Epigenetics 9:1, 152–160; January 2014; © 2014 Landes Bioscience

Prenatal stress changes the glycoprotein GPM6A gene expression and induces epigenetic changes in rat offspring brain

Melisa C Monteleone[1], Ezequiela Adrover[2], María Eugenia Pallarés[2], Marta C Antonelli[2], Alberto C Frasch[1], and Marcela A Brocco[1,*]

[1]Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomus (IIB-INTECH); Universidad Nacional de San Martín (UNSAM); Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) San Martín; Buenos Aires, Argentina; [2]IQUIFIB; Facultad de Farmacia y Bioquímica; Universidad de Buenos Aires; Buenos Aires, Argentina

Keywords: hippocampus, prefrontal cortex, DNA methylation pattern, CpG island, microRNA, target site blocker

Abbreviations: GPM6A, neuronal membrane glycoprotein M6a; PFC, prefrontal cortex; PND, post natal day; PS, prenatal stress; TSB, target site blocker

Prenatal stress (PS) exerts strong impact on fetal brain development and on adult offspring brain functions. Previous work demonstrated that chronic stress alters the mRNA expression of GPM6A, a neuronal glycoprotein involved in filopodium extension. In this work, we analyzed the effect of PS on *gpm6a* expression and the epigenetic mechanisms involved. Pregnant Wistar rats received restraint stress during the last week of gestation. Male offspring were sacrificed on postnatal days 28 and 60. Hippocampus and prefrontal cortex samples were analyzed for gene expression (qPCR for mRNAs and microRNAs), methylation status (bisulfite conversion) and protein levels. Hippocampal neurons in culture were used to analyze microRNA overexpression effects. Prenatal stress induced changes in *gpm6a* levels in both tissues and at both ages analyzed, indicating a persistent effect. Two CpG islands in the *gpm6a* gene were identified. Variations in the methylation pattern at three specific CpGs were found in hippocampus, but not in PFC samples from PS offspring. microRNAs predicted to target *gpm6a* were identified in silico. qPCR measurements showed that PS modified the expression of several microRNAs in both tissues, being microRNA-133b the most significantly altered. Further studies overexpressing this microRNA in neuronal cultures showed a reduction in *gmp6a* mRNA and protein level. Moreover filopodium density was also reduced, suggesting that GPM6A function was affected. Gestational stress affected *gpm6a* gene expression in offspring likely through changes in methylation status and in posttranscriptional regulation by microRNAs. Thus, our findings propose *gpm6a* as a novel target for epigenetic regulation during prenatal stress.

Introduction

Evidence provided by research in animals, as well as retrospective studies in humans, point out that exposure to adverse events in early life like prenatal stress (PS) can alter adult behavior.[1] Stress effects are mediated by hypothalamic-pituitary-adrenal axis. Corticosterone, the main glucocorticoid hormone in the rat, has effects on the growth, differentiation, physiology and viability of hippocampal neurons. Neurotrophins (for example brain derived neurotrophic factor, BDNF) may be mediators of corticosterone action in the hippocampus since these proteins are involved in the function of hippocampal neurons.[2] In animal models, early exposure to stress increases corticosterone responses to mild stressors in adulthood[3] and is associated with a reduction in hippocampal glucocorticoid receptor expression.[4] In humans, depressed and anxious/stressed maternal mood has been related to increased rates of preterm delivery and lower birth weights.[5,6]

Microarray analysis to examine the effects of maternal care on the hippocampal transcriptome of adult offspring have shown changes in the expression of genes related to human neurodegeneration and dementia.[7] In addition, microarray profiling of prefrontal cortex (PFC) samples of PS adult rats showed changes in levels of genes involved in synaptic function.[8] PS increases dopamine and glutamate receptor proteins in adult offspring's hippocampus[8,9] and causes variations in the levels of neurotrophins[10,11] and synaptic proteins.[12] All these reports involve different chronic stress models in which PS animals received an additional stress treatment during adulthood, suggesting that gestational stress increases subject susceptibility to stress later in life.

Variability in the stress response depends on the genetic and epigenetic background of each individual. Epigenetic mechanisms (DNA methylation, histone modifications, and microRNAs) have an important role in many processes in the nervous system, such as neuron development and function, neuronal

*Correspondence to: Marcela A Brocco; Email: mbrocco@iib.unsam.edu.ar and marcelabrocco2002@gmail.com
Submitted: 06/04/13; Revised: 07/25/13; Accepted: 07/27/13; Published Online: 08/19/2013
http://dx.doi.org/10.4161/epi.25925

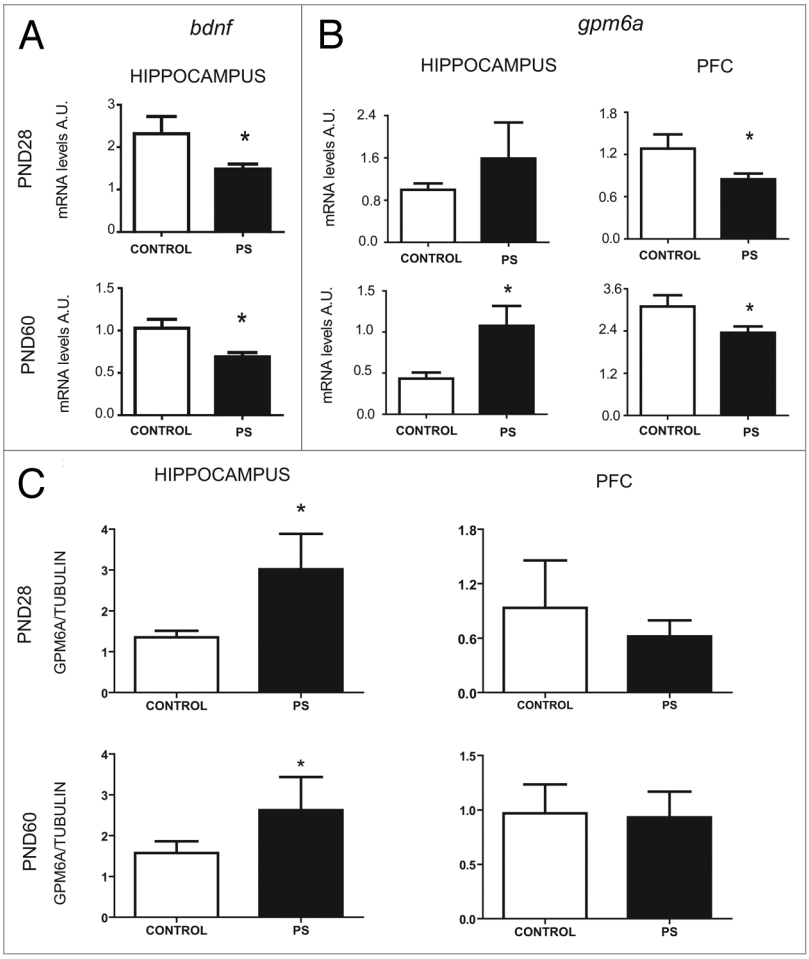

Figure 1. Prenatal stress alters gene and protein expression in the hippocampus and the prefrontal cortex of PS offspring. RT-qPCR measurements of (**A**) *bdnf* mRNA levels in the hippocampus as a positive control of the stress protocol and (**B**) *gpm6a* mRNA levels in the hippocampus and in the PFC in control and PS offspring at PND28 (prepubertal) and PND60 (adults). Relative gene expression levels were determined by the standard curve method using *cyclophilin-a* and *ywhaz* as reference genes. Mann-Whitney test, data are expressed as mean ± SEM, n = 6/group, except for hippocampus PND28 where n = 3; * $p < 0.05$. (**C**) Levels of GPM6A were analyzed by western blot (see **Supplemental Materials** for details) in the hippocampus and prefrontal cortex of PND28 and 60 control and PS offspring. Relative expression was normalized to TUBULIN. Mann-Whitney test, data are expressed as mean ± SEM, n = 6/group, * $p < 0.05$.

