

# MicroRNA 135 Is Essential for Chronic Stress Resiliency, Antidepressant Efficacy, and Intact Serotonergic Activity

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## SUMMARY

The link between dysregulated serotonergic activity and depression and anxiety disorders is well established, yet the molecular mechanisms underlying these psychopathologies are not fully understood. Here, we explore the role of microRNAs in regulating serotonergic (5HT) neuron activity. To this end, we determined the specific microRNA “fingerprint” of 5HT neurons and identified a strong microRNA-target interaction between microRNA 135 (miR135), and both serotonin transporter and serotonin receptor-1a transcripts. Intriguingly, miR135a levels were upregulated after administration of antidepressants. Genetically modified mouse models, expressing higher or lower levels of miR135, demonstrated major alterations in anxiety- and depression-like behaviors, 5HT levels, and behavioral response to antidepressant treatment. Finally, miR135a levels in blood and brain of depressed human patients were significantly lower. The current results suggest a potential role for miR135 as an endogenous antidepressant and provide a venue for potential treatment and insights into the onset, susceptibility, and heterogeneity of stress-related psychopathologies.

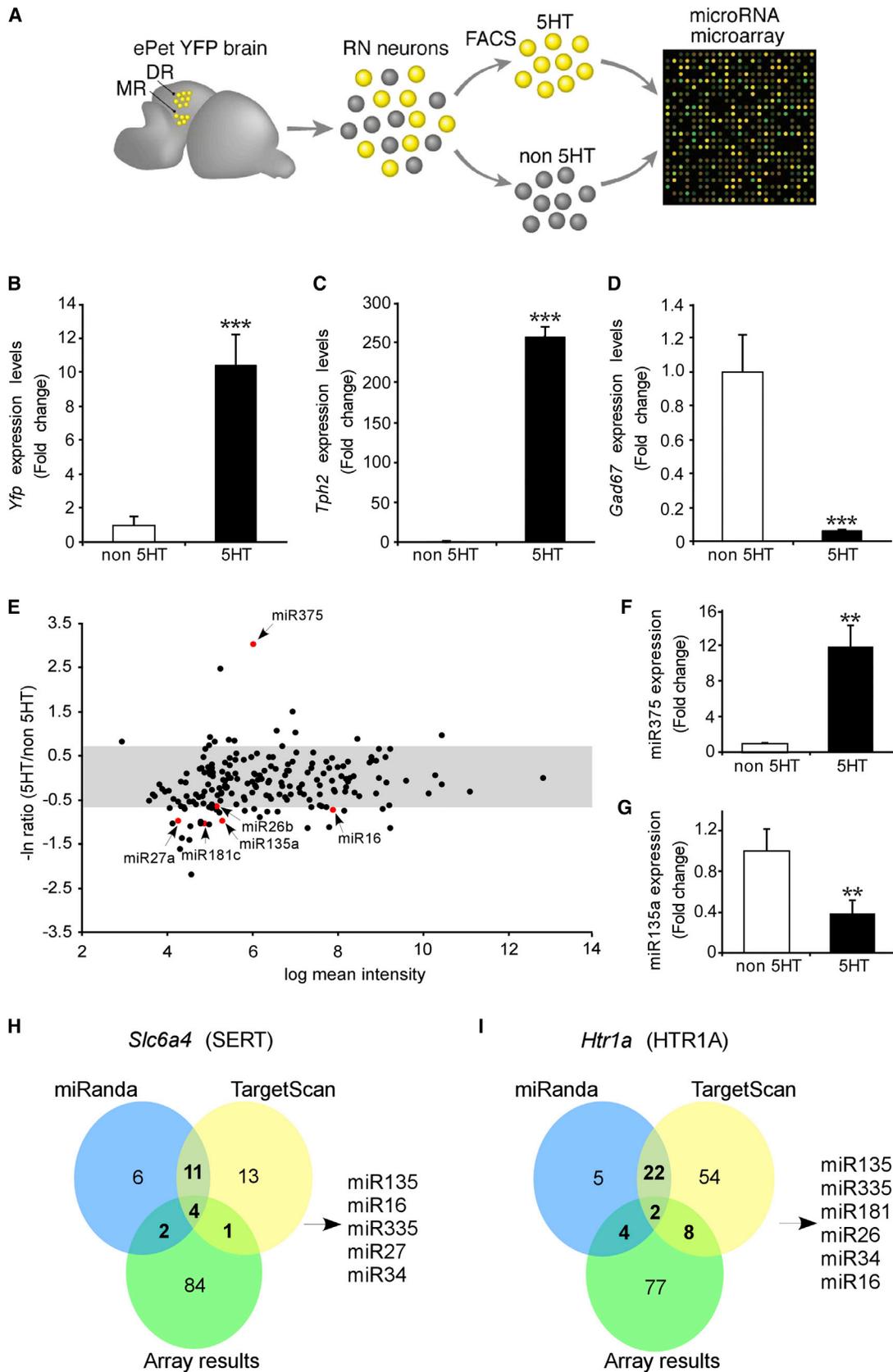
## INTRODUCTION

Mood disorders such as major depression are among the most common health problems worldwide, affecting approximately 10% of the population (Berton and Nestler, 2006; Manji et al., 2001) and are associated with a large global burden of disease, according to the World Health Organization (Ustün et al., 2004). Despite many decades of research, the molecular and cellular

mechanisms that underlie depression onset, susceptibility, and heterogeneity are only partially understood. The treatment efficacy of available antidepressant drugs is low, as 60%–70% of patients do not experience remission and 30%–40% do not show a significant response (Berton and Nestler, 2006; Manji et al., 2001). Furthermore, available antidepressants require long periods of administration before relief of symptoms is observed, and side effects are common and exhibit wide interindividual variability (Wang et al., 2005; Moncrieff and Kirsch, 2005; Masand, 2003). A better understanding of the etiology and pathophysiology of mood disorders is needed to facilitate development of novel and improved therapeutics.

The main current hypothesis regarding the etiology of depression and/or anxiety disorders is of a complex interaction between environmental factors and genetic predisposition, possibly suggesting a mechanistic role for epigenetic processes (Krishnan and Nestler, 2008). Strong comorbidity between anxiety disorders and depression is frequently reported (Müller and Wurst, 2004), and the available pharmacological treatments for both disorders are similar as well (Baldwin et al., 2005).

Serotonin (5HT), a monoamine neurotransmitter, is produced in the brain by neurons in the midbrain raphe nuclei (RN) that project extensively throughout the brain and modulate a variety of cognitive, emotional, and physiological functions. The link between dysregulated serotonergic activity and depression or anxiety disorders is well established and the levels of 5HT, as well as the cellular mechanisms responsible for its production, secretion, reuptake, and deactivation, were reported to be dysregulated in depression (reviewed in Meltzer and Maes, 1995; Albert and François, 2010; Ansorge et al., 2007; Schloss and Henn, 2004). Furthermore, most available antidepressant drugs target the function of 5HT system-related proteins, resulting in increased 5HT levels in the brain (Krishnan and Nestler, 2008). In-depth understanding of the molecular and cellular mechanisms regulating 5HT neuronal functions may contribute to the development of drugs that are more effective, with shorter functional onset and fewer side effects.



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MicroRNAs (miRs) are a subset of endogenous small (~22 nucleotide) noncoding RNA molecules that repress gene expression posttranscriptionally (Bartel, 2004; Friedman et al., 2009). Binding of a miR, primarily to the 3' UTR of mRNAs, leads to direct mRNA destabilization or translational repression, ultimately resulting in reduced protein levels of target genes (Bartel, 2004). Several miR-screening studies have reported that miR levels in various adult rodents or human brain structures are affected by a range of behavioral and pharmacological manipulations (for review, see O'Connor et al., 2012). The fine-tuning of gene expression by miRs can ultimately influence both normal and pathological behavior, offering an exciting new approach for studying control of different normal and pathological behaviors.

Here, we have set out to identify specific miRs that may regulate 5HT neuron-related genes and to dissect the role of such miRs in modulating depression- and anxiety-like behaviors under normal and challenged conditions. We determined the specific miR expression pattern of 5HT neurons and bioinformatically identified miRs that target, among others, two key transcripts in the serotonergic network: the 5HT transporter, *Slc6a4*, and the 5HT autoreceptor, *Htr1a*. Targeting predictions of these genes' 3' UTRs were further verified by *in vitro* assays and mutation studies. miR135 emerged as a prominent regulator of these key 5HT system transcripts and was demonstrated to be upregulated after antidepressant administration. Furthermore, overexpression or knockdown of miR135 in the RN of adult mice supported a role for miR135 as an endogenous antidepressant. Finally, we show data supporting a potential biomarker role for blood miR135a in human depression and treatment responsiveness.

