Amygdalar microRNA-15a is essential for coping with chronic stress

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SUMMARY

MicroRNAs are important regulators of gene expression and associated with stress-related psychiatric disorders. Here, we report that exposing mice to chronic stress led to a specific increase in microRNA-15a levels in the amygdala-Ago2 complex, and a concomitant reduction in the levels of its predicted target, *FKBP51,* which is implicated in stress-related psychiatric disorders. Reciprocally, mice expressing reduced levels of amygdalar microRNA-15a following exposure to chronic stress exhibited increased anxiety-like behaviors. In humans, pharmacological activation of the glucocorticoid receptor as well as exposure to childhood trauma was associated with increased microRNA-15a levels in peripheral blood. Taken together, our results support an important role for microRNA-15a in stress adaptation and the pathogenesis of stress-related psychopathologies.

HIGHLIGHTS

- MiR-15a levels are elevated in the amygdala-Ago2 complex following chronic stress
- MiR-15a targets FKBP51 and affects behavioral responses to stressful challenges
- MiR-15a is elevated in peripheral human blood following dexamethasone exposure
- MiR-15a is elevated in peripheral human blood of patients exposed to childhood trauma

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Volk et al. reveal an important role for microRNA-15a in coping with chronic stress, with amygdala-specific manipulation affecting behavioral responses to stressful challenge. Individuals exposed to childhood trauma exhibit increased levels of miR-15a in their peripheral blood suggesting a novel target for the treatment of stress-related psychopathologies.

INTRODUCTION

Recent studies have linked microRNA (miRNA) expression or biogenesis dysregulation to various psychiatric disorders including anxiety and depression (Dias et al., 2014b; Issler and Chen, 2015; Issler et al., 2014; Lopez et al., 2014; O'Connor et al., 2012; Volk et al., 2014) . However, changes in miRNA expression levels do not necessarily reflect their immediate activity; it is only when a specific miRNA, in the canonical pathway, has matured and been incorporated into the RNA-induced silencing complex (RISC) in the presence of argonaute RISC catalytic component 2 (Ago2) that it becomes truly active (Meister et al., 2004) as a result of its association with its target mRNA.

The amygdala plays a pivotal role in regulating the behavioral responses to stressful challenges (Dunsmoor and Paz, 2015; Duvarci and Pare, 2014; Johansen et al., 2011; Luthi and Luscher, 2014; Maren and Holmes, 2015). Recently, regulation of some amygdalar functions and stress-related behaviors has been attributed to miRNAs. MiR-34c is involved in regulating stress-induced anxiety (Haramati et al., 2011) and miR-34a in fear memory consolidation (Dias et al., 2014). Furthermore, miR-19b plays an important role in memory consolidation following stress by regulating the adrenergic receptor beta 1 (Volk et al., 2014).

In this study, we investigated Ago2-associated miRNAs and transcripts in the amygdala of mice subjected to a chronic social defeat stress. This chronic social stress paradigm consists of 10 consecutive days of short physical encounters between a C57BL/6 mouse and an aggressive ICR (CD1) mouse (Golden et al., 2011; Krishnan et al., 2007). The repeated exposure to stress is considered a model for the induction of chronic stress (Elliott et al., 2010; Elliott et al., 2016; Issler et al., 2014), as well as depression-like behavior (Hollis and Kabbaj, 2014; Malatynska and Knapp, 2005) in mice. Molecular analysis and behavioral studies demonstrate that miR-15a is

recruited to the Ago2 complex following chronic stress and is an essential regulator of an intact behavioral response to chronic stress.

RESULTS

MiR-15a and FKBP51 mRNA are associated with Ago2 in the amygdala following chronic stress

To identify novel miRNAs that are involved in the regulation of the behavioral response to chronically stressful challenges, we immunoprecipitated the Ago2 complex in tissue obtained from the amygdala of mice, 8 days after completion of the chronic social defeat stress. Mice were subjected to the chronic social defeat stress paradigm for 10 consecutive days (Figure 1A) following which they were subjected to a social avoidance test to categorize them as being either "Susceptible" or "Resilient" to the chronic social defeat stress (Figure 1B). The RNA from the Ago2 complex of 3 groups: Susceptible, Resilient and Control, was extracted and analyzed in parallel using 2 distinct microarray platforms; a miRNA and a mRNA expression array (Figure 1C). Initially, we hypothesized that we would observe changes in the miRNA population of the Ago2 complex in the amygdala not only between stressed and control mice but between Susceptible and Resilient mice. However, our analysis revealed that no significant changes were detected between the Susceptible and Resilient mice. For this reason, we combined the 2 groups of mice into 1 that is referred to in the text as 'social defeat'. Analysis of the miRNA array revealed 4 miRNAs that were upregulated and 10 that were downregulated (Figure 1D, Figure S1A). A parallel analysis on the gene array revealed a small number of mRNAs that were either upregulated or down regulated in the Ago2 complex immunoprecipitation (IP) following social defeat (Figure S1B). When we analyzed the mRNAs that were changed following social defeat we focused on stress associated genes that were previously described to be expressed in the amygdala. This is the reason we focused on FK506 binding protein 51 (FKBP51) and miR-15a.

FKBP51 has been previously linked to the pathogenesis of posttraumatic stress disorder and depression (Binder et al., 2008a; Klengel et al., 2013; Lekman et al., 2008; Zannas et al., 2016). MiR-15a levels were raised 1.8 fold (p=0.0002) in the array following exposure to chronic stress (Figure 1E). Interestingly, a parallel increase in FKBP51 mRNA (p=0.001), a predicted target of miR-15a, was observed in the Ago2 complex (Figure 1E). Whereas the levels of miR-15a were also elevated (p=0.039) in the total RNA levels of the amygdala tissue, FKBP51 levels were, as expected, decreased (p=0.008) (Figure 1F), supporting the possibility that FKBP51 is directly down-regulated by miR-15a in the amygdala. Consistently, the protein levels of FKBP51 were measured and a reduction of 25% in its levels was observed (p=0.014) (Figure 1G). Interestingly, the levels of miR-15a were also elevated by 60% in the plasma of mice subjected to chronic stress (p=0.047), whereas the levels of miR-124, an abundant brain miRNA, were unchanged (Figure S2 A-B), implicating miR-15a as a possible marker for chronic stress exposure. These experiments led us to focus on miR-15a and FKBP51 and address their involvement in mediating chronic stress cellular processes.

MiR-15a transcription regulation

MiR-15a is located on chromosome 14 as part of a cluster with miR-16-1 (Figure 2A), indicating that these 2 miRNAs are cotranscribed. Previous studies have demonstrated that the promoter for miR-15a and miR-16-1 is likely to be the promoter for DLEU2, a non-coding gene that contains the transcript for miR-15a (Zhang et al., 2012). Although both miR-15a and miR-16-1 share a seed sequence, their mature miRNA sequence differs in several nucleotides (Figure 2B). In addition, the total levels of miR-16 in most brain areas appear to be higher than that of miR-15a (Figure 2C), possibly since miR-16 has 2 copies in the genome (miR-16-1 on chromosome 14 and miR-16-2 on chromosome 3), which both give rise to a similar mature form of miR-16,

whose genomic origin is indistinguishable. Importantly, the elevation in miR-15a levels observed in our Ago2 IP is specific for this miRNA and not for miR-16 (Figure 2D), implying miR-15a specificity at the level of the Ago2 complex formation.

FKBP51 is a confirmed target of miR-15a

Consistent with direct targeting of FKBP51 by miR-15a, the seed sequence for miR-15a binding at the 3'-UTR of FKBP51 is highly conserved (Figure 3A). Moreover, a luciferase assay in which a construct containing luciferase followed by the 3'-UTR of FKBP51, was constructed and transfected into Huh7 cells expressing either miR-15a or a scramble control for it, showed a robust specific reduction in normalized luciferase levels (Figure 3B, $p<0.001$). Importantly, this reduction was abolished when the miR-15a seed sequence was mutated (Figure 3B). These results support a regulatory role for miR-15a in directly controlling FKBP51 levels.

Over-expression of miR-15a in the basolateral amygdala does not affect anxiety-like behavior

To examine whether increased levels of miR-15a in the amygdala are sufficient to mimic the behavioral effects associated with chronic stress exposure, we designed, constructed and produced lentiviruses over-expressing the precursor of miR-15a or a scramble miR sequence as a control (Figure S3A). The degree of infection and the levels of miR-15a expression were verified using qPCR on RNA samples extracted from amygdala punches obtained from mice injected with these viruses into the BLA. The treated mice showed an approximately 2-fold increase in the level of amygdalar miR-15a compared to scramble control ($p<0.001$; Figure S3B), which is similar to the elevated levels of miR-15a observed following exposure to chronic stress (Figure 1F). To assess the stress-related behavioral changes of mice expressing higher levels of miR-15a, mice were injected bilaterally into the BLA with either miR-15a over-expressing or

control-scrambled viruses under basal or chronic stress conditions (Figure S3 C-E). Behavioral assessment of the injected mice indicated no significant changes between mice over-expressing miR-15a or a control scramble miR in the open field test, or in the elevated plus maze (EPM) test, under baseline (Figure S4A and S4C) or chronic stress (Figure S4B and S4D) conditions. In addition, no changes were observed in the locomotor activity or total time traveled in the open field of mice over-expressing miR-15a compared to control scramble miR under basal (Figure S4 E-G) or chronic stress conditions (Figure S4 H-J). We therefore concluded that over-expression of miR-15a in the BLA is not sufficient to mimic the behavioral effects associated with exposure to chronic social defeat.

Reduced levels of miR-15a in the basolateral amygdala increases anxiety-like behavior following exposure to chronic stress

Next we assessed the requirement of endogenous amygdalar miR-15a levels for the behavioral responses, under baseline and chronic stress conditions. We designed, constructed and produced a viral vector containing multiple binding sites for miR-15a (miR-15a Sponge), which enabled knockdown (KD) of miR-15a levels in the BLA (Figure 4A). The control sponge viral construct was generated by specifically mutating four base pairs on each side of the bulge of the sponge (Figure 4A). Injection of the miR-15a KD or control sponge viruses into the BLA of mice, regardless of their exposure to chronic social defeat, resulted in an approximately 2.5-fold reduction in the levels of miR-15a in the BLA under basal conditions ($p=0.019$) and following social defeat ($p=0.012$) (Figure 4B-C). Whereas a reduction was observed independently of chronic stress, the absolute levels of miR-15a were higher in the chronic social defeat group compared to controls (Figure 4C). This supports our initial observation regarding elevation in miR-15a levels in the amygdala following chronic social defeat. The BLA of an additional group of

mice was injected with miR-15a sponge and a control sponge virus (Figure S5A) and RNA was extracted. The levels of miR-15a were confirmed to be reduced by 40% (p=0.009) using real time PCR (Figure S5B). These samples were also sequenced by Illumina TruSeq Small RNA Library Preparation Kit and no significant changes were observed in the 25 most abundant miRNAs (Figure 4D), thus verifying a specific knockdown for miR-15a. As expected from our luciferase assay, FKBP51 mRNA levels were elevated in the BLA of mice injected with the miR-15a KD virus (p=0.033, Figure 4E). The protein levels of FKBP51 were increased by approximately 50% (p=0.022, Figure 4 F-G) following injection of miR-15a knockdown or control viruses.

