0006417 regulation of translation	4.163386315	1.76E+01
5.	0.000785421	37.8358209	GO:0034063 stress granule assembly	5.241680841	3.10E+00
6.	0.063158655	109.3034826	GO:1990428 miRNA transport	6.772195558	1.20E+00
7.	0.094670904	54.65174129	GO:0098961 dendritic transport of ribonucleoprotein complex	5.772195558	1.02E+00
CELLULAR COMPONENT					
No.	Enrichment FDR	Fold Enrichment	Pathway	Log (2) Fold enrichment	Log (10) FDR
1.	1.05E-31	15.48834774	GO:1990904 ribonucleoprotein complex	3.953111343	30.97867038
2.	1.41E-17	50.15100966	GO:0010494 cytoplasmic stress granule	5.648206841	16.85006248
3.	4.29E-06	21.86069652	GO:0000932 P-body	4.450267463	5.367452283
4.	3.50E-05	22.15611134	GO:0005844 polysome	4.469632788	4.455808899
5.	0.003023363	72.86898839	GO:0070578 RISC-loading complex	6.187233057	2.519509743
6.	0.004478929	3.778737554	GO:0030425 dendrite	1.917904322	2.348825857
7.	0.064160065	2.186069652	GO:0045202 synapse	1.128339368	1.192735204
8.	0.010067571	3.019433221	GO:0036477 somatodendritic compartment	1.594277766	1.997075298
MOLECULAR FUNCTION					
No.	Enrichment FDR	Fold Enrichment	Pathway	Log (2) Fold enrichment	Log (10) FDR
1.	3.31E-41	85.39334577	GO:0003730 mRNA 3-prime-UTR binding	6.416051748	40.48029045
2.	6.05E-17	102.4720149	GO:0035198 miRNA binding	6.679086154	16.21804256
3.	4.45E-09	67.84354092	GO:0008143 poly(A) binding	6.084139564	8.352079057
4.	1.22E-05	13.2680528	GO:0043021 ribonucleoprotein complex binding	3.729884753	4.913597524
5.	3.79E-05	98.37313433	GO:0106222 lncRNA binding	6.620192465	4.42161829

6.	7.53E-05	13.02955422	GO:0045182 translation regulator activity	3.70371582	4.123425998
7.	0.000795929	131.1641791	GO:1990715 mRNA CDS binding	7.035229964	3.0991254
8.	0.197592775	9.368869936	GO:0035255 ionotropic glutamate receptor binding	3.227875042	0.704228941

AUTHOR CONTRIBUTION

D.B. and S.B. conceptualized the project. D.B. performed all experiments. S.S performed spatial memory and anxiety analysis. D.B and S.B. wrote the manuscript.

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