## RESULTS

### microRNA "Fingerprint" of 5HT Neurons

5HT neurons were isolated from the rostral brain of ePet-EYFP mice on embryonic day 12 (E12), and their miR expression profile was compared to non-5HT cells, obtained from the same brain area, using miR microarray (Figure 1A). Cell-sorting validation was performed by comparing the mRNA expression levels of relevant marker genes. *Yfp*, the fluorescent marker for the ePET-positive neurons, was significantly enriched in the 5HT population (Figure 1B), as was tryptophan hydroxylase 2 (*Tph2*), a key enzyme in the production of 5HT (Figure 1C). Glutamate decarboxylase 67 (*Gad67*), the enzyme catalyzing synthesis of GABA, a common non-5HT neurotransmitter in the RN, was abundant

in the non-5HT cells (Figure 1D). The miR "fingerprint" obtained from the microarray (Figure 1E) contained 14 (Table S1 available online) and 27 (Table S2) miRs that were expressed 2-fold more or less, respectively, in 5HT neurons compared to the non-5HT neurons. Representative validation of the array results was performed using real-time PCR for miRs highly expressed in 5HT neurons such as miR375 (Figure 1F) and for miRs expressed at lower levels in 5HT neurons such as miR135a (Figure 1G).

In order to further study the potential role of miRs as modulators of 5HT neurons, extensive bioinformatics analyses were performed in a hypothesis-driven manner. Targeting prediction of known 5HT-related genes expressed in serotonergic neurons that have been previously demonstrated to be associated with psychopathologies were bioinformatically crossed with the microarray results. miR-targeting predictions were performed using two different web-based algorithms, Target Scan (<http://www.targetscan.org>) and MiRanda (<http://www.microrna.org>), and were crossed with the list of 91 miRs altered by at least  $\pm 1.5$ -fold in the 5HT neuron miR array, compared to non-5HT cells. Several protein-coding target genes expressed in 5HT neurons in the RN were selected, including tryptophan hydroxylase 2 (TPH2), the rate-limiting enzyme in 5HT production; monoamine oxidase A (MAOA), an enzyme that metabolizes 5HT the 5HT transporter (SERT), which is responsible for 5HT reuptake (the *Slc6a4* gene); and 5HT inhibitory receptor 1a, HTR1A (the *Htr1a* gene). Based on the miR array data and the bioinformatic analysis, the miR-targeting predictions for *Slc6a4* and *Htr1a* were most promising and chosen for further *in vitro* validation (Figures 1H and 1I).

### miR135 Targets *Htr1a* and *Slc6a4* Transcripts

*In vitro* luciferase assays were performed to test the miR-target interaction between the 3' UTR of the tested 5HT-related genes and the miRs predicted to putatively target these transcripts. miR135 targeting of *Slc6a4* 3' UTR (Figures 2A, 2C, and 2D) and *Htr1a* 3' UTR (Figures 2B, 2E, and 2F) resulted in 30%–50% repression of translation of these transcripts. Additionally, significant repression of *Htr1a* 3' UTR was mediated by miR335, miR181c, and miR26a (Figure 2B). Due to the 30%–50% repression of miR135 on both *Htr1a* and *Slc6a4*, we focused our studies on these miR-target interactions. Further bioinformatic analysis revealed that miR135 has three highly conserved variants: miR135a-1, miR135a-2, and miR135b (Figures S1A, S1B, and S1C). In addition, miR135 seed match sequences in the *Slc6a4* 3' UTR are highly conserved (Figure 2G),

### Figure 1. MicroRNA "Fingerprint" of 5HT Neurons

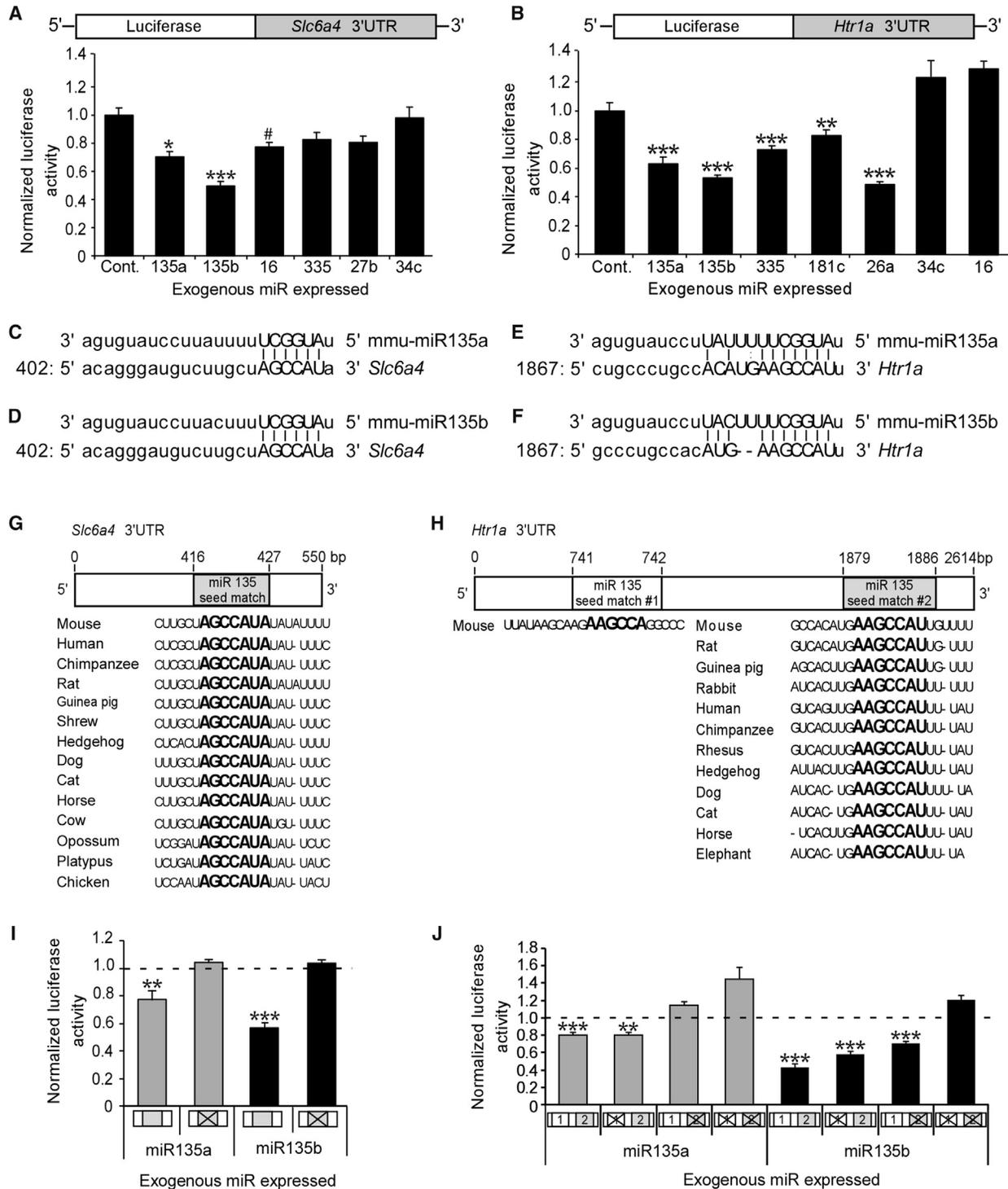
(A) Schematic illustration of the experimental design for determining 5HT neuronal microRNA "fingerprint." ePet-EYFP mouse embryo (E12) hindbrains were dissected and FACS sorted to 5HT-YFP-positive and -YFP-negative cells. miR expression from the two populations was compared using the Agilent microRNA microarray. RN, Raphe nucleus; DR, dorsal Raphe; MR, median Raphe.

(B–D) Validation of cell phenotype by real-time PCR indicating that *Yfp* (B) and *Tph2* (C) were significantly enriched in 5HT compared to non-5HT cells and that *Gad67* a GABAergic marker, was significantly higher in non-5HT cells (D).

(E) Graphic illustration of differentially expressed miRs in 5HT neurons. Lowest normalized values are depicted as  $\ln 2$ -fold change of spot intensity plotted against average log intensities (MA plot). The gray bar represents a fold change of 2.