We next assessed the miR-15a KD mice for anxiety-like behavior using the EPM test. Under baseline conditions (in which mice were not exposed to chronic social defeat) a tendency to main effect was observed (p=0.058). No significant changes were observed in the time spent in the open arms, number of visits to the open arms or distance travelled in the open arms between the miR-15a KD and control groups (Figure 5 A-C). Similarly, the locomotor activity and the total distance traveled in the open field test showed no differences between these groups (Figure H-J). Intriguingly, however, following chronic social defeat a main effect between the behavior of miR-15a KD and control mice was observed (p=0.026). Mice with miR-15a KD spent significantly less time in the open arms (p=0.009) (Figure 5D, G) and traveled less distance in the open arms (p=0.002) (Figure 5F, G) relative to controls (asterisks indicate significance following correction with Bonferroni correction for multiple testing). No differences were observed in the number of visits to the open arms, the locomotor activity between the groups, or the total distance traveled in the open field test (Figure 5E, K-M). These results demonstrate that KD of miR-15a levels in the amygdala specifically impaired the recovery, and behavioral response of mice following their exposure to chronic stress. In the open field test, miR-15a KD mice spent less time in the center of the arena (p=0.032) but no changes were observed in the distance

traveled in the center or the number of visits to the center (Figure S5C). Following chronic social defeat, miR-15a KD and control mice spent similarly less time in the center of the arena suggesting a “floor effect”. However, miR-15a KD mice showed a tendency to travel for less distance in the center of the arena (p=0.060) and made fewer visits to the center of the arena (p=0.041) (Figure S5D). These results are in accordance with Hartmann *et al* (Hartmann et al., 2015) who observed induced anxiety-related behavior following over-expression of FKBP51 in the BLA. Moreover, Attwood *et al* (Attwood et al., 2011) showed that silencing of FKBP51 levels in the BLA by injection of lentiviral short hairpin RNA led to a reduction in anxiety levels in the EPM. Taken together, the current data suggest that amygdalar miR-15a levels are functionally important in regulating the behavioral response to challenge and suggest that this effect is mediated, at least in part, via a reduction of FKBP51 levels.

MiR-15a is regulated by glucocorticoids and trauma in human samples

To examine the potential parallel role of miR-15a in the human stress response, we first analyzed miR-15a expression levels in RNA extracted from peripheral blood cells of young healthy male subjects following administration of the GR agonist dexamethasone (1.5 mg p.o.). We observed a significant up-regulation of miR-15a 3 and 6 hours’ post-treatment (Figure 6A), indicating that miR-15a is potentially regulated by activation of the stress hormone system in humans. In addition, we performed miRNA analyses on peripheral blood cells of subjects with childhood trauma and control subjects matched for age and gender with no history of early life stress. We found that the levels of miR-15a were significantly higher by 32% in subjects exposed to childhood trauma as compared to control subjects who were not exposed (p=0.000, Figure 6B). Taken together, these results support a functional association between the blood levels of miR-15a and psychiatric impairment.

DISCUSSION

The current study reveals an important role for amygdalar miR-15a in regulating the behavioral responses to chronic stressful challenges. miR-15a levels are significantly increased in the amygdala of mice subjected to chronic stress, and amygdala-specific knockdown of miR-15a changes the behavioral responses to chronic stressful challenges. A target of miR-15a, FKBP51, identified in our studies, has been implicated in a number of stress-related psychiatric disorders (Binder et al. 2009;Zannas et al. 2016). FKBP51 is part of the immunophilin protein family and is known to play a role in glucocorticoid receptor (GR) transcriptional activation following elevation of cortisol (Gillespie et al., 2009b). Manipulation of FKBP51 levels in the BLA using small interfering RNA (Attwood et al., 2011) or its over-expression using a viral vector (Hartmann et al., 2015) has been linked to changes in anxiety-like behavior. FKBP51, which is strongly implicated in a number of stress-related psychiatric disorders and currently a leading target for pharmacological manipulation for the treatment of various psychopathologies, is robustly regulated both *in vitro* and *in vivo* by miR-15a. Importantly, miR-15a is upregulated by pharmacological activation of the stress response in humans by dexamethasone treatment as well as exposure to early adverse life events. Therefore, miR-15a might represent an important target for the treatment of stress-related psychopathologies.

Our results imply that in the chronic stress response, miR-15a and its target FKBP51 represent major components for the following reasons: Although miR-15a is bioinformatically predicted to target other stress- and depression/anxiety-related transcripts such as GILZ or Sgk1 (Anacker et al., 2013; Thiagarajah et al., 2014), the mRNA levels of these genes were unchanged in our Ago2 IP array (data not shown) supporting the specificity of the assay. In the current study, we focused exclusively on miR-15a regulation of FKBP51 due to the reported involvement of this gene in stress response regulation and stress-linked psychopathologies (Hartmann et al., 2015;

Hartmann et al., 2012; Scharf et al., 2011). Furthermore, FKBP51 mRNA was detected in the Ago2 complex implicating a direct binding to the RNAi machinery. Finally, a significant decrease in FKBP51 levels was observed in the total RNA samples that concomitantly exhibited elevated miR-15a levels.

While BLA-specific over-expression of miR-15a resulted in no significant behavioral changes, knocking down miR-15a in the BLA caused an anxiogenic phenotype following exposure to chronic stress. The regulation of miR-15a following exposure to chronic stress and the observed anxiogenic phenotype in the BLA-miR-15a knockdown mice following chronic stress exposure, may suggest that miR-15a is specifically involved in regulating the behavioral responses to repeated, or chronic stressful exposure. The lack of an anxiolytic phenotype in the BLA-miR15a over-expressing mice could be explained by either lack of spatial specificity, meaning that the over-expression of miR-15a was not induced in endogenously relevant BLA neurons, or by a possible "ceiling effect", in which increasing levels of miR-15a on top of its endogenous stress-induced elevation is not effective because the stress response has already reached its full capacity. However, preventing the elevation of endogenous miR-15a in the BLA by its KD, resulted in a failure of the mice to mount the required behavioral response when exposed to a chronic stressful challenge.

Finally, the elevation of miR-15a in 2 distinct human stress-linked scenarios, namely, administration of dexamethasone to healthy subjects as well as individuals exposed to childhood trauma, strongly suggests its involvement in human stress conditions. Collectively, the preclinical and human translational results presented in the current study strongly suggest that alterations in miR-15a levels are associated with the behavioral response to chronic or repeated stressful challenges and may be relevant in the pathogenesis of adverse life events and stress-

linked psychiatric disorders such as anxiety. Targeting miR-15a levels might prove to be beneficial in the treatment of these conditions.

EXPERIMENTAL PROCEDURES
(See also ONLINE METHODS)

Chronic social defeat stress

10-week old C57BL/6J male mice were subjected to a chronic social defeat stress protocol as previously described (Krishnan et al, 2007). Briefly, the mice were placed randomly in a home cage of an aggressive ICR mouse and allowed to physically interact for five minutes. During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. A perforated clear Plexiglass divider was then placed between the animals and they remained in the same cage for 24 hours to allow sensory contact. The procedure was then repeated with an unfamiliar ICR mouse for each of the next 10 days.

Immunoprecipitation of Ago2 protein, RNA purification and microarray

Pools of 3 amygdalae taken from 3 mice from the same treatment group (either Social defeat n=18 or Control n=12) were immunoprecipitated using Magnetic protein G beads (Dynabeads, Invitrogen Life Technologies, Carlsbad, CA) and Ago2 monoclonal antibody (WAKO chemicals GmbH, Neuss, Germany).

RNA from the Ago2 immunoprecipitation samples was isolated and analyzed on an Affymetrix miRNA 2.0 array (enriched RNA protocol) and an Affymetrix Mouse Gene 1.0 ST array (Affymetrix, Santa Clara, CA).

Cloning of 3'-UTRs into Psicheck2 luciferase expression plasmid

The 3'-UTR sequence of FKBP51 was PCR amplified from mouse genomic DNA. This mutation replaced the first 4 nucleotides in the miR-15a seed sequence of FKBP51.

Design, construction and validation of miR-15 lentiviruses

The miR-15a over-expression vector was cloned following the human synapsin promoter. The miR scramble control was purchased from GeneCopoeia (Rockville, MD). The H1-miR-15a sponge KD and its control were designed according to Lin *et al.* (Lin et al. 2011).

Stereotactic intracranial injections

A computer-guided stereotaxic instrument and a motorized nanoinjector (Angle Two™ Stereotaxic Instrument, myNeurolab, Leica Biosystems, Buffalo Grove, IL) were used as previously (Elliott et al., 2010; Kuperman et al., 2010; Regev et al., 2012).

Behavioral assessments

All behavioral assessments were performed during the dark (active) phase following habituation to the test room for 2 hours before each test.

Open-field test: The open-field test was performed in a 50 x 50 x 22 cm white box, lit to 120 lux. The mice were placed in the box for 10 minutes. Locomotion in the box was quantified using a video tracking system (VideoMot2; TSE Systems, Bad Homburg, Germany).

EPM test: The apparatus in this test is designed as a plus sign and contains 2 barrier walls and 2 open arms. During the 5-minute test, which is performed in relative darkness (6 lux). Data are scored using a video tracking system (VideoMot2, TSE Systems, Bad Homburg, Germany).

Homecage locomotion: Homecage locomotion was assessed using the InfraMot system (TSE Systems, Bad Homburg, Germany). Measurements of general locomotion consisted of 2 light and 2 dark cycles in the last 48 h, collected at 10 min intervals.

Statistics

Data are expressed as mean ± standard error of the mean and were performed using Statistical Package for the Social Sciences (SPSS) software (SPSS Inc., Chicago, IL).