plasticity and memory formation[13] and their deregulation has been associated with several neurological disorders. Moreover, all of these mechanisms are affected by stress. Thus, the methylation status of the glucocorticoid receptor *NR3C1* is sensitive to prenatal maternal mood;[14] the changes in the state of histone acetylation have been reported as consequence of maternal care[15] and acute and chronic immobilization stress paradigms induce changes in brain microRNA expression levels.[13,16,17]

Previous findings of our group established that social and physical chronic stress modifies the levels of different transcripts, including *gpm6a*, in the hippocampus, and that this effect is prevented by chronic administration of antidepressants.[18,19] We have also studied GPM6A function and shown its involvement in neurite outgrowth and filopodium/spine formation,[20] as well as in filopodium motility and, likely, in synapse formation.[21] These results suggest that GPM6A might participate in the plastic changes found in the hippocampus of stressed/antidepressant-treated animals.

Given this background and the relevance of GPM6A in neural plasticity, we used a prenatal stress model in rats to study the impact of early life adversity on DNA methylation and microRNA expression in the hippocampus and PFC of PS offspring.

Results

Prenatal stress alters *gpm6a* gene and protein expression levels in a persistent way after birth. To evaluate the effects of prenatal stress on *gpm6a* expression, we used chronic restraint stress in pregnant rats and evaluated gene expression in the hippocampus and PFC from control and prenatal stressed male offspring at PND28. Since both pre and postnatal adverse experiences persistently reduce *bdnf* mRNA expression and protein levels in the hippocampus,[22] we included *bdnf* quantification as a positive control of PS. As expected PS, reduced *bdnf* expression in PS offspring's hippocampus (PND28 $P = 0.05$; PND60 $P = 0.015$; **Fig. 1A**).

gpm6a transcript measurements showed higher levels in the hippocampus of PS offspring compared with control ones. In contrast, PS reduced *gmp6a* in the PFC samples from PS offspring (**Fig. 1B**). The difference in *gpm6a* levels between these brain areas was also observed in PND60 offspring, pointing out toward persistent changes in *gpm6a* expression caused by PS (PND28 hippocampus $P = 0.31$, PFC $P = 0.036$; PND60 hippocampus $P = 0.02$, PFC $P = 0.05$; **Fig. 1B**).

In agreement with mRNA levels results, GPM6A protein levels were increased in the hippocampus of PS offspring ($P = 0.04$; **Fig. 1C**). This difference remained until PND60 ($P = 0.04$), reinforcing the notion of the lasting effects of prenatal stress (**Fig. 1C**). In contrast, in the PFC, GPM6A levels showed no changes between stressed and control animals at any of the ages tested (PND28 $P = 0.29$; PND60 $P = 0.46$; **Fig. 1C**) suggesting that different mechanisms operate to control gene expression in each area.

Prenatal stress affects methylation pattern of specific CpGs in *gpm6a* gene. Chromatin changes are introduced and interpreted by different proteins like DNA methyl transferases (DNMT1, DNMT3A, and DNMT3B) and methylated DNA binding proteins (KAISO, MECP2). Hence, we analyzed by qPCR if prenatal stress altered the levels of the mRNAs for those proteins. In the hippocampus, while *dnmt3b* levels were similar in both groups ($P = 0.14$; **Fig. 2A**), *dnmt3a, mecp2,* and *kaiso* expression levels were significantly higher in the PS group (*dnmt3a* $P = 0.03$, *mecp2* $P = 0.02$ and *kaiso* $P = 0.03$; **Fig. 2A**). Under our experimental conditions, there was no amplification for