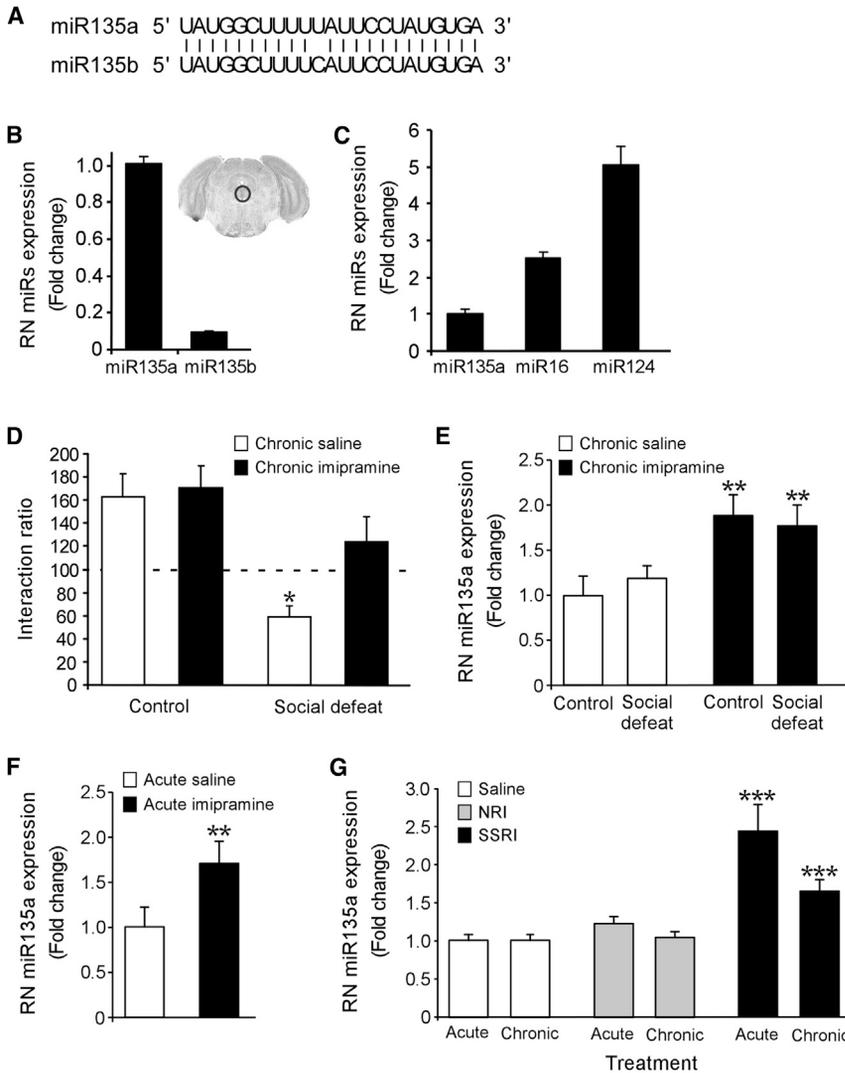
(F and G) Validation of array results by real-time PCR indicating increased levels of miR375 (F) and decreased levels of miR135a (G) in the 5HT neurons compared to non-5HT cells.

(H and I) Venn diagrams representing bioinformatics predictions for *Slc6a4* (H) and *Htr1a* (I) with 5HT microarray data and the miRs chosen for further *in vitro* testing. The numbers represent the amount of miRs predicted to target each gene by the different databases and in the array, individually and overlapping. Error bars represent mean  $\pm$  SEM. \*\*p < 0.01, \*\*\*p < 0.001.



**Figure 2. miR135 Targets *Htr1a* and *Slc6a4* In Vitro**

(A) Luciferase reporter assay results demonstrated that miR135a and miR135b target *Slc6a4* 3' UTR. A strong trend for targeting by miR16 was also observed. (B) Luciferase reporter assay results indicating miR135a, miR135b, miR335, miR181c, and miR26a target *Htr1a* 3' UTR. (C–F) Nucleotide base pairing of miR135a (C) and miR135b (D) with *Slc6a4* 3' UTR, and of miR135a (E) and miR135b (F) with *Htr1a* 3' UTR. (G and H) *Slc6a4* (G) and *Htr1a* (H) 3' UTRs conservation of miR135 seed match. (I) Mutation in miR135 seed match in *Slc6a4* 3' UTR blocked the inhibitory effect of miR135a and miR135b. (J) Mutation in miR135 seed matches in *Htr1a* 3' UTR, individually or both, indicate that miR135b targets *Htr1a* via both seed matches, while miR135a targets *Htr1a* only by seed match number 2. Error bars represent means  $\pm$  SEM. #p < 0.07, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



**Figure 3. miR135a Is Upregulated in Adult Mouse RN after Antidepressant Treatment**

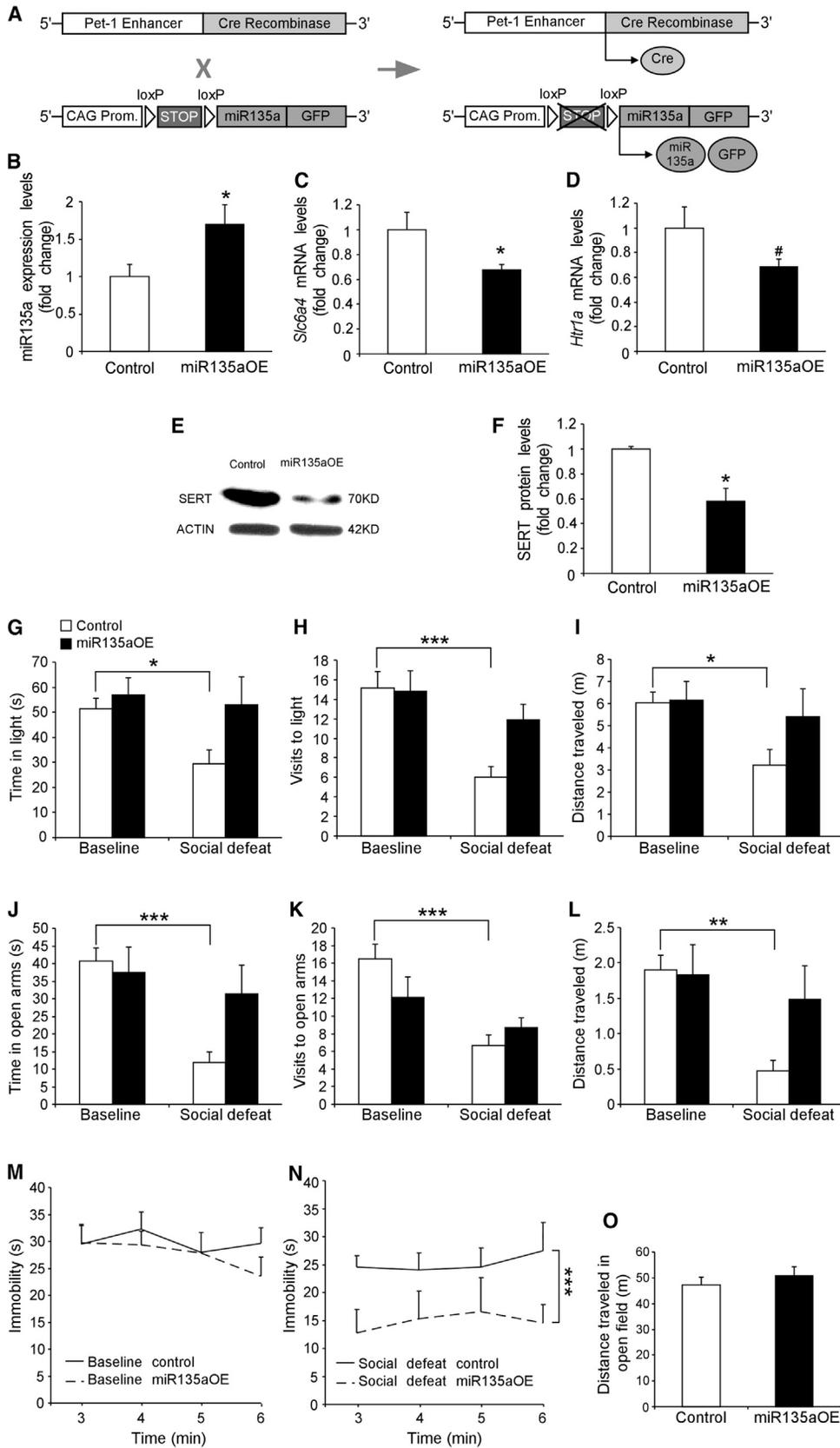
(A) Alignment between mature miR135a and miR135b indicating one nucleotide difference. (B) Expression levels of miR135a and miR135b in the mouse RN demonstrating 10-fold higher levels of miR135a (n = 8). (C) Expression profile of several miRs in adult mouse RN indicating miR135a was approximately five times less abundant than miR124 and 2.5-fold less than miR16 (n = 8). (D) Mice exposed to social defeat demonstrated increased social avoidance unless treated with chronic imipramine. Interaction ratio was calculated as time spent in the zone near the nonfamiliar mouse divided by the time spent in the same zone during habitation multiplied by 100 (n = 8–11 in each group). (E and F) miR135a levels were upregulated in the RN after chronic (E) or acute (F) imipramine administration and were unchanged after exposure to chronic social defeat protocol (n = 8–11 in each group). (G) SSRI and not NRI, after either acute or chronic administration, caused a significant increase in miR135a levels in the RN (n = 7–8 in each group). Error bars represent means ± SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

and in one out of the two seed matches in the *Htr1a* 3' UTR, strong conservation was observed (Figure 2H). Mutation studies on the 3' UTR of the *Slc6a4* transcript, in which the miR135 seed match sequence was removed, revealed that both miR135a and miR135b targeting of *Slc6a4* was mediated via its seed match sequence (Figure 2I). Mutating the *Htr1a* miR135 seed matches, individually or together, revealed that miR135a repressed *Htr1a* 3' UTR via the distal and not the proximal seed match, while miR135b acts via both predicted sites (Figure 2J).