Human studies - Dexamethasone

Dexamethasone unstimulated peripheral blood samples were drawn at 12:00pm followed by oral administration of 1.5 mg of dexamethasone. Subsequently stimulated samples were collected at 1:00pm, 3:00pm, 6:00pm, and at 11:00am the following day.

SUPPLEMENTAL INFORMATION

Supplemental information includes 7 figures and supplemental experimental procedures.

AUTHOR CONTRIBUTIONS

N.V., J.C.P., M.E., A.S.Z., and N.C. conducted the experiments. A.C., E.B.B., and A.C. supervised experiments. N.V., J.C.P., E.B.B., and A.C. wrote the manuscript.

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FIGURE LEGENDS

Figure 1. MiR-15a is elevated following chronic stress and potentially regulates FKBP51.

(A) Schematic illustration of the social defeat paradigm. C57 mice are subjected to 5 minutes of physical contact (left panel) with an aggressive ICR mouse followed by sensory contact for 24 hours (right panel). (B) Social avoidance test. Unstressed mice spend more time in the interaction zone following introduction of an unfamiliar mouse ($t(16)=-3.657$, $p=0.002$). Susceptible mice spend less time in the interaction zone following introduction of an unfamiliar mouse ($t(16)=3.133$, $p=0.006$). Resilient mice spend more time in the interaction zone following introduction of an unfamiliar mouse ($t(16)=-2.358$, $p=0.031$). (**C**) Extracts of the amygdalae of mice subjected to social defeat were used for immunoprecipitation (IP) with anti-Ago2. The bound RNA was analyzed on a miRNA array (4 control arrays, n=12 animals; 6 social defeat arrays, n=18 animals) and a gene expression array (3 control arrays, n=9 animals; 6 social defeat arrays, n=18 animals). (**D**) Log2 miRNA expression analysis. Four miRNAs were elevated and ten miRNAs were decreased in the amygdala Ago2 complex following social defeat. (**E**) miR-15a levels were elevated in the Ago2 precipitate ($t(7)=7.147$, $p=0.0002$) as was FKBP51 mRNA ($t(7)=5.352$, $p=0.0011$). (**F**) MiR-15a was also elevated in total RNA extracted from mice amygdalae (n=5) following social defeat ($t(8)=2.46$, $p=0.039$) whereas FKBP51 levels (n=5) were decreased ($t(8)=3.531$, $p=0.008$). (**G**) FKBP51 protein levels following social defeat. FKBP51 protein levels are downregulated in chronically stressed mice compared to control ($t(6)=3.049$, $p=0.014$).

Figure 2. MicroRNA 15a and 16-1 are differentially expressed following chronic stress.

(**A**) Schematic illustration of the miR-15a and miR-16-1 transcript. (**B**) Alignment of the mature sequence of miR-15a and miR-16. (**C**) The distribution of miR-15a and miR-16 in different brain regions. (**D**) Comparison of the amygdala Ago2 IP results of miR-15a and miR-16.

Figure 3. FKBP51 is regulated by miR-15a *in vitro.*

(**A**) Schematic illustration of FKBP51 3'-UTR indicating the conserved seed match for miR-15a. (**B**) Luciferase assay with luciferase fused to the 3'-UTR of FKBP51 containing intact, or a control of mutated seed site for miR-15a in the presence of miR-15a, or control scramble miR (n=6) showed a 50% decrease in luciferase levels ($t(10)=9.083$, $p=0.000$). This decrease was abolished when the intact FKBP51 3'-UTR contained a miR-15a mutated seed.

Figure 4. Knockdown of miR-15a in the BLA results in increased FKBP51 levels in the BLA.

(**A**) Schematic illustration of lentiviral GFP-labeled constructs of control and sponge used to knockdown (KD) miR-15a. (**B**) Representative microscope image of virally infected basolateral amygdala (BLA) of a 10-week old mouse following injection of lentiviral miR-15a KD with enlargement of the BLA region that corresponds to the injection site (Paxinos and Franklin digital mouse brain atlas). (**C**) left: Decreased miR-15a levels in the BLA (n=4) of mice injected with miR-15a KD relative to control under basal conditions ($t(6)=3.175$, $p=0.019$), or, right: following social defeat ($t(6)=3.528$, $p=0.012$). (**D**) MiRNA sequencing data. No differences are observed in the top 25 most abundant miRNA from mice injected with miR-15a KD virus compared with the control virus. (**E**) Elevated FKBP51 levels in the BLA (n=7) of mice injected with miR-15a KD relative to control ($t(12)=-2.413$, $p=0.033$). (**F**) FKBP51 protein levels following

miR-15a KD virus injection to the BLA. FKBP51 protein levels are upregulated in the BLA of mice injected with miR-15a KD virus compared with control virus (t(6)=-3.060, p=0.022

Figure 5. Mice with virally mediated reduced levels of BLA-miR-15 exhibit increase anxiety-like behavior

(**A-C**) Results from the elevated plus maze (EPM) test of mice injected with miR-15a KD or control viruses (n=11, 12) showing a tendency (F(3,19)=2.971, p=0.058) for differences. No significant differences were observed in the time spent in the open arms (**A**), the number of visits to the open arms (**B**) or the distance travelled in the open arms (**C**) according to Bonferroni correction for multiple testing. (**D-F**) Mice injected with miR-15a KD or control viruses (n=10, 9) which were also subjected to social defeat showed different behavior in the EPM (F(3,15)=4.08, p=0.026) with a significant decrease in the time (**D**) (U=13, p=0.009) and distance (**F**) (U=7, p=0.002) spent in the open arms of the EPM (corrected according to Bonferroni correction for multiple testing). No changes were observed in the number of visits to the open arms (**E**). (**G**) Representative tracking in the EPM of control (upper panel) relative to miR-15a KD mice (lower panel). (**H-M**) No changes were observed in the locomotor activity and total distance traveled in the open field test between miR-15a KD and control mice under basal conditions (**H-J**) or following social defeat (**K-M**).

Figure 6. miR-15a levels in human peripheral blood is higher following acute dexamethasone treatment and exposure to childhood trauma.

(**A**) Relative miR-15a levels in peripheral blood cells of young healthy male subjects following 1.5mg dexamethasone treatment. Repeated measures ANOVA: F(4,22)=4.42 p=0.009. * indicates p<0.05, ** indicates p<0.01. A significant upregulation of miR-15a was observed 3 (t(25)=-2.240, p=0.034) and 6 (t(25)=-3.487, p=0.002) hours post-treatment. (B) Levels of miR-

15a in blood of subjects exposed to childhood trauma as compared to subjects not exposed ($t(29.715) = -13.776$).

FIGURES

Figure 1

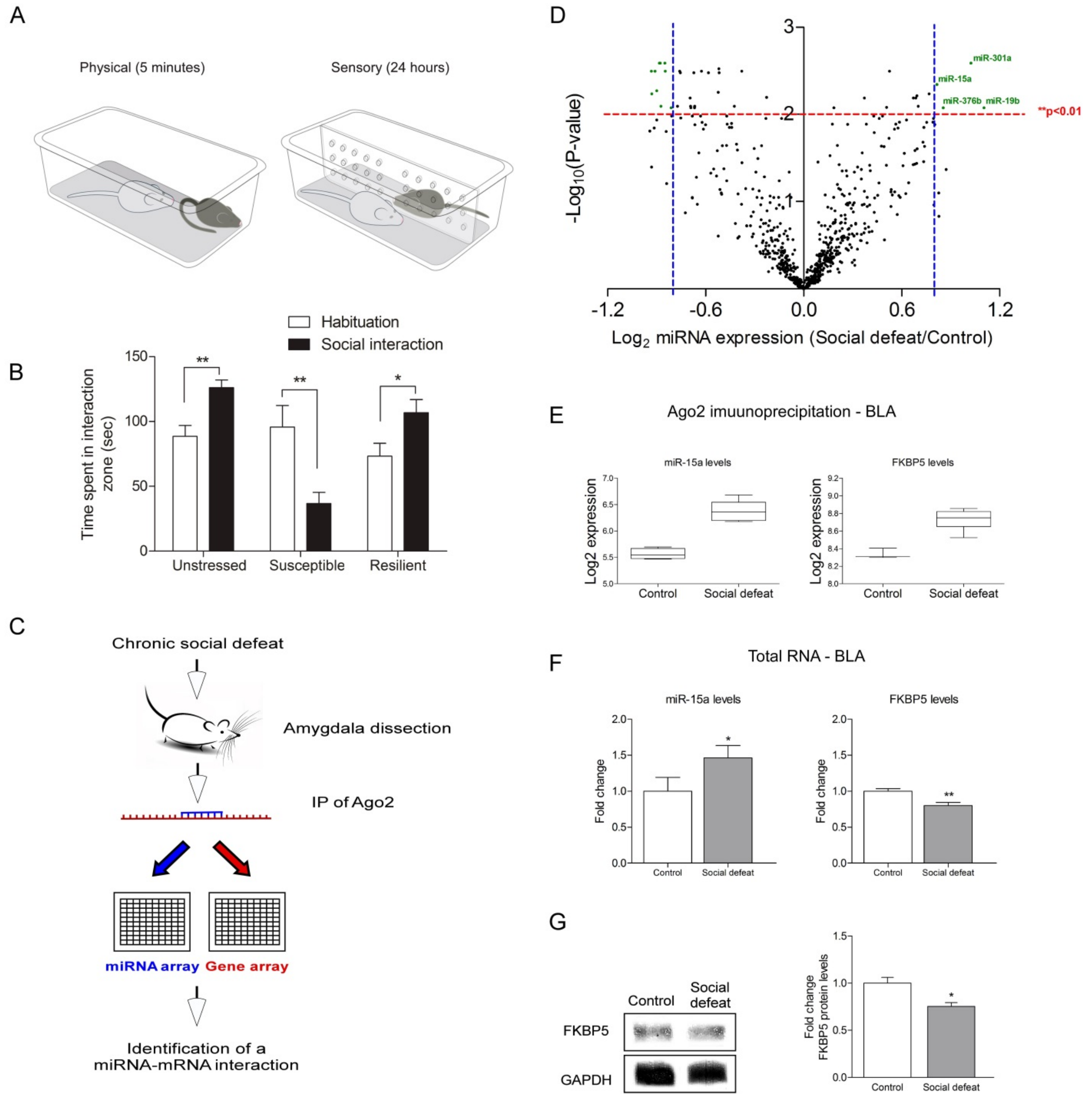
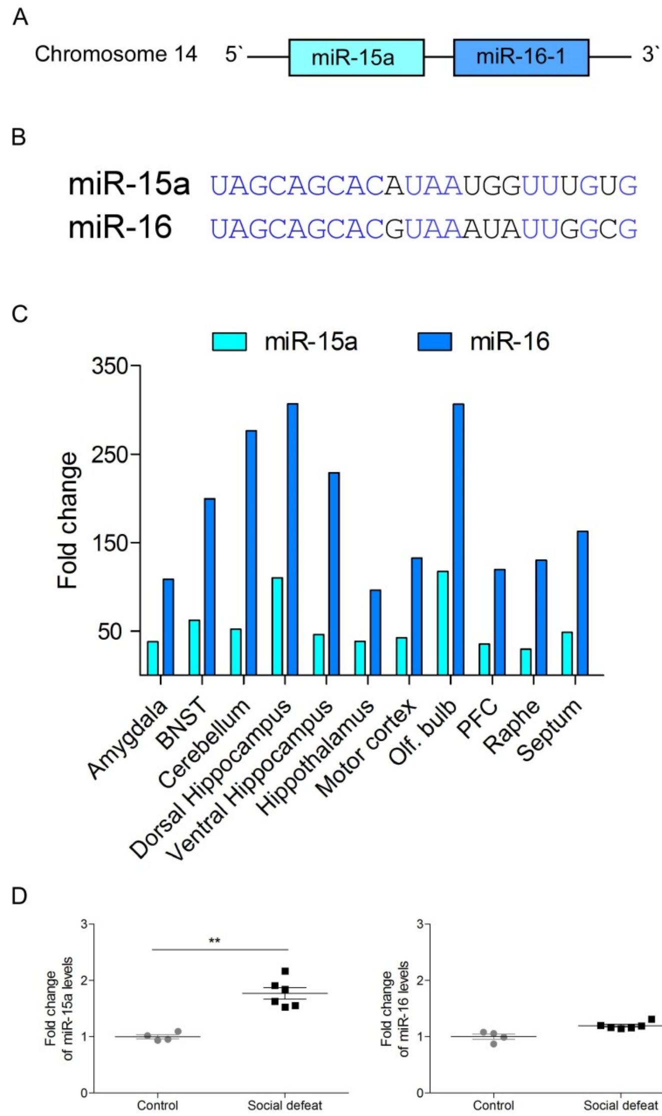