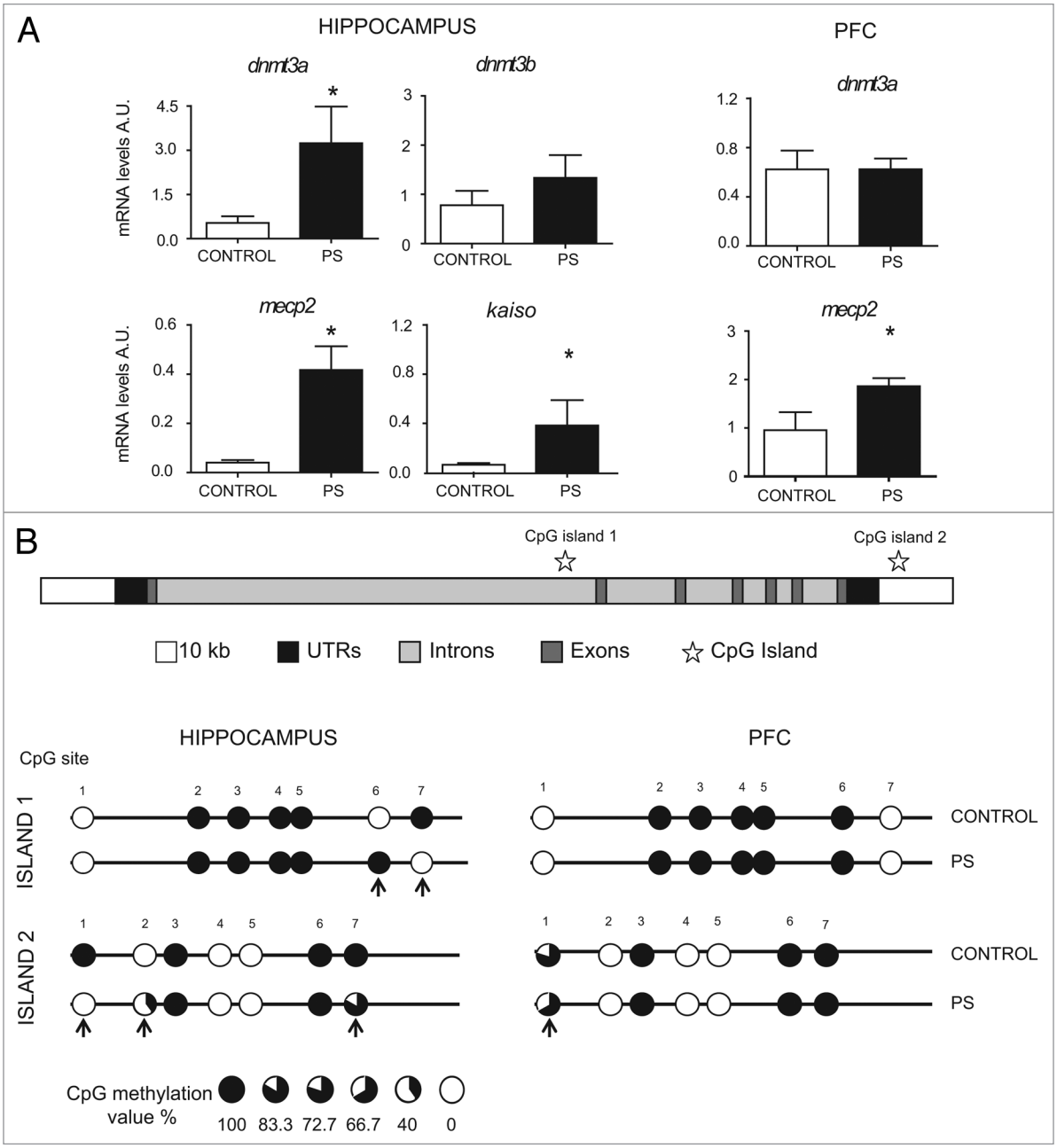

Figure 2. Prenatal stress alters the global and the site-specific methylation pattern of *gpm6a* in PS offspring. (**A**) RT-qPCR measurements of mRNA levels of DNA methyltransferases *dnmt3a* and *dnmt3b* and methyl CpG binding proteins *mecp2* and *kaiso* in the hippocampus and in the PFC in control and PS offspring at PND60 (adults). Relative gene expression levels were determined by the standard curve method using *cyclophilin-a* and *ywhaz* as reference genes. Mann-Whitney test, data are expressed as mean ± SEM, n = 6/group; **P* < 0.05. (**B**) Upper panel: Scheme (not-scaled) of the distribution of introns (gray boxes), exons (dark gray boxes) and UTRs (black boxes) in the *gpm6a* gene. Additional 10 kb (white boxes) up and downstream of the UTRs were also included in the CpG island search. Stars represent putative CpG islands. Lower panel: CpG methylation values (percentage of methylation) for island 1 (in intron 1) and island 2 (~3000 bp downstream *gpm6a* 3'UTR) in genomic DNA samples isolated from the hippocampus and the PFC of control offspring and PS offspring. Differences in methylation were only seen at specific sites (arrows).

dnmt3b and *kaiso* cDNAs in the PFC, suggesting a low mRNA expression. In this tissue *dnmt3a* expression was similar in both groups (*P* = 0.39) and *mecp2* levels were increased in the PS group (*P* = 0.05; **Fig. 2A**).

Taking into account that prenatal stress modified the expression of chromatin remodeler genes, we hypothesized that the differences in *gpm6a* expression could be due to changes in the gene methylation pattern. Thus, we screened for CpG islands in the *gpm6a* gene, including 10 kb up and downstream the initiation transcription site and the polyA addition site, respectively (scheme in **Fig. 2B**). Using bioinformatic tools, we found two putative islands (stars in **Fig. 2B**) located within intron 1 (island 1) and 3132 bp bases after the 3'UTR (island 2).

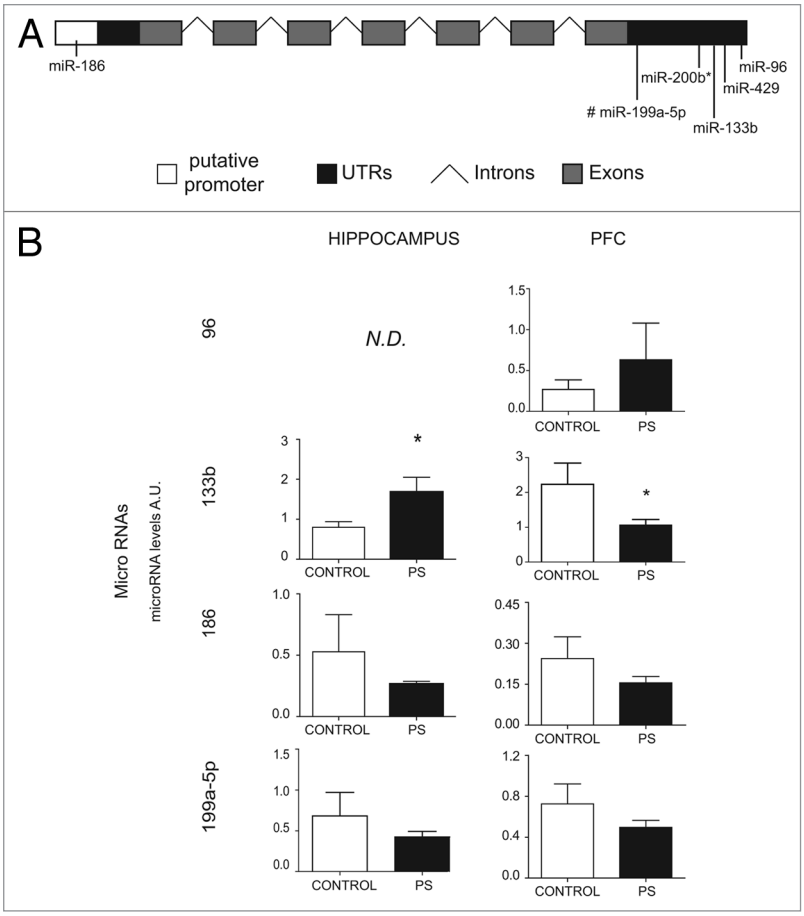

Figure 3. Identification of microRNAs for *gpm6a* and their quantification in PS offspring brain. (**A**) Scheme (not-scaled) of *gpm6a* transcript plus the upstream flanking region assumed as promoter and the binding sites for miRNA families broadly conserved among vertebrates. #Although microRNA 199a-5p belongs to the poorly conserved site group, it was included in the analysis because of its reported expression in rat neural tissues.[46] (**B**) RT-qPCR measurements of miR-96, -133b, -186, and -199a-5p levels in the hippocampus and the PFC of PND60 control and PS offspring. miR-200b* and miR-429 showed such low amplification rates that prevented quantification. ND: Not detectable under our experimental conditions. Relative miRNA expression levels were determined by the standard curve method using U6 as reference gene. Mann-Whitney test, data are expressed as mean ± SEM, n = 6/group, *$P < 0.05$.

The methylation status of these regions was analyzed by bisulfite conversion of genomic DNA purified from the hippocampus of PND60 male rats that were or were not exposed to PS. Percentage of methylation was evaluated by direct sequencing and by subcloning and sequencing. Both methods raised comparable results. In a qualitative analysis, we observed that within the island 1, CpG 6 showed a drastic change as none of the control individuals showed this cytosine methylated and all of the DNA samples from PS animals showed this residue methylated. The opposite case was seen for CpG 7 (**Fig. 2B**). In the case of the island 2, CpG 1 showed 100% methylation in samples from control animals while in all PS samples it was unmethylated (0%). There were also differences in the methylation pattern of CpG 2 (no methylation in samples from control animals vs. 40% for DNA derived from PS rats) and of CpG 7, (100% for control animals vs. 83% for DNA derived from PS rats). We performed the same analysis for DNA isolated from PFC samples, but in this case there were no differences in the methylation pattern between prenatal stress and control samples (**Fig. 2B**). Samples from PND28 individuals showed similar results to those obtained at PND60 for both tissues (not shown).