### RN-miR135a Levels Are Upregulated by Antidepressants

Since both HTR1A (Savitz et al., 2009) and SERT (Murphy et al., 2008) are associated with depression and antidepressant cellular machinery, we sought to examine the regulation of miR135a expression in response to antidepressant treatment. The mature miR135a and miR135b differ by only one nucleotide (Figure 3A), yet were differentially expressed in the RN, as observed in the real-time PCR results conducted on cDNA

higher levels in the RN than miR135b and was also the variant differently altered in the 5HT microarray, we focused our regulation studies on this form. miR135a was expressed also in different brain nuclei, with relatively high levels in the RN, the hypothalamus, prefrontal cortex, septum, and ventral hippocampus (Figure S2). Future studies should assess the role of miR135 endogenously expressed in other brains regions associated with depression and anxiety such as the hippocampus and prefrontal cortex. Next, we tested the levels of miR135a in mice exposed to chronic social defeat, an environmental model used for the induction of anxiety- and depression-like behaviors (Krishnan et al., 2007) and to chronic treatment with the tricyclic antidepressant imipramine. Using the social avoidance test, we verified that social defeat can cause social avoidance and antidepressant administration can reverse this, as reported previously (Berton et al., 2006) (Figure 3D). Indeed, only mice exposed to social defeat and injected with saline and not those that received imipramine developed social avoidance, as implied by an interaction ratio lower than 100% (Figure 3D). Interestingly, chronic social defeat stress did not alter miR135a levels in the



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RN; however, imipramine administered chronically (Figure 3E) or acutely (Figure 3F), both in stressed and nonstressed mice, significantly increased miR135a expression levels in the RN. Since imipramine is not a specific 5HT reuptake inhibitor, we further tested the effects of both acute and chronic administration of the selective serotonin reuptake inhibitor (SSRI), fluoxetine, and the noradrenaline reuptake inhibitor (NRI), reboxetine, and found an increase in miR135a levels in the RN after both acute and chronic SSRI treatment, yet no differences were observed after the NRI treatment (Figure 3G). The reduction in SERT and HTR1A levels after antidepressant administration was described elaborately in the literature and therefore was not tested in this study (SERT reviewed in [Schloss and Henn, 2004] and HTR1A reviewed in [Albert and François, 2010]).

### miR135a Overexpression Specifically in 5HT Neurons Reduces Anxiety- and Depression-like Behaviors after Social Defeat

To further explore the role of 5HT-miR135 *in vivo*, we established a mouse model that specifically overexpresses miR135a in 5HT neurons of the RN (miR135a OE). Mice expressing Cre recombinase specifically in the RN 5HT-positive neurons (ePet-Cre) (Scott et al., 2005) were crossbred with a transgenic mouse line carrying a conditional miR135a cassette (Anderegg et al., 2013) (Figure 4A). As controls, we used mice positive for the miR135a overexpression transgene and negative for the ePet-Cre. miR135a expression level in the RN of miR135a OE mice was tested by real-time PCR and was demonstrated to be overexpressed by approximately 2-fold compared to control mice (Figure 4B). Overexpression levels of miR135a in this mouse model were similar to those measured in the RN of mice after SSRI administration. Additionally, the levels of miR135 target genes SERT (Figures 4C, 4E, and 4F), and to a lesser extent HTR1A (Figure 4D) were reduced in the RN of miR135a OE mice compared to control mice, demonstrating *in vivo* repression of miR135 target genes.

The miR135a OE and their littermate controls were behaviorally characterized in tests for anxiety- and depression-like behaviors, under “baseline” conditions or after chronic social defeat protocol (different groups of mice). Under “baseline”

conditions, no differences were observed between miR135a OE and control mice in tests for anxiety- and depression-like behaviors (Figures 4G–4L, left bars, and Figure 4M). However, miR135a OE mice demonstrated significant resiliency to the effects of chronic social defeat. In the dark-light transfer test, miR135a OE mice exposed to social defeat spent more time in light (Figure 4G), visited the lit compartment more frequently (Figure 4H), and traveled longer distance in light (Figure 4I) relative to control mice. The behavioral performance of the miR135a OE mice did not significantly differ after the social defeat protocol. In contrast, control mice demonstrated significant increases in anxiety-like behaviors in all measured parameters of the dark-light test after social defeat (Figures 4G–4I). Similar results were observed in the elevated plus-maze test, as control mice that were exposed to social defeat spent less time (Figure 4J), had fewer visits (Figure 4K), and traveled less distance (Figure 4L) in the open arms compared to miR135a OE mice tested under the same conditions. No significant differences between “baseline” and stress conditions were observed in the miR135a OE group. Similar results were observed in tests assessing depression-like behaviors. While no differences were observed under “baseline” conditions (Figure 4M), when tested after chronic social defeat, miR135a OE mice exhibited significantly less immobility time in the forced swim test compared to controls (Figure 4N), which is interpreted as decreased depression-like behavior or increased coping-like behavior. Interestingly, control mice tested after social defeat showed immobility time similar to those of mice tested under “baseline” stress conditions, a phenomenon reported by others (Krishnan et al., 2007). These differences could not account for changes in locomotor activity, since the distance traveled in the open field was similar in both genotypes (Figure 4O). Taken together, overexpression of miR135a specifically in 5HT neurons protected against the adverse effect of chronic stress on anxiety- and depression-like behaviors.

### Knockdown of miR135 in Adult Mice RN Increased Anxiety-like Behaviors and Decreased the Response to Antidepressants

To determine the importance of miR135 endogenous levels in mediating anxiety- and depression-like behaviors and response to antidepressant treatment, we established a lentiviral-based

#### Figure 4. Overexpression of miR135a Specifically in 5HT Neurons Cause Behavioral Resiliency to Social Defeat

(A) Schematic illustration of miR135a conditional overexpression mouse model. Transgenic mice with floxed transcriptional STOP sequence upstream to miR135a and GFP sequences were crossed with the ePet-Cre recombinase mice. Double transgenic mice overexpress miR135a specifically in 5HT-positive neurons. Littermate mice carrying only the transgene for miR135a served as controls.

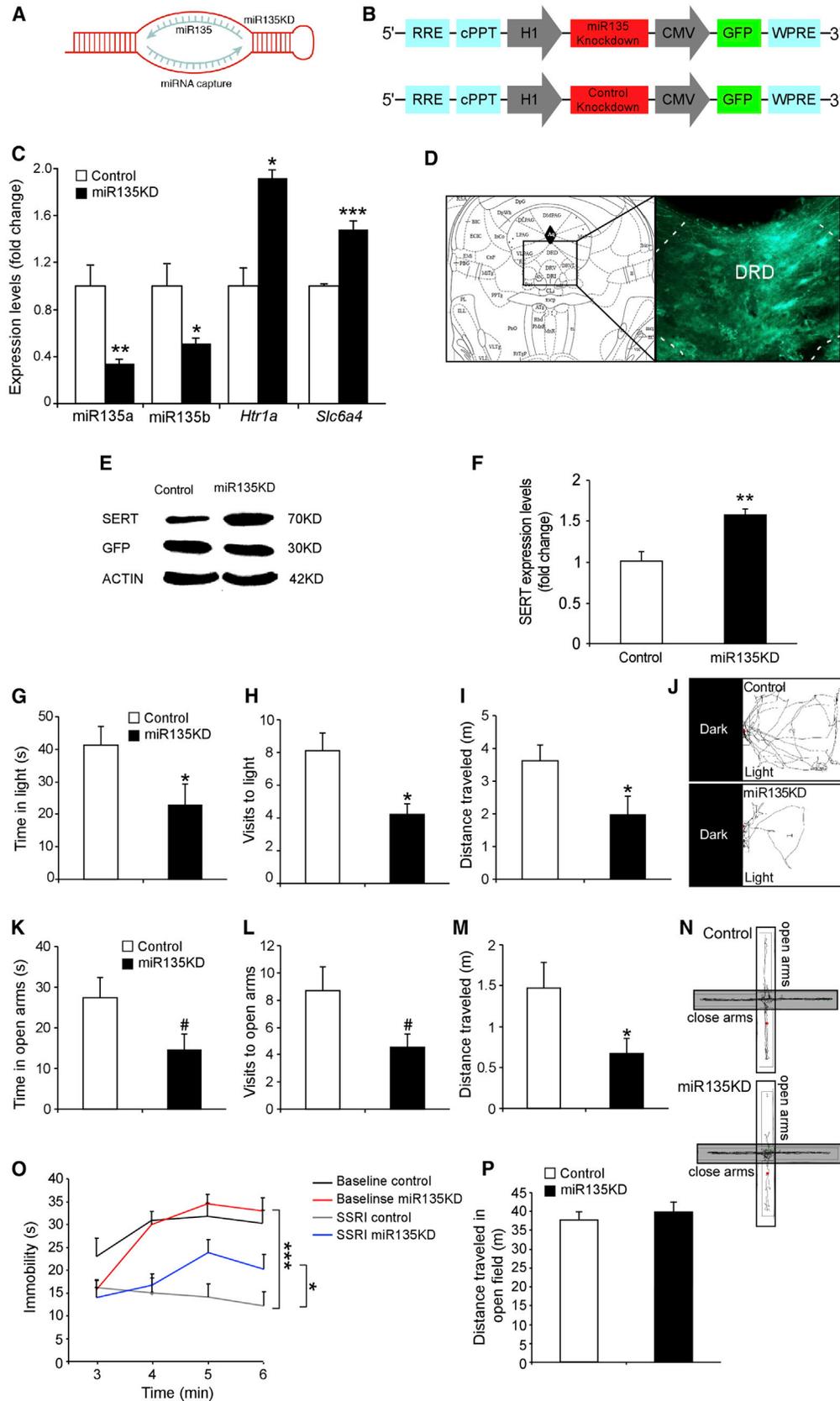
(B) miR135a expression levels in the RN were upregulated by approximately 2-fold in miR135a overexpressing (OE) mice compared to controls.