Figure 2



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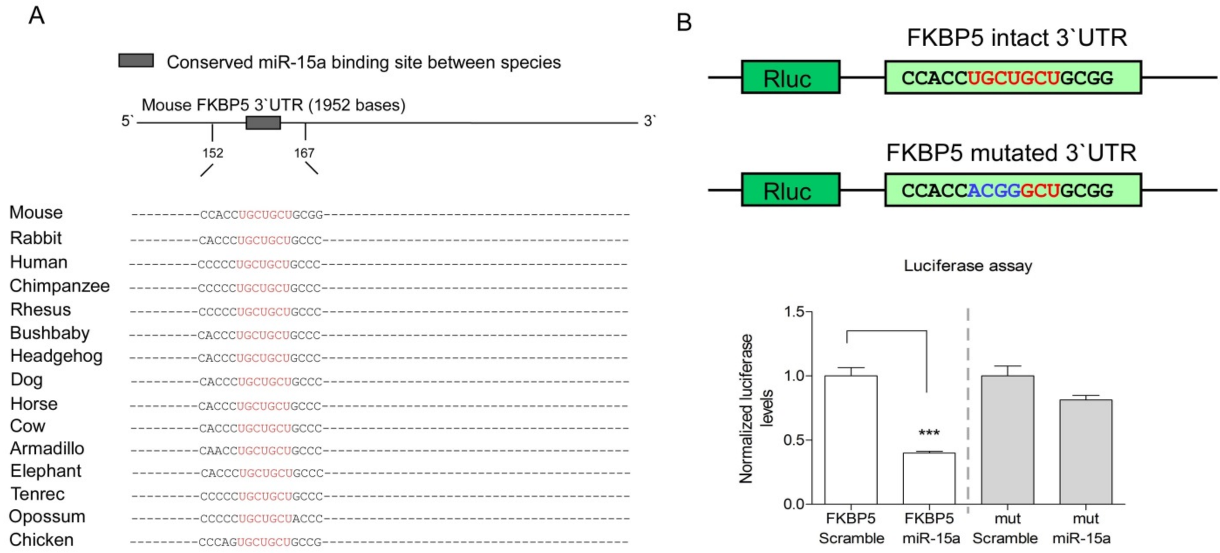

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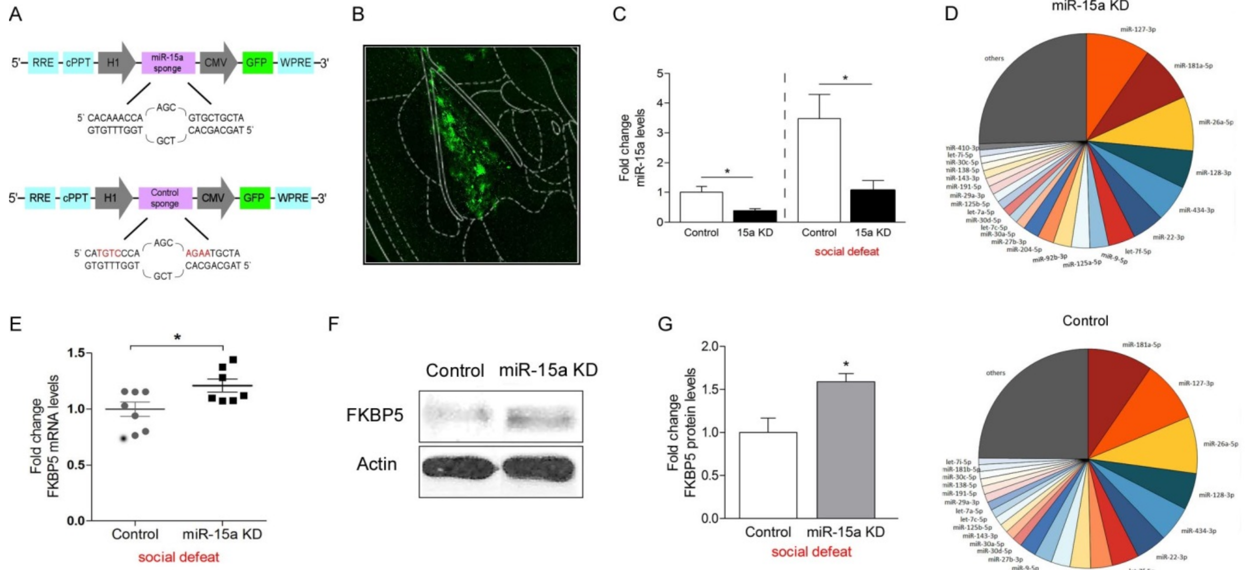

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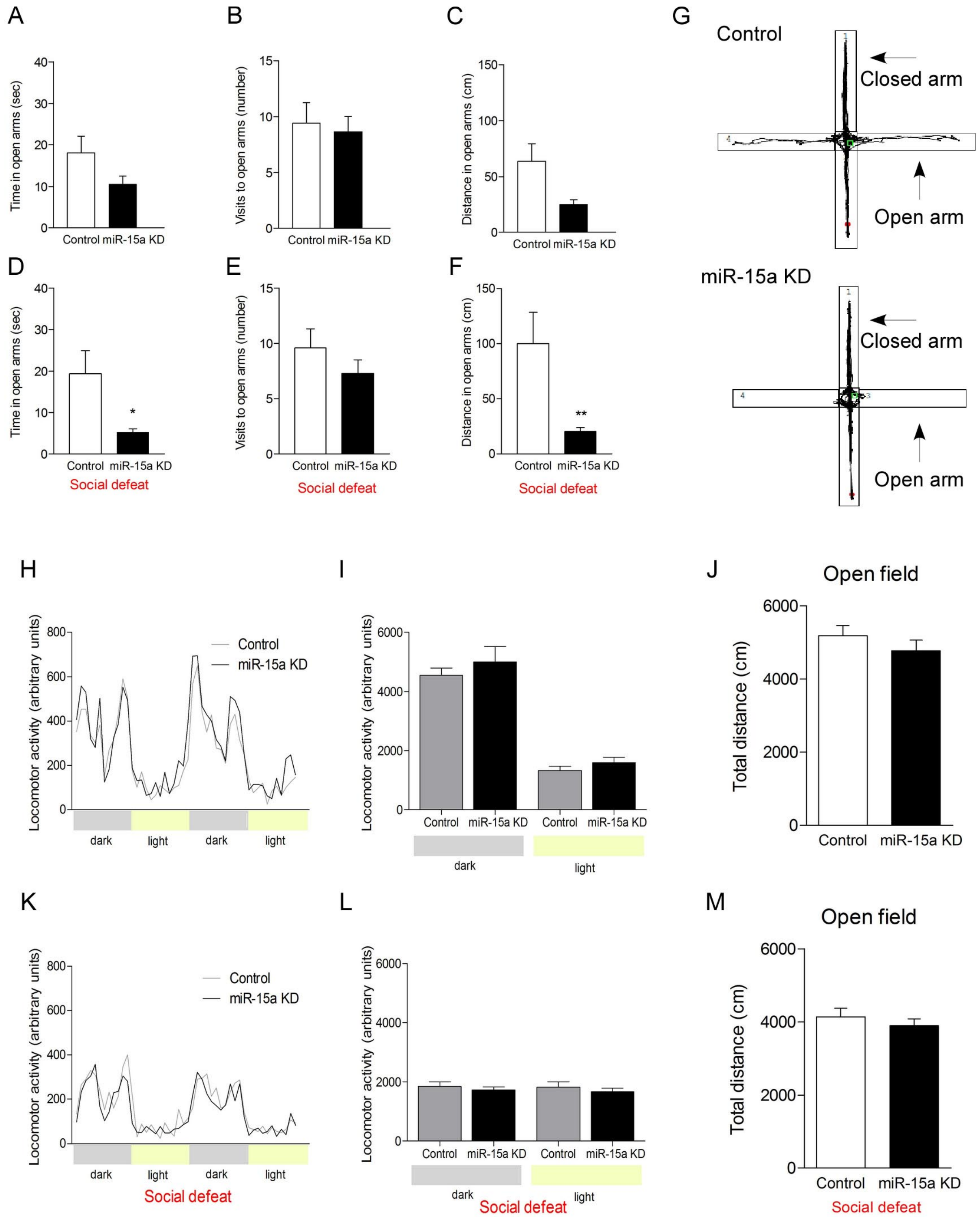