Interestingly, the methylation pattern of CpGs sites 6 and 7 from island 1 was opposite between hippocampus and PFC, i.e., in all hippocampus control samples, the CpG site 6 was unmethylated while in all PFC samples, it was methylated. The opposite occurred for CpG site 7 (**Fig. 2B**). In contrast, such differences were not observed when comparing samples from PS animals, since samples from both tissues showed the same methylation pattern for the island 1. No differences were observed for island 2, where methylation patterns were similar between the hippocampus and the PFC.

Prenatal stress affected expression of microRNAs that could modulate *gpm6a* expression. In addition to the methylation status, prenatal stress may affect *gpm6a* expression through another mechanism that involves posttranscriptional regulators such as microRNAs. To test this hypothesis, we first looked for predicted miRNA binding sites on the *gpm6a* sequence, using different databases. Among the microRNAs predicted to target *gpm6a* we chose miR-96, -133b, -186, -199a-5p, -200b*, and -429, because of seed length, probability of preferential conservation, reported brain expression and/or association with neuropsychiatric diseases.[13,16,23-26] All interaction sites for these miRNAs are located in the *gpm6a* 3'UTR (schema in **Fig. 3A**), except for miR-186, which is within the region assumed as a promoter.

The levels of the selected microRNAs were evaluated in the hippocampus and in the PFC from control and prenatally stressed PND60 rats. Although a significant reduction in expression levels of miR-200b* and miR-429 has been previously reported in rat frontal cortex,[26] we observed such low amplification rates that it was not possible to carry out a reliable quantification for these microRNAs in any of the tissues analyzed. In the case of miR-96, while in the hippocampus it was undetectable, in the PFC it seems to be increased in PS animals compared with control samples, although this difference was not statistically significant ($P = 0.45$; **Fig. 3B**). miR-186 and 199a-5p levels appeared to be slightly reduced in both tissues (miR-186 hippocampus $P = 0.39$, PFC $P = 0.46$; miR-199a-5p hippocampus $P = 0.39$, PFC $P = 0.39$; **Fig. 3B**). In the case of miR-133b, its expression was significantly upregulated in the hippocampus ($P = 0.05$) and downregulated in the PFC ($P = 0.05$) of PS animals compared with control ones (**Fig. 3B**).

miR-133 interaction with *gpm6a* is specific and affects mRNA expression and protein levels and function. TargetScan, the most robust tool to predict microRNA targets,[27] reports a miR-133abc

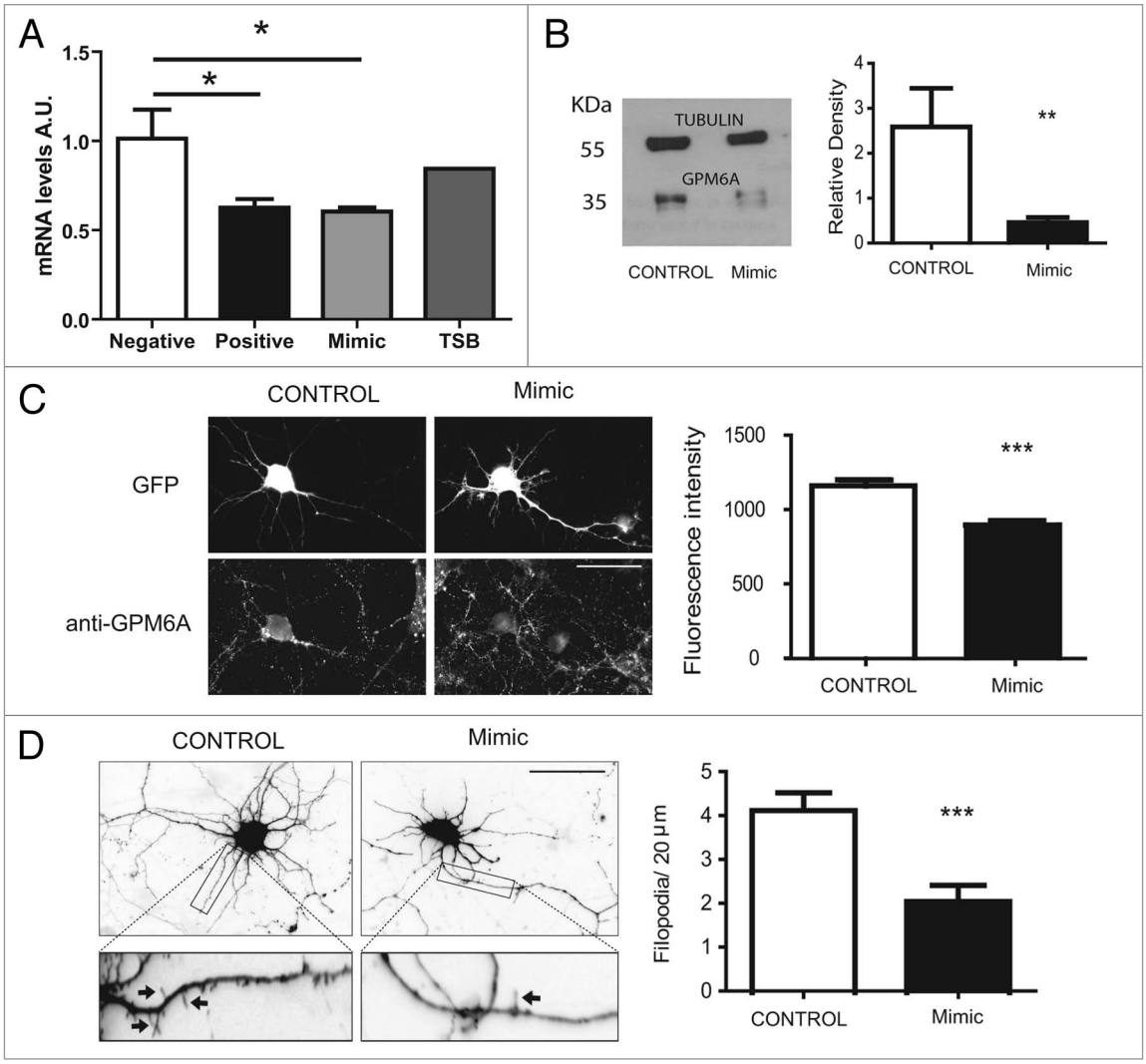

Figure 4. miR-133 interaction with *gpm6a* mRNA is specific and affects its expression and function. (**A**) RT-qPCR measurements of transcript levels of *gpm6a* in 7 DIV hippocampal neurons transfected with: AllStars negative control (Negative); miR-133 mimic (Mimic, overexpression), or with miR-133 mimic + Target Site Blocker (TSB). Levels of *klf15* were measured as positive control since it is a validated target of miR-133.[28] While Mimic reduced *gpm6a* expression in treated cells compared with control ones, TSB blocked miR-133 effect. Relative gene expression levels were determined by the ΔΔCt method. *cyclophilin-a* was used as reference gene. ANOVA test followed by Tukey post test, data are expressed as mean ± SEM, n = 3 plates/condition; **P* < 0.05. (**B**) Western blot of GPM6A using a protein extract of 10 DIV hippocampal neurons transfected with miR-133 mimic (Mimic) or with transfection reagent only (Control). Relative expression was normalized to TUBULIN (Mann-Whitney test, mean ± SEM, n = 3 plates/condition; **P* < 0.05. (**C**) Immunofluorescence intensity analysis in neurons transfected with GFP plasmid alone (positive control of transfection) or with miR-133 mimic + GFP plasmid (Mimic). GPM6A was labeled with anti-GPM6A antibodies and rhodamine-conjugated secondary antibodies. Fluorescence intensity was quantified with ImageJ software (Mann-Whitney test,mean ± SEM, n = 30; ***P* < 0.0001). (**D**) Effects of miR-133 mimic (Mimic) on GPM6A function were assessed by filopodium density analysis. The number of protrusions per 20 μm neurite length was quantified in 10 DIV hippocampal neurons. Arrows indicate protrusions (Mann-Whitney test, mean ± SEM, n = 45; ***P* < 0.0001).