(C–F) miR135 target genes, SERT mRNA (C) and protein (E and F) and HTR1A mRNA (D) were downregulated in miR135a OE mice compared to control littermates ( $n = 7–11$  for mRNA,  $n = 3–4$  for SERT protein).

(G–I) In the dark-light transfer test, no differences were observed between miR135a OE mice and their control littermates under “baseline” conditions; however, after chronic social defeat, control mice demonstrated increased anxiety-like behavior and spent less time in light (G), visited the lit compartment less frequently (H), and traveled a shorter distance in light (I). The behavioral performance of the miR135a OE mice did not significantly differ following the social defeat protocol. (J–L) Similarly, in the elevated plus-maze test, control mice that were exposed to social defeat spent less time (J), had a smaller number of visits (K), and traveled less distance (L) in the open arms, compared to control mice tested under “baseline” conditions. No significant differences between “baseline” and stress conditions were observed in the miR135a OE group.

(M and N) In the forced swim test, no significant differences were observed between the groups when tested under “baseline” conditions (M); however, when tested after chronic social defeat, miR135a OE mice demonstrated decreased immobility compared to control littermates (N).

(O) No differences in locomotion activity were observed between the miR135a OE and control littermates as measured by total distance traveled in the open-field test ( $n = 7–11$  in each group in behavioral data). Error bars and line graphs represent means  $\pm$  SEM. #  $p \leq 0.07$ ; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



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system to specifically knock down (KD) endogenous miR135 in the RN of wild-type mice.

Expression plasmid containing a miR135 inhibitor (miRNA capture; Figure 5A) or control sequence were subcloned into a lentiviral construct containing the H1 promoter and the GFP reporter (Figure 5B) to allow constitutive expression of the miR135 capture sequence. The efficiency of expression plasmid containing a miR135 inhibitor to suppress the target gene was tested in vitro using luciferase assay. A miR135 inhibitor expression plasmid was cotransfected into HEK293 cells with a miR135 precursor expression plasmid and a miRNA target sequence expressing *Slc6a4* 3' UTR (Figure S3A) or *Htr1a* 3' UTR (Figure S3B) downstream to a luciferase sequence. The result showed that miR135 suppressed the luciferase activity from the *Slc6a4* 3' UTR (Figure S3B) and the *Htr1a* 3' UTR (Figure S3B), and this suppression effect was blocked by the introduction of the inhibitor against miR135. Additionally, the efficiency of the lentiviruses, produced from these constructs, to suppress the expression of the target genes *Htr1a* and *Slc6a4* was tested in vitro by infecting RN46A cells, which endogenously express *Htr1a*, *Slc6a4*, and miR135. RN46A cells infected with miR135 KD lentiviruses expressed significantly lower levels of both miR135a and miR135b and higher levels of *Htr1a* and *Slc6a4* mRNA, as tested by real-time PCR, compared to cells infected by the KD control lentiviruses (Figure 5C).

Wild-type adult mice RN were infected with either miR135 KD or control lentiviruses. Infection accuracy was subsequently verified using GFP immunohistochemistry (Figure 5D). Furthermore, miR135 KD lentivirus infection led to increased SERT protein levels in vivo (Figures 5E and 5F), as measured by western blot analysis performed on RN tissue. The accuracy of injection site and of the microdissection was confirmed by immunoblotting for GFP coexpressed by the lentiviruses for miR135 KD (Figure 5E).

After a recovery period, mice injected with the miR135 KD viruses were assessed behaviorally using tests for anxiety- and depression-like behaviors. In the dark-light transfer test, the miR135 KD mice demonstrated a significant increase in anxiety-like behavior compared to control-injected mice (Figures 5G–5J). The miR135 KD mice spent less time (Figure 5G), visited less (Figure 5H), and walked shorter distances in the lit compart-

ment (Figures 5I and 5J). Similarly, in the elevated plus-maze test, the miR135 KD mice demonstrated increased anxiety-like behaviors compared to control-injected mice. The miR135 KD mice showed a tendency to spend less time (Figure 5K), visit less (Figure 5L), and travel significantly less distance (Figures 5M and 5N) in the open arms of the maze (Figures 5M and 5N).

Depression-like behaviors of the miR135 KD mice were tested both under “baseline” conditions and after SSRI treatment. In the forced swim test, no differences were observed between the groups under “baseline” conditions (Figure 5O). However, after SSRI administration to the same group of mice, miR135 KD mice were significantly more immobile compared to control-injected mice (Figure 5O), suggesting an important role for endogenous RN-miR135 levels in mediating SSRI-induced antidepressant effects. The incomplete block of miR135 KD of the antidepressant effect could be explained by the partial reduction in miR135 levels (knockdown but not knockout). Reduced levels of miR135 in the RN did not affect the locomotor activity of these mice as measured by total distance traveled in the open-field test (Figure 5P).

#### miR135a Overexpression Altered 5HT Levels and Metabolism

To evaluate whether changes in miR135a levels are also reflected in the tissue concentrations of central 5HT and its turnover, we microdissected the RN subdivisions and the brain regions innervated by these areas from the miR135a OE mouse model and control littermates. Figures 6, S4, and S5 depict tissue concentrations of 5HT and the 5HT metabolism (5HIAA/5HT ratio) in these mice under “baseline” conditions and after the social defeat protocol.

Tissue concentrations of 5HT and the 5HT metabolism within an anxiety- and depression-related neural circuit were influenced by the miR135a genotype, as well as the social defeat manipulation. miR135a OE mice had decreased tissue 5HT concentrations and increased 5HT metabolism, a pattern consistent with increased 5HT turnover, in brain regions implicated in regulation of anxiety-related behavior (Gardner et al., 2005; Hale and Lowry, 2011; Lowry, 2002) and stress resilience (Deakin and Graeff, 1991; Graeff et al., 1996), such as the prelimbic cortex (PrL), infralimbic cortex (IL), basolateral amygdala (BLA), CA1

#### Figure 5. Knockdown of miR135 in the RN of Adult Mice Caused Increased Anxiety-like Behavior and Attenuated Response to Antidepressants

(A) Schematic illustration of “miR135 capture” structure.

(B) Schematic illustration of miR135 KD and control viral vectors.

(C) miR135 KD lentivirus infection decreased miR135a and miR135b levels and increased *Htr1a* and *Slc6a4* mRNA expression levels in RN46a cells that endogenously express these genes.

(D) Brain section map showing the site of injection, adapted from the mouse brain atlas (Paxinos and Franklin, 1997) (left) and GFP immunostaining in DRD of adult mice infected with miR135 KD lentiviruses (right).

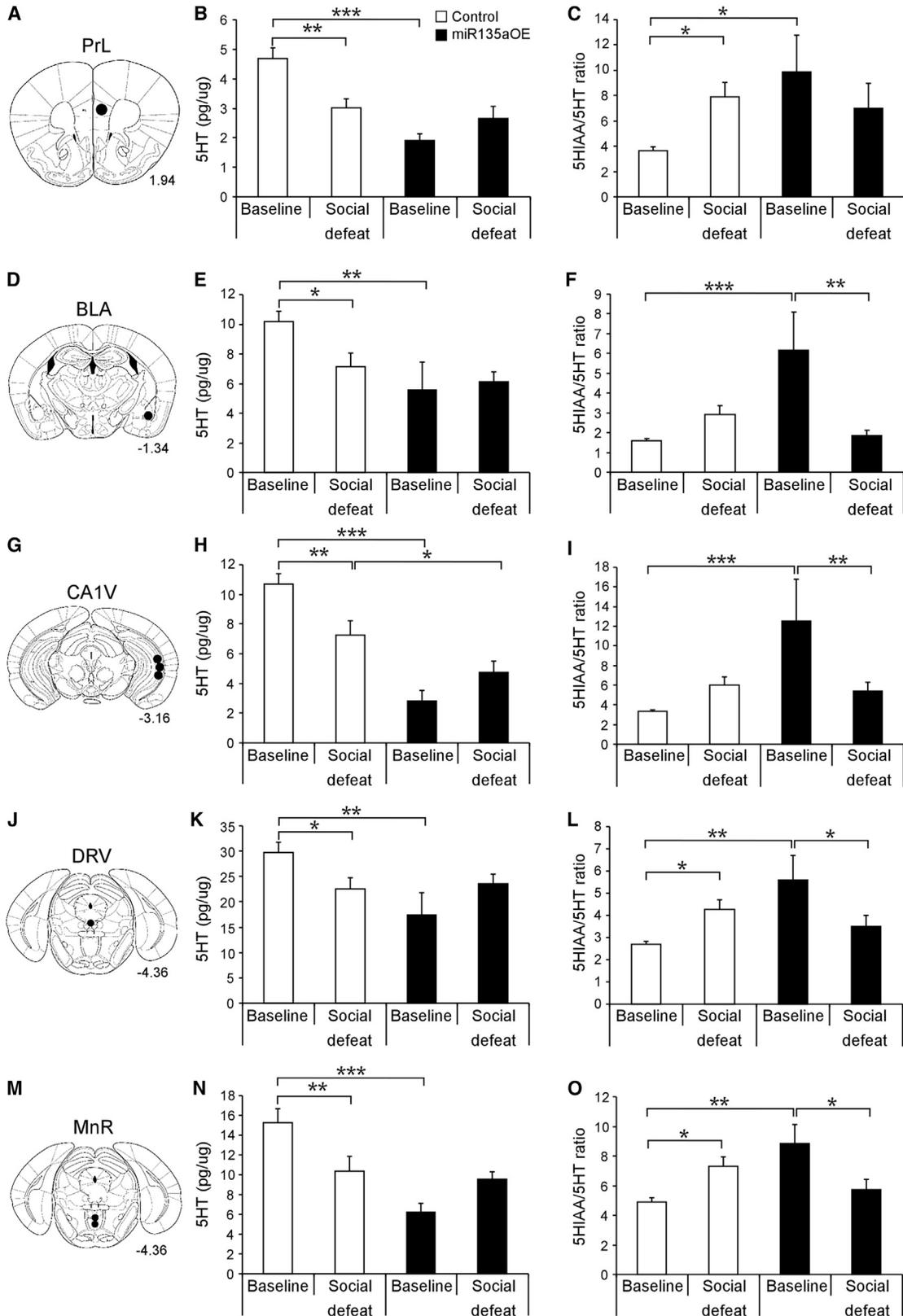
(E and F) miR135 KD in the RN of adult mice lead to an increase in SERT protein (E and F) as measured in GFP-positive samples (E) (n = 4–5 in each group).