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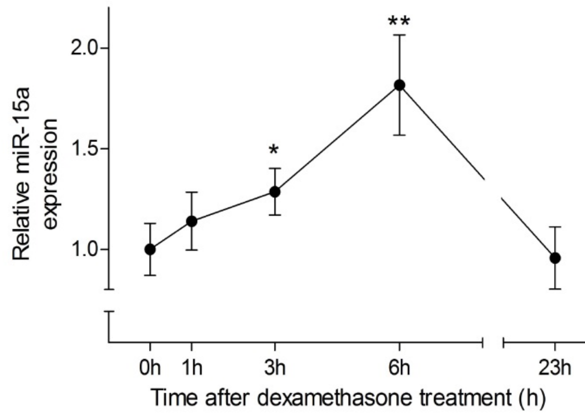
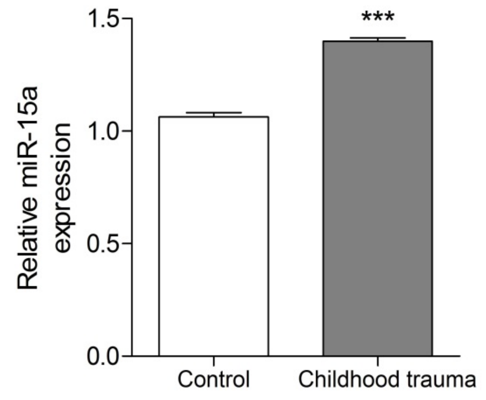




SUPPLEMENTAL INFORMATION

Figure S1: miRNA and gene array results

(A) A list of miRNAs that were significantly (step up p value<0.01) up and down regulated in RNA from IP of amygdala Ago2 complex following chronic stress in mice. **(B)** A list of genes that were significantly (p value<0.05) up and down regulated in RNA from IP of amygdala Ago2 complex following chronic stress in mice.

Figure S2: miR-15a levels are elevated in mice plasma following social defeat.

Real time PCR analysis. (**A**) The levels of miR-15a (n=6) were increased by 60% (t(10)=-2.265, p=0.047) in plasma of mice subjected to the chronic social defeat paradigm whereas (**B**) the levels of miR-124 (n=7,6) remained unchanged.

Figure S3: Construction and validation of miR-15a over-expressing lentiviruses.

(**A**) Schematic illustration of the Syn-miR-15a over-expression (OE) and scramble control lentiviral constructs. (**B**) miR-15a levels in the basolateral amygdala (BLA) (n=6) of mice injected with miR-15a OE or control (scramble) lentiviruses under basal conditions (t(10)=-6.147, p=0.000). (**C**) Schematic representation of the site of delivery. Adapted from Paxinos and Franklin digital mouse brain atlas. (**D**) Enlargement of the BLA region corresponding to the injection site. (**E**) Representative microscope image of a virally infected BLA following miR-15a injection of a 10-week old mouse.

Figure S4: Anxiety and depression-like behavioral tests and locomotor activity in mice over-expressing miR-15a

(**A, B**) No significant differences were observed in the open field test between BLA miR-15a OE and control mice under basal conditions (**A**) or following chronic social defeat (**B**). (**C, D**) No significant differences were observed in the EPM test between BLA miR-15a OE and control injected mice under basal conditions (**C**) or following chronic social defeat (**D**). (**E-J**) No significant differences were observed in the locomotor activity or total distance traveled in the open field test between BLA miR-15a OE and control mice under basal conditions or (**H-J**) following chronic social defeat.

Figure S5: Knockdown of miR-15a results in increased anxiety-like behavior in the open-field test

(A) Illustration of miR-15a KD and control virus injection sites. Blue circles represent the punch area used for RNA extraction. **(B)** Real time PCR analysis of miR-15a levels (n=5). The levels of miR-15a were significantly decreased following injection of miR-15a KD virus compared to control virus ($t(8)=-3.445$, $p=0.009$). **(C)** Open-field test for mice injected with miR-15a KD virus relative to control (n=11, 12). The mice spent significantly less time in the center of the arena ($F(1,22)=5.27$, $p=0.032$). No changes were observed in the distance spent in center or visits to center. **(D)** Open-field test for mice injected with miR-15a KD or control viruses (n=10, 9) and were also subjected to social defeat. There were no differences in the time the mice spent in the center of the arena but they showed a tendency to travel less distance in the center of the arena ($U=22$, $p=0.060$) and had fewer visits to the center of the arena ($U=20$, $p=0.041$).

Figure S1

A

Upregulated	log2(x)	stepuplog10(y)
mmu-miR-19b_st	1.103437211	2.076686023
mmu-miR-301a_st	1.023376015	2.589425203
mmu-miR-376b_st	0.853955738	2.076686023
mmu-miR-15a_st	0.815886886	2.344322875

Downregulated	log2(x)	stepuplog10(y)
mmu-miR-491_st	-0.811076393	2.076686023
mmu-miR-744_st	-0.847844614	2.495934396
mmu-miR-423-5p_st	-0.849815159	2.589425203
mmu-miR-770-3p_st	-0.874521579	2.093004858
mmu-miR-210_st	-0.878568412	2.589425203
mmu-miR-346_st	-0.883534799	2.589425203
mmu-miR-667_st	-0.899353539	2.271560179
mmu-miR-320_st	-0.909412145	2.495934396
mmu-miR-668_st	-0.929609229	2.235314375
mmu-miR-139-3p_st	-0.93265839	2.495934396

p value (step up)	stepuplog10(y)
0.05	1.301029996
0.01	2
Fold change cut off	log2(x)
1.75	0.807354922
0.571428571	-0.807354922

B

RefSeq	Gene name	Fold change	p value	Full name
NM_175314	Adamts9	1.65039	0.000496	Mus musculus a disintegrin-like and metallopeptidase
NM_010220	Fkbp5	1.30443	0.001009	Mus musculus FK506 binding protein 5
NM_001166737	Vmn1r103	1.56482	0.003005	vomeronasal 1 receptor 103
NM_001005568	Olfr1281	1.32074	0.003176	olfactory receptor 1281
NM_001081064	Pdzd2	1.31115	0.003384	PDZ domain containing 2
NM_001081391	Csmd3	1.38291	0.003796	CUB and Sushi multiple domains 3
NM_001105189	Vmn2r78	1.34524	0.005359	vomeronasal 2, receptor 78
NM_146649	Olfr1160	1.3222	0.00763	lfactory receptor 1160
NM_001011822	Olfr787	1.32773	0.006516	olfactory receptor 787
NM_172800	Sdk2	1.32533	0.008346	Sidekick Cell Adhesion Molecule 2
NM_175473	Fras1	1.40663	0.009796	Fraser syndrome 1

RefSeq	Gene name	Fold change	p value	Full name
XR_032386	Gm4804	-1.33326	0.001287	glyceraldehyde-3-phosphate dehydrogenase pseudogene
ENSMUST00000065297	Lonrf1	-1.31542	0.002133	LON Peptidase N-Terminal Domain And Ring Finger
NM_001177750	Gm10767	-1.39142	0.002899	predicted gene 10767
NR_004051	Btnl5	-1.59032	0.003562	Mus musculus butyrophilin-like 5 (Btnl5), non-coding RNA
XR_001896	Gm8174	-1.68049	0.004361	glyceraldehyde-3-phosphate dehydrogenase pseudogene
XR_033497	Gm3809	-1.37672	0.005489	glyceraldehyde-3-phosphate dehydrogenase pseudogene
XR_031132	Gm5210	-1.35348	0.004779	glyceraldehyde-3-phosphate dehydrogenase pseudogene
NR_029580	Mir194-1	-1.3076	0.006629	microRNA 194-1
XR_033575	Gm7712	-1.34359	0.006891	predicted gene 7712