binding site in *gpm6a* 3'UTR as the only and longest (8mer) site with higher probability of preferential conservation. In addition, we observed a significant differential effect of prenatal stress on miR-133b expression. Therefore, we deepened the analysis of mir-133 in vitro. First, we assessed by qPCR if miR-133 overexpression modulates *gpm6a* levels in primary neuronal cultures. Indeed, miR-133 reduced *gpm6a* mRNA levels compared with negative control (≈ 60% of control, $P < 0.05$, **Fig. 4A**). The mRNA for the potassium channel *klf15*, a validated miR-133 target,[28] was included in the analysis as a positive control. As expected, *klf15* expression was reduced in miR-133 transfected neurons when compared with negative control ($P < 0.05$, **Fig. 4A**). Cells that were treated with an unrelated small RNA (negative control) showed no reduction in *gpm6a* levels ($P > 0.05$, **Fig. 4A**). To test the specificity of the miR-133-*gpm6a* interaction, we used a target site blocker (TSB) that hybridizes to the target site preventing miRNA pairing, thus disrupting the interaction. Co-transfection of 7 DIV neurons with miR-133 mimic and TSB revealed no effect on *gpm6a* expression ($P > 0.05$, **Fig. 4A**). These results suggest that miR-133 can specifically modulate *gpm6a* expression.

Further confirmation that the miR-133 overexpression affected *gpm6a* expression was revealed by both western blot and immunofluorescence intensity analysis. Western blot semi-quantification demonstrated a reduction in GPM6A content in miR-133 treated cells ($P = 0.004$, **Fig. 4B**). As well, analysis in cells co-transfected with the GFP plasmid and miR-133, showed reduced fluorescence intensity compared with cells transfected with GFP only ($P < 0.0001$, **Fig. 4C**).

We next evaluated if miR-133 overexpression affected GPM6A function. Filopodium density was compared between miR-133 treated and untreated cells. Cells overexpressing miR-133 revealed a marked decrease ($\approx 50\%$, $P = 0.0002$) in filopodium number compared with control ones (**Fig. 4D**). Altogether, these findings indicate that in vitro miR-133 reduced not only *gpm6a* mRNA and protein levels, but also affected GPM6A function.

Discussion

In this work we showed that prenatal stress modifies *gpm6a* mRNA and GPM6A protein levels in the hippocampus and in the PFC in offspring and that these differences are maintained at least until adulthood. The results point to changes in methylation patterns and in miR-133b levels as putative epigenetic mechanisms contributing to regulate *gpm6a* expression during prenatal stress. GPM6A was previously shown to promote filopodium/spine formation and was suggested to be involved in synaptogenesis.[20,21] Altogether these findings further support the idea that environmental stress during pregnancy might result in alterations in the offspring including neuronal connectivity-plasticity. Therefore, PS-induced changes in *gpm6a* levels may reflect a homeostatic mechanism to counteract stress damage to neuronal cells.

qPCR measurements demonstrated that stress upregulated *gpm6a* mRNA levels in the hippocampus, whereas the opposite occurred in the PFC. This tissue dependent expression for *gpm6a* has already been observed after chronic stress exposure in adult male rats[29] and might be interpreted as a compensatory mechanism. Previous work from our group showed that chronic social and physical stress decreases *gpm6a* mRNA levels in the hippocampus[18,19] which suggests that GPM6A participates in plastic hippocampal changes observed in stressed treated animals. However, we found that *gpm6a* levels were upregulated in the hippocampus of PS animals. The different stress paradigms compared (chronically stressed adult animal vs. PS offspring) point out that the nervous system might exhibit alternative responses depending on the developmental stage.

Since all the changes in gene expression were observed in the PS offspring at adulthood, we asked how PS effects were maintained over time. In that sense, we found higher mRNA expression for DNA methyl transferases and methylated DNA binding proteins in the hippocampus of PS offspring, suggesting global changes in methylation status. An appropriate DNA methylation within the nervous system is vital for its proper functioning and several neurological disorders arise in response to mutations in the proteins involved in this mechanism (e.g., mutated MECP2 in Rett syndrome). Weaver et al.,[30] showed that particular patterns of maternal care have epigenetic effects such as an altered methylation pattern in a region of the glucocorticoid receptor *Nr3c1* promoter responsible to control its hippocampal expression. A decreased DNA methylation of the corticotrophin-releasing-factor gene and an increased methylation of the glucocorticoid receptor gene have also been demonstrated in the hypothalamus of adult male mice exposed to gestational stress.[31] Despite there were not differences in the overall methylation level in the CpG islands analyzed for *gpm6a*, we did find drastic changes in CpGs sites 6 and 7 from the island 1, particularly in the hippocampus. These results indicate that specific variations in the methylation pattern are highly sensitive to environment and minimum changes may account for differences in gene expression. Moreover, when we analyzed GPM6A protein levels we found that only in the hippocampus, where the major methylation differences were observed, the protein levels were significantly higher in PS animals compared with control ones. This suggests that methylation variations may control *gpm6a* expression in hippocampus but not in the prefrontal cortex.

In prenatally stressed offspring, the expression of some miRNAs (miR-186 and -199a-5p in the hippocampus and miR-96 in the PFC) appeared to show an inverse correlation with *gpm6a* mRNA levels suggesting that the classical regulatory mechanisms of translational repression and/or mRNA degradation were activated in response to gestational stress. However, these changes were subtle. On the other hand, in both tissues, PS significantly altered miR-133b levels which accompanied *gpm6a* levels, suggesting a different mechanism of action. This type of regulation has been previously described for microRNAs expressed together with their targets in the brain and is referred as Type I circuits[32] or incoherent regulation[33] because target is expressed in the same fashion as the microRNA. Many genes regulated by this kind of mechanism are involved in cell differentiation, morphogenesis and synaptic transmission,[34] processes in which GPM6A has also been implicated.[20,21,35] These type of circuits could provide fine tuning and maintenance of protein steady-states and emphasize the role of miRNAs not only as simply transcript inhibitors but as participants of regulatory networks operating in nervous system homeostasis.[32]

In addition, here we showed that miR-133 overexpression affected *gpm6a* mRNA and GPM6A protein levels and that the addition of a specific target site blocker reverted these effects. Therefore, we postulate *gpm6a* as a new candidate target for miR-133b in the nervous system. Interestingly, another microRNA, miR-124 has recently been proposed as a human *GPM6A* regulator and has been associated to claustrophobia.[17]

Although the understanding of maternal stress effects on the gene expression of newborn/adult descendants is a relatively new research area, it has central implications for development during life. Given that GPM6A is involved in neurite extension, filopodium/spine formation and, likely, in synaptogenesis, our results further support the magnitude of PS effects on neuronal development. Epigenetic mechanisms, like the ones described in this work, might provide the link between environmental factors (PS) and gene expression.