(G–J) In the dark light transfer test, miR135 KD mice spent less time (G), had fewer visits (H), and traveled less distance (I and J) in the light compartment, compared to control injected mice.

(K–N) In the elevated plus-maze test, miR135 KD mice demonstrated a tendency to spend less time (K), had fewer visits (L), and travel significantly less distance (M and N) in the open arms.

(O) In the forced swim test, miR135 KD mice did not differ in their immobility time from control mice when tested under “baseline” conditions; however, when tested 30 min after SSRI administration, miR135 KD mice demonstrated increased immobility time, indicating attenuated response to antidepressants.

(P) No significant differences in locomotor activity between miR135 KD and control mice were observed as measured by total distance traveled in the open-field test (n = 10–11 in each group in behavioral data). Error bars represent means ± SEM. #p < 0.07, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



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region of the ventral hippocampus (CA1V), subiculum (S), bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), dorsal, ventral, caudal, and interfascicular parts of the dorsal raphe nucleus (DRD, DRV, DRC, DRI), and median raphe nucleus (MnR) (Figures 6, S4, and S5). These results are in line with the decrease of *Htr1a* and *Slc6a4* expression in miR135a OE mice (Figure 4) under “baseline” conditions, effects that would be expected to result in increased serotonergic neuronal firing rates and serotonergic signaling, respectively. Social defeat decreased tissue 5HT concentrations and increased 5HT metabolism in anxiety-related brain regions in control mice, a pattern consistent with increased 5HT turnover, including the PrL and BNST (Figures 6 and S5), persistent with previous studies demonstrating social defeat-induced activation of anxiety-related subsets of serotonergic neurons in the DRD and DRC (Gardner et al., 2005). These effects of social defeat were prevented in miR135a OE mice, suggesting a mechanistic explanation for the behavioral resiliency to chronic stress observed in these mice (Figure 4).

#### miR135a Levels in the Blood and Brain Are Downregulated in Depressed Patients

Since circulating miR levels were shown to correlate with disease states (Reid et al., 2011), we tested whether blood miR135a levels are altered in depressed human patients. Relative levels of miR135a and miR16 were tested in two sets of human blood samples, in which their clinical manifestations and response to treatment were previously reported. The first compared depressed patients to matched healthy controls (detailed description in Menke et al., 2012) and the other measured changes in miRs levels over time within depressed patients receiving either 3 months of SSRI treatment or cognitive behavioral therapy (CBT) (detailed description in Dunlop et al., 2012). miR135a levels were robustly reduced in currently depressed patients (mean Hamilton Depression Rating Scale [HRDDS] = 24.3 [SD: 5.3], i.e., with moderate-to-severe depressive symptoms) compared to controls (Figure 7A), while a nonsignificant reduction in miR16 levels was observed (Figure 7B). Comparing miR135a blood levels in depressed patients before and after 3 months of SSRI treatment or CBT revealed a significant increase in miR135a levels after CBT (Figure 7C). No effect was observed in the same blood samples for miR16 levels (Figure 7D). These results suggest miR135a levels in human blood as a possible biomarker for depression state and response to treatment, yet further studies with larger patient cohorts are needed.

Moreover, in order to test whether miR135 is dysregulated in the depressed human brain, we tested miR135 levels in different subnuclei of the raphe in postmortem tissue obtained from depressed suicide victims and controls described in detail in Merali et al. (2006). miR135a and miR16 levels were tested in five subnuclei: the dorsal raphe (RN), raphe magnus (RM), pontine raphe, median raphe, and the medullary raphe (raphe pallidus and obscurus combined). Differences were detected only in the RN (Figures 7E and 7F) and the RM (Figures 7G and 7H), where miR135a (Figures 7E and 7G) and miR16 (Figures 7G and 7H) were significantly lower in suicide victims compared to controls. These results suggest that miR135 might play a role also in the raphe of the human brain in depression.

#### DISCUSSION

In the current study, we elucidated the role of a specific microRNA in regulating central 5HT system activity, under “baseline” and challenged conditions. We determined the unique “fingerprint” of miRs expression in serotonergic neurons and bioinformatically identified several 5HT-linked target genes. In vitro luciferase assays and mutation studies revealed a strong repressive effect for miR135 on both *Slc6a4* and *Htr1a* transcripts. Intriguingly, miR135a levels in the RN were robustly upregulated after acute or chronic SSRI administration. Genetically modified mouse models, expressing higher or lower levels of miR135, demonstrated major alternations in anxiety- and depression-like behaviors, 5HT levels and metabolism, and behavioral response to antidepressant treatment. Finally, miR135a levels in the blood and brain of depressed human patients and response to treatment were presented.

The use of the ePet-EYFP mouse model for the isolation of 5HT and non-5HT cells from the mouse RN allowed us to determine the specific miRs profile of serotonergic neurons. This approach was successful and informative, yet in order to efficiently sort the 5HT-positive neurons from the mouse RN, we used embryonic and not adult brain tissue. Therefore, part of the miRs presented in the 5HT miRs profile may be relevant to developmental processes and not adult 5HT neuronal functions. Interestingly, miR375, commonly associated with pancreatic beta cell differentiation, was robustly expressed in 5HT neurons compared to non-5HT cells, supporting the suggested common developmental path of these tissues (Baroukh and Van Obberghen, 2009; Ohta et al., 2011).

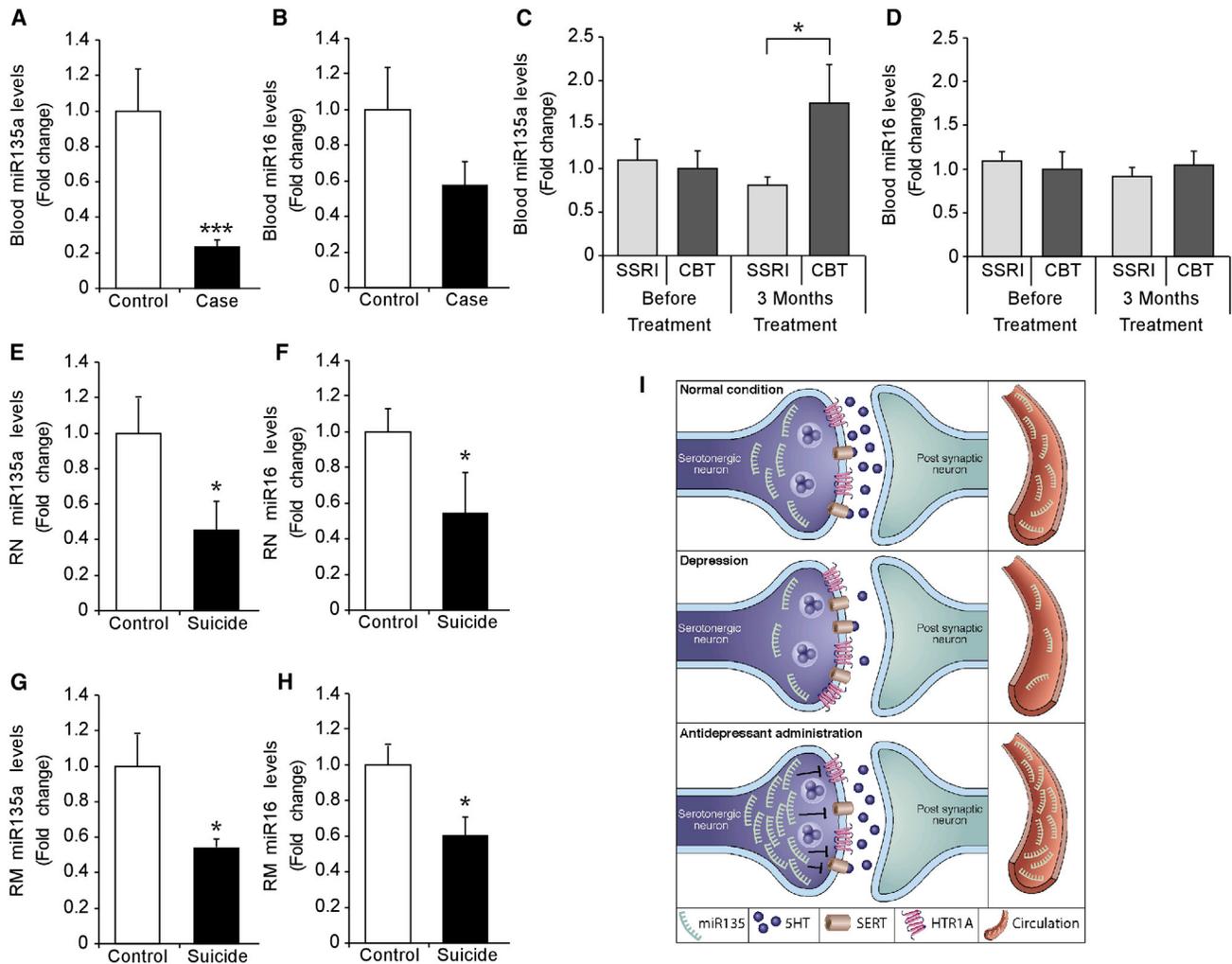
Bioinformatic analysis suggested several putative miR-target interactions between the *Htr1a* and *Slc6a4* 3' UTRs and miRs