P value
0.01
Fold change cut off
1.3, -1.3

Figure S2

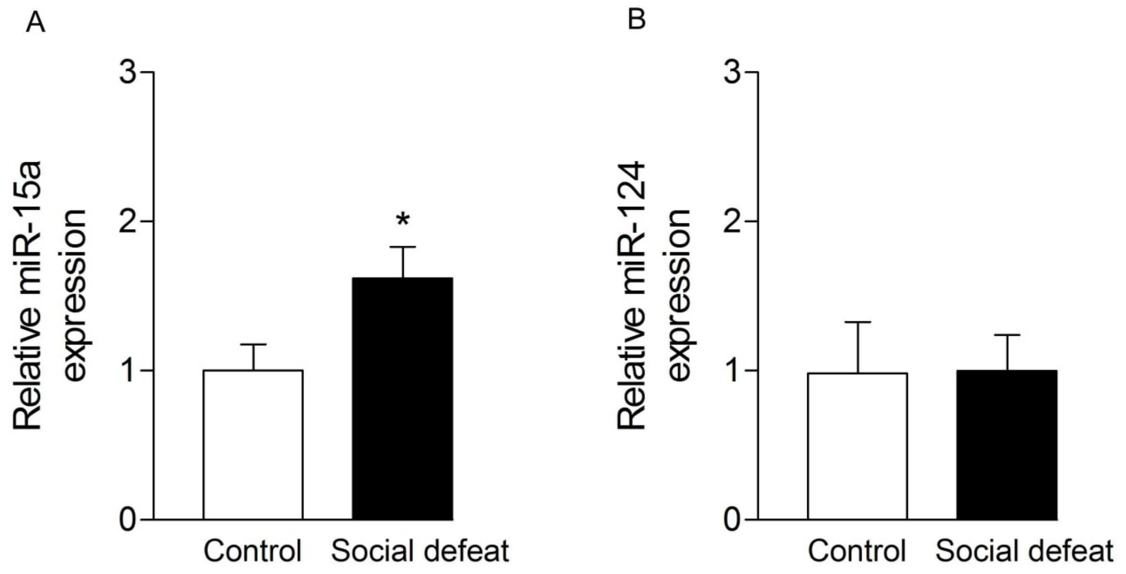


Figure S3

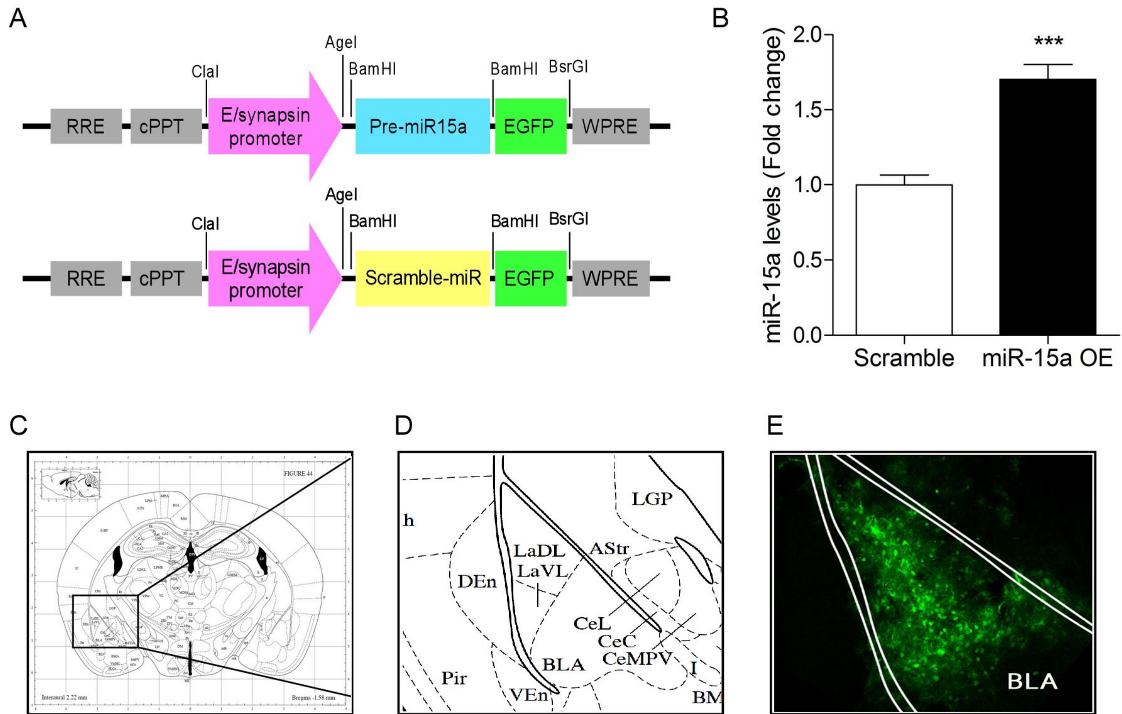


Figure S4

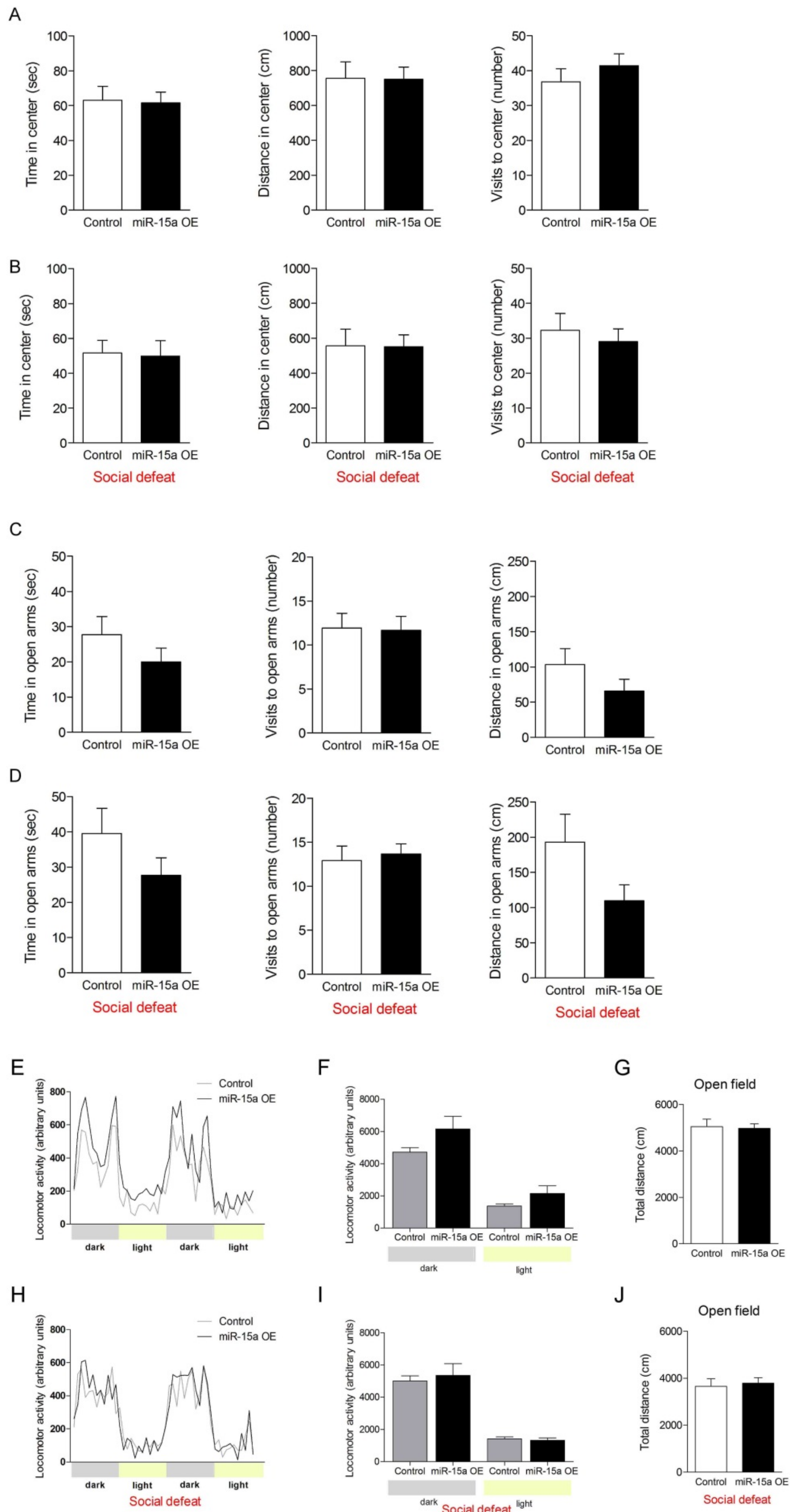
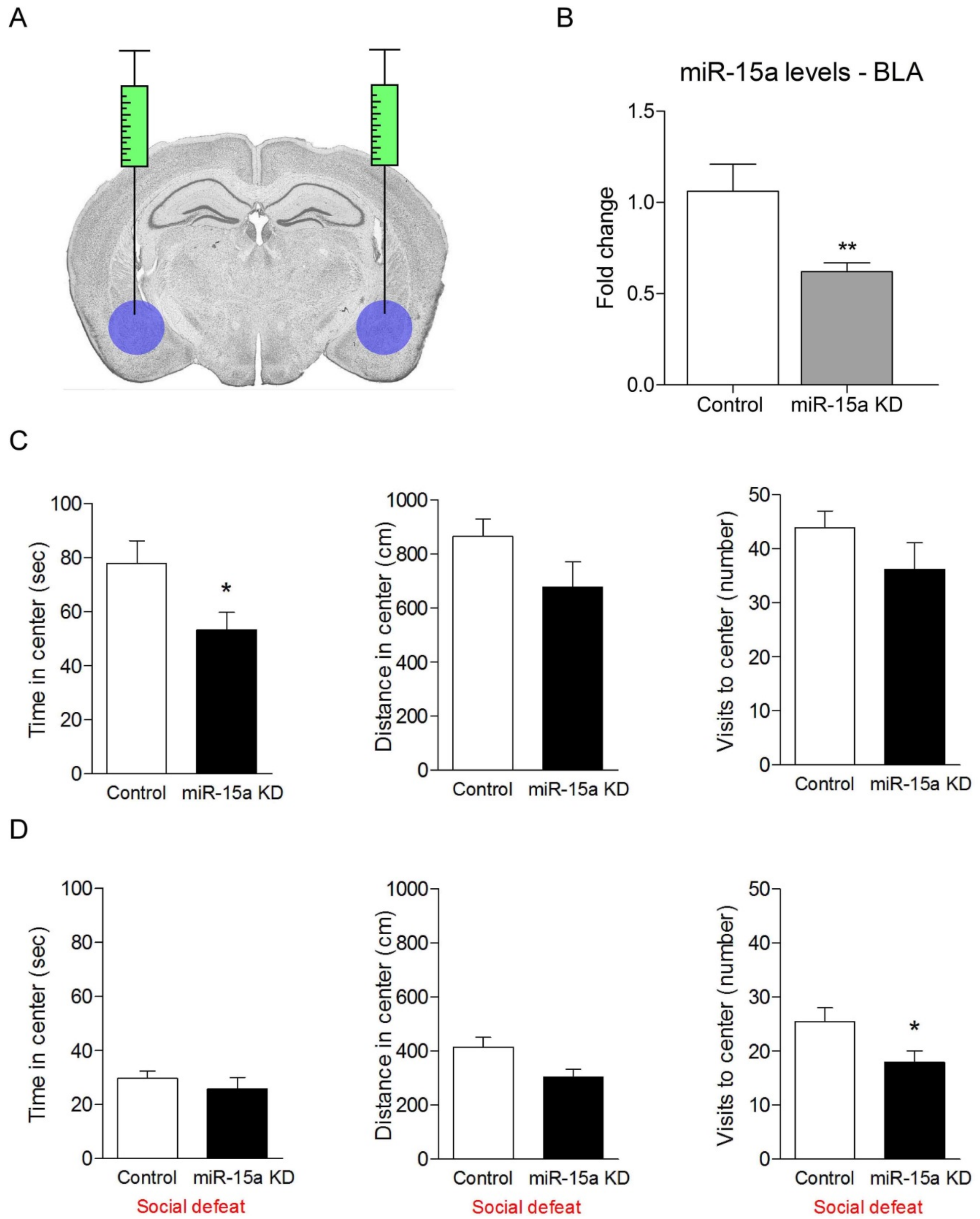


Figure S5



ONLINE METHODS

Animals

C57BL/6J mice and ICR mice (outbred mice strain, also known as CD1) (Harlan Israel, Kiryat Weizmann, Rehovot) were maintained in a pathogen-free temperature-controlled (22 ± 1°C) mouse facility on a reverse 12 h light-dark cycle at the Weizmann Institute of Science, according to institutional guidelines. Food (Harlan Israel, Kiryat Weizmann, Rehovot) and water were given *ad libitum*. C57BL/6J mice were housed 4 per cage whereas ICR mice were single caged. The total number of animals used for the Ago2 IP was 30 (18 social defeat and 12 controls). The total number of animals used for the lentiviruses experiment was 40 in total (10 per group).