Materials and Methods

Prenatal stress protocol. Pregnant Wistar dams were randomly assigned to either the control or the PS group and were individually housed with ad libitum access to food and water. A constant light/dark cycle was maintained at a temperature of 21–25 °C. Control rats (n = 6) were left undisturbed in the home cage, while PS dams (n = 6) were subjected to a restraint stress procedure, which involved rats being transferred to an experimental room where the stressor was applied. Pregnant females were individually placed into a transparent plastic restrainer fitted closely to body size for three 45 min periods per day (09:00 AM, 12:00 PM, and 04:00 PM) between days 14 and 21 of pregnancy. The restrainer had ventilation holes and dimensions appropriate for a 350 g pregnant rat. This type of stressor has an indirect influence on the fetuses via a direct stress on the mother.[36] No other subjects were present in the experimental room during the stress exposure. At the end of the stress session, the rats were returned to the animal housing room where they were individually housed with free access to food and water. Only male offspring were used for further experiments. To avoid litter effects, one pup from each litter was tested for each experiment.

Prenatal stress protocol is in accordance with the guidelines laid down by the Committee for the Care and Use of Animals for Experimentation (CICUAI-University of Buenos Aires #121/2013). On postnatal day 28 (PND28, prepubertal) or 60 (PND60, adult) animals were euthanised by decapitation. The PFC and the hippocampus were surgically removed and immediately homogenized in TRIzol® Reagent (Life Technologies). Total RNA, genomic DNA and proteins were isolated from these homogenates according to manufacturer's instructions.

RNA Isolation, cDNA Synthesis and Quantitative Polymerase Chain Reaction (qPCR). Total RNA was isolated with DirectZol RNA Miniprep (Zymo Research) following manufacturer's instructions. cDNA was synthesized by retrotranscription using oligodT and SuperScriptTM II Reverse Transcriptase enzyme (Life Technologies) according to the manufacturer's instructions. qPCR reactions were achieved with Kapa SYBR fast qPCR kit (KAPA Biosystems) in triplicate. For details and primer sequences, see **Supplemental Materials**. Data were analyzed using the linear standard curve method. For datum normalization, we measured mRNA levels for the reference genes *cyclophilin-a* and tyrosine 3-monooxygenase/tryptophan5-monooxygenase activation protein, zeta polypeptide *(ywhaz)*. Normalization with both reference genes resulted in almost identical data.

CpG islands prediction, amplification and analysis. The *gpm6a* complete gene sequence was downloaded from Ensemble, release 70. We selected the region 16:39660750–39793384 which included 10 kb up- and down-stream of the transcription initiation site and of the polyA addition site, respectively. This region was screened using the CpG island Searcher software[37] and the CPGREPORT algorithm[38] with a sliding window of 100 bp and a step increment of 1. The limit values were: length > 100 bp, G+C content > 50%, observed/expected CpG ratio > 0.6. Two CpG islands were predicted, one in the first intron (island 1) and a second one approximately 3 kb downstream the 3'UTR (island 2).

Bisulfite conversion. Isolated genomic DNA was bisulfite treated (Epitect Bisulfite Kit, Qiagen) following the manufacturer's instructions. Briefly, 2 μg of DNA were used for each reaction. Conversion was performed on a thermocycler as follows: 99 °C for 5 min, 60 °C for 25 min, 99 °C for 5 min, 60 °C for 85 min, 99 °C for 5 min, 60 °C for 175 min. The bisulfite-treated DNA was recovered by EpiTect spin column, amplified and subsequently sequenced. We chose bisulfite conversion since it is considered the gold standard method for DNA methylation analysis.

The primers for bisulfite sequencing PCR were selected using the Methprimer software:[39] Island 1 F: 5'-TTTTTGATTT TTTTAATGGG GTTT-3'; R: 5'-ATTCAAAACC TACCCCACAT ATAAC-3'; Island 2 F: 5'-GTTTTGATTT TAGTATTAGG AATTATAATT-3', R 5'-AAAATACCCA CCTAACCAAA C-3'

PCR products were purified with DNA clean and concentrator (Zymo Research) according to manufacturer's instructions and sequenced in an ABI 3130 sequencer (Applied Biosystem). Sequences were analyzed with QUMA software.[40]

Alternatively, clonal analysis[41] of 10 clones (pGEM-T-Easy Vector and Rapid Ligation Buffer System, Promega) from pooled PCR fragments was performed.

microRNA target prediction. Integration of various computational methods is a common approach to improve prediction accuracy. To identify putative miRNA target sites located within the *gpm6a* transcript, we used the miRWalk database[42] which major function is to report predicted miRNA-mRNA interactions on the 3' UTRs of known genes. Among the programs available within the miRWalk database, TargetScan (TargetScan software version 6.2), was selected for being a well-established algorithm of seed and sequence complementarity with conservation of binding sites across multiple species.[43]

Retrotranscription and qPCR to quantify microRNA levels. Reverse transcription of microRNAs (20 ng total RNA template isolated with DirectZol RNA Miniprep as mentioned before) was performed using miRCURY LNA Universal RT microRNA PCR Universal cDNA Synthesis kit (Exiqon) according to manufacturer's instructions. cDNA was diluted 1/80 and qPCR was performed using the miRCURY LNA Universal RT microRNA PCR SYBR Green master mix (Exiqon) in triplicate. Values for each treatment were normalized to U6 and RNU5G (reference genes) and resulted in almost identical patterns.

Cell Transfections. Hippocampal neurons in culture (See Supplemental Information for details) were transfected 7 d after plating. Transfections were performed with Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. For 35 mm dishes (RT-qPCR and western blot assays), 5 μl Lipofectamine diluted in OptiMEM were combined for 20 min with (1) 200 nM rno-miR-133 (miScript miRNA Mimics, Qiagen) to overexpress miR-133; (2) 200 nM AllStars Negative Control (Qiagen), a siRNA that has no homology to any known mammalian gene as a negative control; (3) Target Site Blocker (TSB Exiqon) + 200 nM rno-miR-133. TSB is predicted to bind

selectively to a sequence overlapping the miR-133 site in the 3'UTR of *gpm6a*, interfering though with the mRNA-miRNA interaction. Cells were incubated with the transfection mix for 4 h at 37 °C. Finally, media were changed and replaced for fresh serum-free medium. For immunofluorescence intensity or filopodium density analysis, cells were seeded in 24-well plates and 2 μl of Lipofectamine were combined with 1 μg/well of the plasmid encoding the enhanced green fluorescent protein (pEGFP-C1, from Clontech) and 200 nM rno-miR-133 (co-transfection). The rno-miR-133 was omitted in controls. Three days after transfection, cells were homogenized in 1 ml TRIzol® Reagent for mRNA and protein quantification or fixed with paraformaldehyde for immunocytochemistry. qPCR data was analyzed with the ΔΔCt method[44] and *cyclophilin-a* was used as reference gene. For mRNA and protein quantification, one 35 mm dish per condition from 3 independent experiments was used.