#### Figure 6. Overexpression of miR135a in 5HT Neurons Alters 5HT Levels and Metabolism across the Brain and Blocks Social Defeat-Induced 5HT Reduction

(A, D, G, J, and M) Schematic illustration of microdissection sites for miR135a OE and control mouse brains under baseline conditions or after chronic social defeat. (PrL, prelimbic cortex; BLA, basolateral amygdala; CA1V, hippocampus CA1 ventral; DRV, dorsal raphe nucleus, ventral part; MnR, median raphe nucleus. Section map adopted from Paxinos and Franklin [1997].)

(B, E, H, K, and N) 5HT levels as measured by HPLC in different brain sites revealed decreased 5HT levels in miR135a OE mice compared to controls in baseline stress conditions. Additionally, 5HT levels were downregulated in control mice exposed to social defeat compared to baseline stress conditions, an effect not observed in miR135a OE 5HT mice.

(C, F, I, L, and O) 5HT metabolism calculated as the ratio between the levels of the metabolite 5HIAA to 5HT levels was upregulated in miR135a OE mice compared to controls in baseline stress conditions. Furthermore, 5HT metabolism was reduced in the BLA, CA1V, DRV, and MnR in miR135a OE 5HT exposed to chronic social defeat compared to control mice from the same genotype. In the PrL, DRV and MnR 5HT metabolism was upregulated in control mice exposed to social defeat compared to baseline conditions (n = 5–11 in each group). Error bars represent means ± SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



**Figure 7. Lower Levels of miR135a in the Blood and Brain of Human Depressed Patients**

(A) miR135a levels in total blood of depressed human patients were reduced compared to those of healthy controls. (B) miR16 blood levels did not differ significantly between depressed to controls ( $n = 11-12$  in each group). (C) Depressed patients treated for 3 months with cognitive behavioral therapy (CBT) showed a significant increase in total blood miR135a levels compared to patients receiving SSRI medication. (D) miR16 levels were similar in both time points in all groups of patients ( $n = 11$  in each group). (E–H) miR135a (E and G) and miR16 (F and H) in the dorsal raphe RN (E and F) and raphe magnus RM (G and H) were lower in suicide victims compared to controls ( $n = 4-9$  in RN and  $n = 6-11$  in RM in each group). (I) Schematic representation of a suggested model describing the involvement of miR135 in regulating serotonergic synapse components under normal conditions (top), depression (middle), and following antidepressant administration (bottom). Error bars represent means  $\pm$  SEM. \* $p < 0.05$ , \*\*\* $p < 0.001$ .

differentially expressed in the 5HT microarray. HTR1A and SERT have been shown to play a major role in the serotonergic system function, in depression and anxiety disorders, and in the response to antidepressants (reviewed by Murphy et al., 2008; Savitz et al., 2009). HTR1A is an inhibitory G protein-coupled receptor that is expressed as an autoreceptor on 5HT-producing cells and postsynaptically across the brain of 5HT projection sites. Stimulation of HTR1A autoreceptors inhibits serotonergic neuronal firing and the release of serotonin and has been postulated to be one of the causes for the therapeutic lag that is commonly reported for most serotonergic antidepressants such as SSRIs (Savitz et al., 2009). SERT is a plasma membrane

transporter that terminates 5HT action by recycling it from the synaptic cleft into presynaptic neurons, in a sodium-dependent manner (Homberg and Lesch, 2011). SERT is the direct target of most commonly used antidepressants, either the former generation of tricyclic antidepressants that inhibit different monoamine reuptake transporter activities including SERT or the more specific SSRIs (Schloss and Williams, 1998). Decreased activity of both SERT and the presynaptic HTR1A would be expected to increase 5HT levels in the brain, which are consistent with antidepressant action and decreases in depressive symptoms (reviewed in Albert and François, 2010; Ansorge et al., 2007; Meltzer and Maes, 1995; Schloss and Henn, 2004).

Luciferase assays confirmed miR135 variants as significant repressors of both *Slc6a4* and *Htr1a* transcripts. Mutation studies further demonstrated the importance of miR135 seed binding sites in the *Htr1a* and *Slc6a4* 3' UTRs in mediating the observed miR135 repressive effects. SNPs in the 3' UTR of human *Slc6a4* and *Htr1a*, previously reported for these genes (Piva et al., 2010), are not within miR135 seed match sequences.

Several microRNA screening studies have reported that microRNA levels in various adult rodent or human brain structures are affected by a range of behavioral and pharmacological manipulations (Kocerha et al., 2009; Kye et al., 2011). Stressful challenges were shown to alter miR expression in different brain sites using different paradigms (Rinaldi et al., 2010; Smalheiser et al., 2011; Uchida et al., 2008). We previously demonstrated the involvement of miR34 in the regulation of anxiety-like behaviors (Haramati et al., 2011), while miR-22, miR-138-2, miR-148a, and miR-488 were associated with panic disorder (Muiños-Gimeno et al., 2011). Studies using mice, presented in the current manuscript, revealed a clear upregulation of miR135a after antidepressant administration. Further comparison of SSRI and NRI antidepressants demonstrated an SSRI- but not NRI-specific effect, further suggesting a role for miR135a in the biology of 5HT neurons. While chronic stress is associated with increased susceptibility to the development of depression, surprisingly, chronic stress conditions did not affect miR135a levels in the RN. miR16 was shown to target *Slc6a4* and to have a role in antidepressant response (Baudry et al., 2010), while lithium administration was shown to alter miRs expression (Creson et al., 2011; Zhou et al., 2009). An association was found between variants in miR182 (Saus et al., 2010) and miR30e (Xu et al., 2010) in major depression patients, and miRs expression was altered in the prefrontal cortex of patients with suicidal depression (Smalheiser et al., 2012). Additionally, a polymorphism in serotonin receptor 1B moderates the regulation by miR96 and associates with aggressive behaviors (Jensen et al., 2009).

To further support a role for miR135 as an endogenous antidepressant, we conducted a series of experiments in which we manipulated miR135 levels in vivo and assessed the effects on animal behavior. The transgenic mouse model that overexpresses miR135a specifically in 5HT neurons, in levels equivalent to those observed after antidepressant treatment, showed a strong protective effect from the adverse behavioral effects of chronic social defeat. These results resembled the effect observed when HTR1A (Bortolozzi et al., 2012) or SERT (Thakker et al., 2005) were knocked down using siRNA approaches, showing reduced depression-like behaviors. In contrast, the developmental knockout mouse models for HTR1A (reviewed in Savitz et al., 2009) and SERT (reviewed in Holmes et al., 2003) showed paradoxical increases in anxiety- and depression-like behaviors, which were suggested to be mediated by developmental compensatory changes. In addition to HTR1A and SERT, it is bioinformatically apparent that miR135a may affect other transcripts that are associated with the serotonergic system functions, which possibly contribute to the observed phenotypes. Among others, the predicted miR135 targets include the following 5HT and affective disorder-related genes: inositol monophosphatase (IMPA1), glycogen synthase kinase-3beta (GSK3B), ankyrin 3 (ANK3), glutamate receptor,

ionotropic, AMPA3 (GRIA3), potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 (KCNN3), and nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP) (see Table S6 for additional putative miR135 target genes associated with stress, the 5HT system, and neuropsychiatric disorders).

Using a complementary approach, we knocked down the levels of miR135 specifically in the RN of adult wild-type mice using lentiviruses and assessed the effects on mouse behavior. In contrast to the behaviors observed by the mice that overexpress miR135a, reduced levels of this miR caused a robust increase in anxiety-like behavior and an attenuated response to antidepressants. These results support an important role for basal miR135 endogenous levels in maintaining intact responses to challenge under "baseline" conditions and its essential role in the mechanism of antidepressant action (Richardson-Jones et al., 2010). These findings are in agreement with studies describing a polymorphism in the human *Htr1a* gene that was associated with higher HTR1A autoreceptor binding and increased anxiety and depression (Fakra et al., 2009). In contrast, lower expression levels of SERT, due to a shorter promoter variant, were reported to be associated with increased anxiety and depression and reduced responses to antidepressants (Homberg and Lesch, 2011).