Chronic social defeat

10-week old C57BL/6J male mice were subjected to a social defeat protocol as previously described (Krishnan et al. 2007). Briefly, the mice were placed randomly in a home cage of an aggressive ICR mouse and allowed to physically interact for five minutes. During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. A perforated clear Plexiglas® divider was then placed between the animals and the mice remained in the same cage for 24 h to allow sensory contact. The procedure was then repeated with an unfamiliar ICR mouse for each of the 10 consecutive days. Control mice were housed in the same room as the social defeat mice but were taken out of the room during the five-minute interaction with the ICR. Control mice were handled daily and housed 2 per cage with a perforated clear Plexiglas® divider placed between the 2 mice. The cage used for the social defeat is a type II long cage for mice (W x D x H) 15.59 x 8.46 x 6.77 inch. The bedding used during the social defeat was Aspen Sami bedding 17304. Mice were not lethally injured although

superficial marks were observed. We find that within a period of 5 minutes the mice tend to avoid major injuries.

Microdissection of brain sites for Ago2 IP

Amygdala samples were collected from social defeat and control mice 8 days after the end of the chronic social defeat protocol. Tissue collection and processing was performed as previously described (Lebow et al., 2012; Sztainberg et al., 2010). Briefly, after removing the brain and placing it on an acryl 1 mm brain matrix (Stoelting Co., Wood Dale, IL, cat# 51380), 2 mm slices were taken using standard razor blades (GEM, 62-0165) based on designated anatomical markers. Blunted syringes of different diameters were used to punch out the amygdala from slices removed from the matrix.

Immunoprecipitation of Ago2 protein

Pools of 3 amygdalae taken from 3 mice from the same treatment group (either Social defeat n=18 or Control n=12) were homogenized in NP40 buffer, which was supplemented with RNase inhibitor, protease inhibitor and phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN). The samples were constantly agitated for 2 h at 4°C. Samples were then centrifuged for 20 min at 12,000 rpm at 4°C in a microcentrifuge; the supernatant was placed in a fresh tube, kept on ice and the pellet was discarded. Magnetic protein G beads (Dynabeads, Invitrogen Life Technologies, Carlsbad, CA) were incubated with the Ago2 monoclonal antibody (WAKO chemicals GmbH, Neuss, Germany) with rotation at room temperature for 10 minutes. After several washes, the samples were added to the Ago2-coated protein G beads and incubated overnight at 4°C under agitation. The following day the beads were washed 3 times with PBS. For RNA purification, the beads were homogenized in RLT buffer (RNeasy kit, QIAGEN, Hilden,

Germany - miRNA supplementary protocol). For western blot analysis, the beads were boiled in sample buffer to release the protein from the beads.

RNA purification and microarray

RNA from the Ago2 immunoprecipitation samples was isolated using the RNeasy plus kit (QIAGEN, Hilden, Germany) following QIAGEN's supplementary Protocol 1: Purification of total RNA containing miRNA. RNA for all other purposes was isolated from frozen brain punches using the miRNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendation. RNA derived from tissues of stressed mice following Ago2 immunoprecipitation was further analyzed on an Affymetrix miRNA 2.0 array (enriched RNA protocol) and an Affymetrix Mouse Gene 1.0 ST array (Affymetrix, Santa Clara, CA).

Microarray analysis

For the miRNA analysis, we used 4 arrays for the control mice and 6 arrays for the social defeat mice (each array consists of a pool of 3 mice). We used step up correction for multiple testing correction. The fold change threshold was 1.75 with a p value of $p < 0.01$. For the gene analysis, we used 3 arrays for the control mice and 6 arrays for the social defeat mice (each array consists of a pool of 3 mice). The fold change threshold was 1.3 with a p value of $p < 0.05$.

Bioinformatic analysis of microRNA microarray results

miRNAs and genes were tested in 3 different web based programs in search for a seed match between a miRNA and a 3'-UTR: Target Scan (http://www.targetscan.org), Miranda (http://www.microrna.org), (Betel et al., 2008) and Pictar (http://pictar.mdc-berlin.de).

Cloning of 3'-UTRs into Psicheck2 luciferase expression plasmid

The 3'-UTR sequence of FKBP51 was PCR amplified from mouse genomic DNA using a forward primer: CCAACTCAGGACTGAACAGT and a reverse primer: GTTCCTTAGGCTGTGGAGAA. The DNA sequence for the mutated form of FKBP51 was generated by site directed mutagenesis using the original cloning primers of FKBP51 and 2 new primers: FKBP51-SDM-F ATGACCACCACGGGCTGCGG and FKBP51-SDM-R CCGCAGCCCGTGGTGGTCAT. This mutation replaced the first 4 nucleotides in the miR-15a seed sequence of FKBP51 from TGCT to ACGG. The 3'-UTR fragments were then ligated into pGem-T easy vector (Promega, Madison, WI) according to the manufacturer's guidelines, and further subcloned into a single *NotI* site at the 3' end of luciferase in the Psicheck2 reporter plasmid (Promega, Madison, WI). Cloning orientation was verified by diagnostic cuts and sequencing.

Transfections and luciferase assay

Huh7 cells were grown on poly-L-lysine coated 48-well plates to 70-85% confluence and transfected using polyethylenimine with the following plasmids: 5 ng of Psicheck2- 3'-UTR plasmid and 215 ng of EGFP over-expressing vector for either a specific miRNA, or a miR-scramble EGFP plasmid. 24 h following transfection, cells were lysed and luciferase reporter activity was assayed as previously described (Chen et al. 2005). Renilla luciferase values were normalized to control luciferase levels (transcribed from the same vector but not affected by the 3'-UTR tested) and averaged across eight-well repetitions per condition.

miRNA RT-qPCR expression analysis

Quantitative miRNA expression was acquired and analyzed using a step one thermocycler (Applied Biosystems, Waltham, MA), using miRCURY LNA Universal RT microRNA PCR primers

(Exiqon, Vedbaek, Denmark) or miScript primer assay (QIAGEN, Hilden, Germany). RNA samples were assessed using miRCURY Universal cDNA Synthesis kit II and miRCURY ExiLENT SYBR Green (Exiqon, Vedbaek, Denmark) or miScript II RT kit and miScript SYBRgreen PCR kit (QIAGEN, Hilden, Germany), according to the manufacturer's guidelines. U6, 5S rRNA were used as internal controls. Gene expression was obtained using the High Capacity kit and SYBR green PCR master mix (Applied Biosystems, Waltham, MA). The real-time PCR primers for FKBP51 were: forward: ATGACTACTGATGAGGGCAC and reverse: GACATAAACTTTGTCACCAAAC.

Design, construction and validation of miR-15 lentiviruses

The miR-15a over-expression vector was cloned as follows: the enhanced form of human synapsin I promoter (Hioki et al. 2007) was PCR amplified (forward primer: tttttatcgatctcgagtagttattaatagtaatc, reverse primer: ttttttaccggtggcgcgcccgccgcagcgcagatggt) from pENTR1A-E/SYN-GFP-WRPE1 (Kindly provided by Dr. Takeshi Kaneko, Department of Morphological Brain Science, Graduate School of Medicine, Kyoto University, Kyoto, Japan) and inserted between ClaI and AgeI restriction sites to replace the CMV promoter in pCSC-SP-PW-GFP (kindly provided by Dr. Inder Verma, The Salk Institute for Biological Studies, La Jolla, CA). Following the Synapsin promoter, the precursor for miR-15a was inserted: gcacataccagtgttagatttttttcaaacatagattttatgtgttctactttttcctaaaaagccttttctgtaaattactattgaggtgctagg agttttcaaaaccaacccttggagtaaagtagcagcacataatggtttgtggatgttgaaaaggtgcaggccatactgtgctgcctcaaaa tacaaggacctgatcttctgaagagagtacctgtctttttattcatagctcctatgatagcaatgtc.

The miR scramble control was purchased from GeneCopoeia (Rockville, MD) and sub-cloned into pCSC-SP-PW-SYN-GFP plasmid. The miR-15a sponge KD and its control were designed according to Lin *et al.* (Lin et al. 2011) and inserted following an H1 promoter in the p156RRL-CMV-GFP viral plasmid. The sequence for miR-15a sponge was:

CGCGGATCTAGCTAGCCACAAACCAAGCGTGCTGCTAATCGCACAAACCAAGCGTGCTGCTAATCGCACAAACCAAGCGTGCTGCTAATCGCACAAACCAAGCGTGCTGCTAGCTAGATCGATCTTCTAGAAAGATCCAAACCAAGCGTGCTGCTAATCGCACAAACCAAGCGTGCTGCTAATCGCACAAACCAAGCGTGCTGCTAATCGCACAAACCAAGCGTGCTGGCTAGCTAGCCTAGATCCGCGTCGTTAATTAACCTTAGGGCTTAGCGCTAGC. The sequence for miR-15a sponge control was: CTAGCTAGCCATGTCCCAAGCAGAATGCTAATCGCATGTCCCAAGCAGAATGCTAATCGCATGTCCCAAGCAGAATGCTAATCGCATGTCCCAAGCAGAATGCTA. High titer lentiviruses were produced as previously described (Tiscornia et al. 2006). Briefly, recombinant lentiviruses were produced by transient transfection in HEK293T cells. Infectious particles were harvested at 48 and 72 h post-transfection, filtered through 0.45 µm-pore cellulose acetate filters, concentrated by ultracentrifugation, re-dissolved in sterile HBSS, aliquoted and stored at –80°C.

Stereotactic intracranial injections

A computer-guided stereotaxic instrument and a motorized nanoinjector (Angle Two™ Stereotaxic Instrument, myNeurolab, Leica Biosystems, Buffalo Grove, IL) were used as previously described (Elliott et al., 2010; Kuperman et al., 2010; Regev et al., 2012). 10-week old male mice were randomly selected and anesthetized using 1.5% isoflurane and 1 µl of the lentiviral preparation was delivered to each BLA using a Hamilton syringe connected to a motorized nanoinjector system at a rate of 0.2 µl per min (coordinates relative to bregma: AP = –1.58 mm, L = ±3.3 mm, H = –4.6 mm). Following a 2-week recovery period, mice were subjected to behavioral studies and later anesthetized and perfused with 4% PFA. The fixed brains were serially sectioned and immunohistochemically stained in order to confirm the location of the injection site, as previously described (Regev et al., 2011). The antibodies that were used were: Goat biotinylated anti-GFP (Abcam, Cambridge, UK; ab6658) and Alexa Fluor

488 Streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA; 016-540-084). 3 repetitions were used for the immunohistochemical analysis.