Image analysis. Fluorescent images of immunolabeled neurons (see **Supplemental Materials** for details) were acquired with a Nikon Eclipse 80i microscope (Plan APO 60X oil, 1.4 NA, 0.13 mmWD, DIC objective) equipped with CoolLED pE excitation system. Thirty cells from each condition were analyzed. Using ImageJ software (plot profile tool), a line of 150 pixels length was drawn across the GFP-transfected neuron soma and the information of the intensity from each pixel was obtained. At least 20 neurons from three independent experiments were used.

Filopodium density (number of protrusions per 20 μm neurite length measured within 50 μm from the soma) was quantified as previously described.[20,45] At least 3 coverslips from three independent experiments were used and 45 neurites (3 neurites per neuron) per group were analyzed.

Statistical analysis. Statistical analysis and graphs were performed with GraphPad Prism Version 5.00. When two groups were compared the non-parametric Mann-Whitney U-test was used because of data distribution. One-way analysis of variance (ANOVA) test followed by Tukey test was performed in order to compare more than 3 groups. For this analysis IS version 2010 (Infostat software, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina) was used.

Results were reported as mean ± SD. For all tests, a $P < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Dr Silvia Billi for her technical assistance. MCM, EA and MEP are recipients of a doctoral fellowship from the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina (CONICET). MCA, ACF, and MAB are researchers from CONICET. This work was supported by grants to ACF and to MCA from the ANPCyT and to ACF from the UNSAM and by grants to MAB from Fundación F Fiorini and from International Society for Neurochemistry (ISN)—Committee for Aid and Education in Neurochemistry (CAEN).

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/25925

References

1. Weinstock M. Alterations induced by gestational stress in brain morphology and behaviour of the offspring. Prog Neurobiol 2001; 65:427-51; PMID:11689280; http://dx.doi.org/10.1016/S0301-0082(01)00018-1
2. Schaaf MJM, de Jong J, de Kloet ER, Vreugdenhil E. Downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. Brain Res 1998; 813:112-20; PMID:9824681; http://dx.doi.org/10.1016/S0006-8993(98)01010-5
3. Clarke AS, Schneider ML. Effects of prenatal stress on behavior in adolescent rhesus monkeys. Ann N Y Acad Sci 1997; 807:490-1; PMID:9071378; http://dx.doi.org/10.1111/j.1749-6632.1997.tb51947.x
4. Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky PM, Meaney MJ. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science 1997; 277:1659-62; PMID:9287218; http://dx.doi.org/10.1126/science.277.5332.1659
5. Wadhwa PD, Garite TJ, Porto M, Glynn L, Chicz-DeMet A, Dunkel-Schetter C, Sandman CA. Placental corticotropin-releasing hormone (CRH), spontaneous preterm birth, and fetal growth restriction: a prospective investigation. Am J Obstet Gynecol 2004; 191:1063-9; PMID:15507922; http://dx.doi.org/10.1016/j.ajog.2004.06.070
6. Van den Bergh BRH, Mulder EJH, Mennes M, Glover V. Antenatal maternal anxiety and stress and the neurobehavioural development of the fetus and child: links and possible mechanisms. A review. Neurosci Biobehav Rev 2005; 29:237-58; PMID:15811496; http://dx.doi.org/10.1016/j.neubiorev.2004.10.007
7. Weaver ICG, Meaney MJ, Szyf M. Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. Proc Natl Acad Sci U S A 2006; 103:3480-5; PMID:16484373; http://dx.doi.org/10.1073/pnas.0507526103
8. Kinnunen AK, Koenig JI, Bilbe G. Repeated variable prenatal stress alters pre- and postsynaptic gene expression in the rat frontal pole. J Neurochem 2003; 86:736-48; PMID:12859686; http://dx.doi.org/10.1046/j.1471-4159.2003.01873.x
9. Berger MA, Barros VG, Sarchi MI, Tarazi FI, Antonelli MC. Long-term effects of prenatal stress on dopamine and glutamate receptors in adult rat brain. Neurochem Res 2002; 27:1525-33; PMID:12512957; http://dx.doi.org/10.1023/A:1021656607278
10. Fumagalli F, Bedogni F, Slotkin TA, Racagni G, Riva MA. Prenatal stress elicits regionally selective changes in basal FGF-2 gene expression in adulthood and alters the adult response to acute or chronic stress. Neurobiol Dis 2005; 20:731-7; PMID:15967670; http://dx.doi.org/10.1016/j.nbd.2005.05.005
11. Fumagalli F, Bedogni F, Perez J, Racagni G, Riva MA. Corticostriatal brain-derived neurotrophic factor dysregulation in adult rats following prenatal stress. Eur J Neurosci 2004; 20:1348-54; PMID:15341606; http://dx.doi.org/10.1111/j.1460-9568.2004.03592.x
12. Bogoch Y, Biala YN, Linial M, Weinstock M. Anxiety induced by prenatal stress is associated with suppression of hippocampal genes involved in synaptic function. J Neurochem 2007; 101:1018-30; PMID:17254015; http://dx.doi.org/10.1111/j.1471-4159.2006.04402.x
13. Babenko O, Golubov A, Ilnytskyy Y, Kovalchuk I, Metz GA. Genomic and epigenomic responses to chronic stress involve miRNA-mediated programming. PLoS One 2012; 7:e29441; PMID:22291890; http://dx.doi.org/10.1371/journal.pone.0029441
14. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics 2008; 3:97-106; PMID:18536531; http://dx.doi.org/10.4161/epi.3.2.6034
15. McGowan PO, Suderman M, Sasaki A, Huang TCT, Hallett M, Meaney MJ, Szyf M. Broad epigenetic signature of maternal care in the brain of adult rats. PLoS One 2011; 6:e14739; PMID:21386994; http://dx.doi.org/10.1371/journal.pone.0014739
16. Meerson A, Cacheaux L, Goosens KA, Sapolsky RM, Soreq H, Kaufer D. Changes in brain MicroRNAs contribute to cholinergic stress reactions. J Mol Neurosci 2010; 40:47-55; PMID:19711202; http://dx.doi.org/10.1007/s12031-009-9252-1
17. El-Kordi A, Kästner A, Grube S, Klugmann M, Begemann M, Sperling S, Hammerschmidt K, Hammer C, Stepniak B, Patzig J, et al. A single gene defect causing claustrophobia. Transl Psychiatry 2013; 3:e254; PMID:23632458; http://dx.doi.org/10.1038/tp.2013.28
18. Alfonso J, Pollevick GD, Van Der Hart MG, Flügge G, Fuchs E, Frasch ACC. Identification of genes regulated by chronic psychosocial stress and antidepressant treatment in the hippocampus. Eur J Neurosci 2004; 19:659-66; PMID:14984416; http://dx.doi.org/10.1111/j.1460-9568.2004.03178.x