Further support for a role of miR135 in 5HT circuits emerged from the HPLC data indicating a robust alteration in 5HT levels and its metabolism across the brain of the miR135a OE mice. 5HT levels were lower, while 5HT metabolism was higher, in the miR135a OE mice compared to controls under "baseline" stress conditions both in subnuclei of the raphe where 5HT is synthesized and in the projection sites important for controlling anxiety- and depression-like behaviors. This pattern of change in 5HT levels and 5HT metabolism is consistent with increased serotonergic neuronal firing and increased serotonergic signaling in miR135a OE mice. These differences could be a result of compensatory changes associated with the overexpression of miR135a from development through adulthood. However, despite the low "baseline" 5HT levels, the mice demonstrate normal behaviors under "baseline" conditions, probably due to a more active 5HT system, as can be depicted by their higher 5HT metabolism at "baseline" conditions. Conceivably, lower expression levels of SERT and HTR1A that function as inhibitors of 5HT secretion in the RN enable the mice to function normally with lower levels of 5HT. Interestingly, chronic stress caused a decrease in 5HT levels accompanied by an increase in 5HT metabolism in some brain areas of control mice, as expected, while in the miR135a OE mice these effects were not observed. These changes may provide a mechanistic explanation for the behavioral resiliency to chronic stress observed in the miR135a OE mice.

The possible use of circulating miRs as a noninvasive biomarker for pathological conditions is a rising field and is supported by relatively high levels and stability of miRs in the circulation. While little is known about the role and origin of the extracellular miRs, circulating miRs have been associated with pathophysiological states, such as different types of cancer, heart diseases, oxidative liver injury, sepsis, pregnancy, and more (Reid et al., 2011). Few studies have reported correlations

between peripheral miRs expression levels and human depression (Belzeaux et al., 2012; Bocchio-Chiavetto et al., 2013; Li et al., 2013). In the current study, the levels of miR135a in the blood of depressed patients were determined and a decrease in miR135a levels in the blood of depressed patients, compared to match controls, was observed. These findings are in line with our data from animal models indicating miR135 to be an endogenous regulator of mood and vulnerability to depression and suggest miR135a as a possible biomarker for depression state and possibly for response to treatment. While the observed increase in miR135a with treatment in peripheral blood of patients is promising, the lack of congruence between the treatments indicates that additional studies in larger patient cohorts are needed.

Further support for the possible role of miR135 in human depression arrived from the postmortem analysis of subregions of the raphe nuclei, demonstrating lower levels of both miR135 and miR16 in the RN and MR subnuclei of suicide victims compared to controls. Interestingly, pathophysiology of the serotonergic RN was evident in depressed suicide patients, while the other raphe subregions, which did not show alteration in miRs levels, are not directly associated with the 5HT-depression circuit (Hornung, 2003).

Additional intriguing support for the possible role of miR135 in the etiology and pathophysiology of human mood disorders rises from genomic data indicating that miR135a1, miR135a2, and miR135b genes are all located within loci for susceptibility to bipolar affective disorder and major depressive disorder. The *miR135a1* gene is located on chromosome 3p21.1 in proximity to the SNPs rs2251219 and rs1042779 identified in genome-wide associations and meta-analysis of bipolar disorder (McMahon et al., 2010; Moskvina et al., 2009; Scott et al., 2009). Interestingly, the miR135a2 gene (chr 12q23.1) is also located within a highly replicated locus for bipolar disorder, recurrent major depression, and neuroticism (Curtis et al., 2003; Dawson et al., 1995; Green et al., 2003). Finally, miR135b (chr 1q32.1) is located within a third locus for bipolar disorder (Detera-Wadleigh et al., 1999; Ekholm et al., 2003; Turecki et al., 1995).

To conclude, we propose that miR135 is an essential regulatory element responsible for maintaining intact serotonergic tone under normal conditions and essential for the brain response to antidepressants. Increased levels of miR135 repress an array of 5HT system-related transcripts, including SERT and presynaptic HTR1A levels, causing an increase in 5HT in the synaptic cleft, which is associated with decreases in depressive symptoms (see schematic model in Figure 7E). These findings may pave the way to better understanding of the psychopathologies that are associated with dysregulation of 5HT systems and may lead to the development of more effective treatments and/or biomarkers.

## EXPERIMENTAL PROCEDURES

Methods and materials are described in detail in the [Supplemental Experimental Procedures](#).

### Mice

For the microRNA microarray of 5HT neurons ePet-EYFP mice (Wylie et al., 2010) were used. For miR135aOE in 5HT neurons, ePet-Cre mice (Scott

et al., 2005) were crossed with miR135aOE mice (Andereggi et al., 2013). Adult C57BL/6 male mice were used for the lentiviral experiments and regulation studies.

### Luciferase Assays for Identifying miR-Target Interactions

Normal and mutated 3' UTRs sequences of *Slc6a4* and *Htr1a* were subcloned into the psiCHECK-2 reporter plasmid (Promega) as previously described (Haramati et al., 2011). HEK293T cells were transfected with psiCHECK-2 plasmid containing the 3' UTR and the overexpressing vector for a specific miRNA. Twenty-four hours after transfection, cells were lysed and luciferase reporter activities were assayed as previously described (Kuperman et al., 2011).

### Chronic Social Defeat and Antidepressant Treatment

Mice were subjected to a social defeat protocol as previously described (Elliott et al., 2010). Mice received intraperitoneal injection of the tricyclic, imipramine, the SSRI, fluoxetine, or the NRI, reboxetine (20 mg/kg in saline), or saline.

### Brain Microdissection, RNA Extraction, and Real-Time PCR

Brain samples were taken from mice raphe nuclei using an acryl brain matrix (Stoelting, 51380) and blunted 14G. mRNAs including microRNAs were isolated using miRNeasy mini kit (QIAGEN) and treated using miScript reverse transcription kit to generate cDNA. Samples were then analyzed using miScript SYBR Green PCR kit (QIAGEN) according to the manufacturer's guidelines in AB 7500 thermocycler (Applied Biosystems).

### Western Blot

Protein was purified in RIPA buffer, separated by electrophoresis on 10% SDS-polyacrylamide gel, and transferred onto nitrocellulose membranes. Membrane was probed with antibodies against SERT, GFP, or  $\beta$ ACTIN and incubated with peroxidase labeled antibodies followed by visualization with ECL and quantifications using ImageJ software.

### Behavioral Assessments

The open-field, dark-light transfer, and elevated plus-maze tests for anxiety-like behaviors were performed as previously described (Sztainberg et al., 2011). Mice locomotion was quantified using a video tracking system (VideoMot2; TSE). Depression-like behaviors were assessed using the forced swim and social avoidance tests performed as previously described (Krishnan et al., 2007) and automatically scored using EthoVision XT (Noldus).

### miR135 Knockdown Lentiviral Production and Intracerebral Injections

miR135 knockdown (KD) plasmid and commercial scrambled control were purchased from GeneCopeia (USA). Recombinant lentiviruses were produced by transient transfection in HEK293T cells, as described previously (Tiscornia et al., 2006). For surgery and lentiviral delivery, mice were placed on a stereotaxic apparatus under general anesthesia as previously described (Lebow et al., 2012) and the lentiviral preparation was delivered to the DR: ML 0 mm; AP -4.6 mm; DV -3.9 mm in 30° tilt. After recovery, mice were subjected to behavioral studies and later anesthetized and perfused with 4% PFA. The fixed brains were serially sectioned in order to confirm the location of the injection site, using immunohistochemistry as described previously (Regev et al., 2011).

### HPLC-ED Analysis of 5HT and 5-HIAA Concentrations

Microdissections followed by high-performance liquid chromatography with electrochemical detection (HPLC-ED) as previously described (Neufeld-Cohen et al., 2010; Evans et al., 2008).

### Human Sample Studies

#### Case Control Study

Patients recruited from the Max-Planck Institute of Psychiatry were selected as described in Menke et al. (2012). Total RNA was isolated using the PAXgene Blood RNA Kit (QIAGEN). The studies were conducted in accordance with the Declaration of Helsinki and its amendments and approved by Max-Planck Institute of Psychiatry.

### Cognitive Behavioral Therapy versus SSRI Study

Patients treated with cognitive behavioral therapy (CBT) or escitalopram had blood drawn for RNA at baseline and after treatment as previously described (Dunlop et al., 2012). Whole blood was extracted using the Tempus Spin RNA Isolation Reagent kit (Applied Biosystems). Declaration of Helsinki and its amendments and approved by Emory University School of Medicine.

### Suicide versus Control Study

Brains obtained from the University Medical School in Budapest as previously described (Merali et al., 2006). Declaration of Helsinki and its amendments and approved by Colorado Institutional Review Board.

### Statistical Analysis

To test for statistical significance, we used Student's t test, one-way ANOVA, two-way ANOVA, and repeated-measure analysis as appropriate using Jmp7 software.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight tables and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2014.05.042>.

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