Behavioral assessments

All behavioral assessments were performed during the dark (active) phase following habituation to the test room for 2 hours before each test. Behavioral tests were conducted as previously described (Haramati et al., 2011; Lebow et al., 2012) in the following order, from the least stressful procedure to the most and ending with home cage locomotor testing: Open-field, EPM and home cage locomotion.

Open-field test: The open-field test was performed in a 50 x 50 x 22 cm white box, lit to 120 lux. The mice were placed in the box for 10 minutes. Locomotion in the box was quantified using a video tracking system (VideoMot2; TSE Systems, Bad Homburg, Germany).

EPM test: This apparatus in this test is designed as a plus sign and contains 2 barrier walls and 2 open arms. During the 5-minute test, which is performed in relative darkness (6 lux), the number of entries, the distance traveled and the time spent in the open arms is automatically scored using a video tracking system (VideoMot2, TSE Systems, Bad Homburg, Germany).

Homecage locomotion: Homecage locomotion was assessed using the InfraMot system (TSE Systems, Bad Homburg, Germany). Mice were housed individually for 72 h, in which the first 24 h were considered habituation to the individual housing conditions. Measurements of general locomotion consisted of 2 light and 2 dark cycles in the last 48 h, collected at 10 min intervals.

Statistics

Data are expressed as mean ± standard error of the mean (Binder et al.). Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software (SPSS Inc.,

Chicago, IL). All data sets' distributions were tested for normality using Shapiro-Wilks test in order to determine which statistical tests should be applied. In cases where indices of 2 groups were compared and the data's distribution was normal, a 2-sided students t-test was used, where the data departed from normal distribution, the Mann- Whitney U test was applied. All data sets were also tested for variance similarity between compared groups. For the miRNA array results, a q-value correction was performed. Mice were excluded from the analysis if they had values higher than AVG+2*SD or lower than AVG-2*SD

Human studies - qPCR analysis

Samples: For this study, 26 males of Caucasian origin aged between 19 and 30 years were recruited (mean age = 25.58 +/- 2.64SD). All participants were free of a history of psychiatric disorders as well as major neurological and general medical disorders. Further exclusion criteria were regular use of medical drugs, as well as excessive alcohol or caffeine consumption. All subjects gave written informed consent. Procedures were approved by the Ethics Committee of the Ludwig Maximilians University, Munich, Germany, in accordance with the Declaration of Helsinki.

Study Design: Unstimulated peripheral blood samples were drawn at 12:00pm followed by oral administration of 1.5 mg of dexamethasone. Subsequently stimulated samples were collected at 1:00pm, 3:00pm, 6:00pm, and at 11:00am the following day. PAXgeneTM (QIAGEN, Hilden, Germany) whole blood RNA collection tubes were used at each time point for whole blood collection.

RNA extraction: Total RNA was extracted using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) with the QIAGEN method for column purification of nucleic acids (PreAnalytiX, Hombrechtikon, Switzerland) according to the manufacturer's instructions. For

RNA quality and quantity, extracted samples were subsequently run on the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). All samples had an RNA integrity number (RIN) ≥7.

Quantitative real time polymerase chain reaction: Total RNA was reverse transcribed using the miRCURY LNA™ Universal RT miRNA PCR cDNA synthesis kit (Exiqon, Vedbaek, Denmark). CDNA was diluted and mixed with the Exiqon microRNA LNA PCR primers as well as the Exiqon SYBR Green master mix and assayed in 10 µl reactions. qPCR experiments were performed using the Roche 480 LightCycler system (Roche Applied Science, Roche Diagnostics, Indianapolis, IN) in 384-well plates. Each sample was run in technical triplicates. Normalization of the results was performed against SNORD38 using the delta Ct method.

Western blot analysis

Frozen brain samples were homogenized in RIPA buffer supplemented with proteinase inhibitors (Sigma-Aldrich, St. Louis, MO) and were incubated on ice for 10 min. After 10 min centrifugation, the supernatant was transferred to a new tube and sample buffer was added to the sample, which was then boiled for 5 min and placed on ice. The samples were separated in a 10% polyacrylamide gel electrophoresis. Transfer was performed using an assembly of nitrocellulose membrane and Whatman paper. The transfer was performed at 100v, 280 mAmp for 1h and 40 minutes. After washes with PBST (PBS + 20% Tween 20) membrane was blocked with 10% milk for 1 h. The first antibody was added (goat anti FKBP51 – Santa Cruz 11518, mouse anti-GAPDH – abcam 8245 or mouse Anti-b-Actin- Sigma-Aldrich, St. Louis, MO; A1978) to PBST and placed on constant shaking at 4°C overnight or 1 h at room temperature. The second antibody (anti-goat HRP – abcam 6885, anti-mouse HRP – cell signal 7076) was added in

10% milk for 1 h. Each step was separated by additional washes with PBST. Finally, ECL was added to the membrane which was then exposed to film.

Social avoidance test

The test is performed in an open field design with a small neighboring chamber that is separated from the open field with a divider with small open slits, allowing full sensory contact between the 2 fields. The mice are allowed to habituate to the open field for 3 minutes, and then an unfamiliar ICR mouse is placed in the neighboring chamber, and they are allowed to interact for 3 minutes. The entire session is videotaped and analyzed with Ethovision software (Noldus, Wageningen, Netherlands). The space next to the small neighboring chamber is deemed the interaction zone. The time a mouse spends in the interaction zone with the unfamiliar ICR mouse is divided by the time the mouse spent in the interaction zone without the unfamiliar ICR mouse and then multiplied by 100. Mice were categorized into 3 groups: Control, "Susceptible" and "Resilient". Mice that received below 100 in this analysis were characterized as "Susceptible". Mice that received above 100 in this analysis were characterized as "Resilient". Only control mice that received above 100 were further used.

miRNA Sequencing analysis

RNA samples were quantified by Qubit and 400 ng RNA used for library preparation using the Illumina TruSeq Small RNA Library Preparation Kit following the standard protocol and size-selected on DNA-PAGE to a size of 145-155nt. QC was carried out using BioAnalyzer High Sensitivity DNA chips and libraries quantified using the KAPA library Quantification Kit for Illumina (Kapa Biosystems Inc., Wilmington, MA) on a Roche Lightcycler480. Libraries were multiplexed in euqimolar pools and sequenced single-end 50nt on an Illumina MiSeq at MPI of

Psychiatry, München, using v3 chemistry to a depth of minimum 5Mio reads per sample (% >=Q30 higher then 96).

Fastq raw sequence reads were quality-checked using FASTQC* and reads between 15-40nt length containing the Illumina Small RNA Adapter selected and adapter-trimmed using Cutadapt*. Reads were aligned to miRbase v21 and normalized to counts per million using sRNAbench* (library mode using Bowtie*). Differential expressed microRNAs were queried using the Bioconductor - DESeq2 package* applying a minimum cut-off of 5 read counts in all samples.

Subjects exposed to early life trauma

Control subjects were available at the IRCCS Fatebenefratelli Institute, Brescia (Italy). Individuals presenting a history of neurological disease, prior electro-convulsivant treatment, prior traumatic brain injury, or mental retardation (IQ<70) were excluded from the study. Written informed consent was obtained by participants after receiving a complete description of the study, which has received approval by the local ethics committee.

The absence of psychiatric disorder was ascertained via 2 schedules: the Mini International Neuropsychiatric Interview (M.I.N.I. Plus, Bonora et al., 1995), to exclude any psychiatric disorder in Axis I; and the Structured Clinical Interview for DSM disorders (SCID-II, Spitzer et al., 1993) to exclude any psychiatric disorder in Axis II.

The list of traumatic events includes loss of a biological parent due to death or separation for at least 6 months, including being taken into local authority care, severe physical, sexual abuse by a parental figure and neglect. Physical abuse includes incidents that meet at least 2 of the following criteria: a) the abuse consisted of being hit with a belt or stick, or being punched or kicked; b) the abuse resulted in an injury, including broken limbs, black eyes or bruising; and c)

the perpetrator was considered to be out of control. Sexual abuse was defined as unwanted or illegal sexual experiences prior to age 17 years with any adult or an individual at least 5 years older than the recipient, not necessarily limited to the immediate family; moreover, these experiences have to meet at least 2 of the following criteria: a) the perpetrator was known to the individual; b) the perpetrator was a relative; c) the perpetrator lived in the same household; d) the unwanted sexual experience occurred more than once; e) the perpetrator touched the child's genitals; f) the perpetrator forced the child to touch the perpetrator's genitals; and g) the abuse involved sexual intercourse.

Neglect was defined in terms of parents' disinterest in material care (feeding and clothing), health, schoolwork and friendships. Neglect was quantified for both mother and father.

The most conservative cut-off points published by Bifulco et al. (2005) were used to dichotomise these responses into a yes/no answer. For this study, we performed miRNA analyses using qPCR in a group of 20 subjects who reported at least one type of abuse (physical abuse, sexual abuse, physical, parents separation or loss and neglect) and 20 subjects matched for age and gender with no history of early life stress (mean age ± SD: 38.1 ± 6.1 and 37.5 ± 6.7 respectively in the subjects with and without childhood trauma, $p<0.05$); percentage of females of 55% and of 54% respectively in the subjects with and without childhood trauma, $p<0.05$).

Blood samples were collected by using PaxGene Blood Tubes. After collection, blood samples were then kept at room temperature for 2 hours, then at -20°C for 2 days and then at -80°C until their processing.

<u>miRNA isolation from the blood of subjects</u>

Total RNA was extracted from 2.5 mL of blood with the PAXGene Blood miRNA Kit (QIAGEN, CA, USA), designed for the simultaneous isolation of small and large RNAs; RNA concentration and quality were assessed through a NanoDrop spectrophotometer (Thermo Scientific, MA, USA).

Real-time PCR analyses

We looked specifically at the expression of hsa-miR-15a-5p by Real Time PCR (RT-PCR). RT-PCR was conducted using TaqMan MicroRNA Assays (Applied Biosystems, CA, USA), following the manufacturer's instructions and the reactions were run on the StepOnePlus instrument (Applied Biosystems, Waltham, MA). The Ct values were normalized according to the deltaCt method on the endogenous controls RNU44 and RNU48.