19. Alfonso J, Frick LR, Silberman DM, Palumbo ML, Genaro AM, Frasch AC. Regulation of hippocampal gene expression is conserved in two species subjected to different stressors and antidepressant treatments. Biol Psychiatry 2006; 59:244-51; PMID:16140276; http://dx.doi.org/10.1016/j.biopsych.2005.06.036
20. Alfonso J, Fernández ME, Cooper B, Flugge G, Frasch AC. The stress-regulated protein M6a is a key modulator for neurite outgrowth and filopodium/spine formation. Proc Natl Acad Sci U S A 2005; 102:17196-201; PMID:16286650; http://dx.doi.org/10.1073/pnas.0504262102
21. Brocco MA, Fernández ME, Frasch AC. Filopodial protrusions induced by glycoprotein M6a exhibit high motility and aids synapse formation. Eur J Neurosci 2010; 31:195-202; PMID:20074218; http://dx.doi.org/10.1111/j.1460-9568.2009.07064.x
22. Roceri M, Hendriks W, Racagni G, Ellenbroek BA, Riva MA. Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. Mol Psychiatry 2002; 7:609-16; PMID:12140784; http://dx.doi.org/10.1038/sj.mp.4001036
23. Mouillet-Richard S, Baudry A, Launay J-M, Kellermann O. MicroRNAs and depression. Neurobiol Dis 2012; 46:272-8; PMID:22226785; http://dx.doi.org/10.1016/j.nbd.2011.12.035
24. Juhila J, Sipilä T, Icay K, Nicorici D, Ellonen P, Kallio A, Korpelainen E, Greco D, Hovatta I. MicroRNA expression profiling reveals miRNA families regulating specific biological pathways in mouse frontal cortex and hippocampus. PLoS One 2011; 6:e21495; PMID:21731767; http://dx.doi.org/10.1371/journal.pone.0021495
25. Kye MJ, Neveu P, Lee Y-S, Zhou M, Steen JA, Sahin M, Kosik KS, Silva AJ. NMDA mediated contextual conditioning changes miRNA expression. PLoS One 2011; 6:e24682; PMID:21931811; http://dx.doi.org/10.1371/journal.pone.0024682
26. Smalheiser NR, Lugli G, Rizavi HS, Zhang H, Torvik VI, Pandey GN, Davis JM, Dwivedi Y. MicroRNA expression in rat brain exposed to repeated inescapable shock: differential alterations in learned helplessness vs. non-learned helplessness. Int J Neuropsychopharmacol 2011; 14:1315-25; PMID:21275079; http://dx.doi.org/10.1017/S1461145710001628
27. Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature 2008; 455:64-71; PMID:18668037; http://dx.doi.org/10.1038/nature07242
28. Horie T, Ono K, Nishi H, Iwanaga Y, Nagao K, Kinoshita M, Kuwabara Y, Takanabe R, Hasegawa K, Kita T, et al. MicroRNA-133 regulates the expression of GLUT4 by targeting KLF15 and is involved in metabolic control in cardiac myocytes. Biochem Biophys Res Commun 2009; 389:315-20; PMID:19720047; http://dx.doi.org/10.1016/j.bbrc.2009.08.136
29. Cooper B, Fuchs E, Flügge G. Expression of the axonal membrane glycoprotein M6a is regulated by chronic stress. PLoS One 2009; 4:e3659; PMID:19180239; http://dx.doi.org/10.1371/journal.pone.0003659
30. Weaver ICG, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ. Epigenetic programming by maternal behavior. Nat Neurosci 2004; 7:847-54; PMID:15220929; http://dx.doi.org/10.1038/nn1276
31. Mueller BR, Bale TL. Sex-specific programming of offspring emotionality after stress early in pregnancy. J Neurosci 2008; 28:9055-65; PMID:18768700; http://dx.doi.org/10.1523/JNEUROSCI.1424-08.2008
32. Tsang J, Zhu J, van Oudenaarden A. MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals. Mol Cell 2007; 26:753-67; PMID:17560377; http://dx.doi.org/10.1016/j.molcel.2007.05.018
33. Shkumatava A, Stark A, Sive H, Bartel DP. Coherent but overlapping expression of microRNAs and their targets during vertebrate development. Genes Dev 2009; 23:466-81; PMID:19240133; http://dx.doi.org/10.1101/gad.1745709
34. Liu H, Kohane IS. Tissue and process specific microRNA-mRNA co-expression in mammalian development and malignancy. PLoS ONE 2009; 4:e5436; PMID:19415117; http://dx.doi.org/10.1371/journal.pone.0005436.
35. Michibata H, Okuno T, Konishi N, Wakimoto K, Kyono K, Aoki K, Kondo Y, Takata K, Kitamura Y, Taniguchi T. Inhibition of mouse GPM6A expression leads to decreased differentiation of neurons derived from mouse embryonic stem cells. Stem Cells Dev 2008; 17:641-51; PMID:18522499; http://dx.doi.org/10.1089/scd.2008.0088
36. Maccari S, Piazza PV, Kabbaj M, Barbazanges A, Simon H, Le Moal M. Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. J Neurosci 1995; 15:110-6; PMID:7823121
37. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A 2002; 99:3740-5; PMID:11891299; http://dx.doi.org/10.1073/pnas.052410099
38. Harte N, Silventoinen V, Quevillon E, Robinson S, Kallio K, Fustero X, Patel P, Jokinen P, Lopez R; European Bioinformatics Institute. Public web-based services from the European Bioinformatics Institute. Nucleic Acids Res 2004; 32(Web Server issue):W3-9; PMID:15215339; http://dx.doi.org/10.1093/nar/gkh405
39. Li L-C, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002; 18:1427-31; PMID:12424112; http://dx.doi.org/10.1093/bioinformatics/18.11.1427
40. Kumaki Y, Oda M, Okano M. QUMA: quantification tool for methylation analysis. Nucleic Acids Res 2008; 36(Web Server issue):W170-5; PMID:18487274; http://dx.doi.org/10.1093/nar/gkn294
41. Clark SJ, Statham A, Stirzaker C, Molloy PL, Frommer M. DNA methylation: bisulphite modification and analysis. Nat Protoc 2006; 1:2353-64; PMID:17406479; http://dx.doi.org/10.1038/nprot.2006.324
42. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform 2011; 44:839-47; PMID:21605702; http://dx.doi.org/10.1016/j.jbi.2011.05.002
43. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120:15-20; PMID:15652477; http://dx.doi.org/10.1016/j.cell.2004.12.035
44. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-8; PMID:11846609; http://dx.doi.org/10.1006/meth.2001.1262
45. Fuchsova B, Fernández ME, Alfonso J, Frasch AC. Cysteine residues in the large extracellular loop (EC2) are essential for the function of the stress-regulated glycoprotein M6a. J Biol Chem 2009; 284:32075-88; PMID:19737934; http://dx.doi.org/10.1074/jbc.M109.012377
46. Hua Y-J, Tang Z-Y, Tu K, Zhu L, Li Y-X, Xie L, Xiao H-S. Identification and target prediction of miRNAs specifically expressed in rat neural tissue. BMC Genomics 2009; 10:214; PMID:19426523; http://dx.doi.org/10.1186/1471-2164-10-214