Published in final edited form as:

Neurobiol Dis. 2015 August ; 80: 42–53. doi:10.1016/j.nbd.2015.05.006.

Elevated microRNA-181c and microRNA-30d levels in the enlarged amygdala of the valproic acid rat model of autism

N.F.M. Olde Loohuis^a, K. Kole^b, J.C. Glennon^a, P. Karel^a, G. Van der Borg^b, Y. Van Gemert^b, D. Van den Bosch^b, J. Meinhardt^b, A. Kos^a, F. Shahabipour^b, P. Tiesinga^b, H. van Bokhoven^{a,e}, G.J.M. Martens^c, B.B. Kaplan^d, J.R. Homberg^a, A. Aschrafi^{b,*}

^aDepartment of Cognitive Neuroscience, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Centre, Nijmegen, The Netherlands ^bDepartment of Neuroinformatics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen, Nijmegen, The Netherlands ^cDepartment of Molecular Animal Physiology, Donders Institute for Brain, Cognition and Behavior, Nijmegen Centre for Molecular Life Sciences (NCMLS), Radboud University Nijmegen, Nijmegen, The Netherlands ^dLaboratory of Molecular Biology, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892, USA ^eDepartment of Human Genetics, Radboud University Medical Centre, Nijmegen, The Netherlands

Abstract

Autism spectrum disorders are severe neurodevelopmental disorders, marked by impairments in reciprocal social interaction, delays in early language and communication, and the presence of restrictive, repetitive and stereotyped behaviors. Accumulating evidence suggests that dysfunction of the amygdala may be partially responsible for the impairment of social behavior that is a hallmark feature of ASD. Our studies suggest that a valproic acid (VPA) rat model of ASD exhibits an enlargement of the amygdala as compared to controls rats, similar to that observed in adolescent ASD individuals. Since recent research suggests that altered neuronal development and morphology, as seen in ASD, may result from a common post-transcriptional process that is under tight regulation by microRNAs (miRs), we examined genome-wide transcriptomics expression in the amygdala of rats prenatally exposed to VPA, and detected elevated miR-181c and miR-30d expression levels as well as dysregulated expression of their cognate mRNA targets encoding proteins involved in neuronal system development. Furthermore, selective suppression of miR-181c function attenuates neurite outgrowth and branching, and results in reduced synaptic density in primary amygdalar neurons *in vitro*. Collectively, these results implicate the small non-coding miR-181c in neuronal morphology, and provide a framework of understanding how

*Corresponding author at: Department of Neuroinformatics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands. a.aschrafi@donders.ru.nl (A. Aschrafi).

Author contributions

Conceived and designed the experiments: AA, NOL, JH. Performed the experiments: NOL, PK, GvB, YvG, AK, FS, KK, DvB, JM, AK, AA. Analyzed the data: AA, HvB, GJM, BBK, JH, PT. Wrote the paper: AA, GJM, BBK, JG, JH, NOL.

The authors have declared that no competing interests exist.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nbd.2015.05.006>.

dysregulation of a neurodevelopmentally relevant miR in the amygdala may contribute to the pathophysiology of ASD.

Keywords

Autism spectrum disorder; Valproic acid; Amygdala; MicroRNA; Gene networks; Neurodevelopmental disorder; Rat model of autism

Introduction

Autism spectrum disorders (ASD) constitute a heterogeneous group of severe neurodevelopmental disorders with a genetic predisposition and involvement of multiple environmental factors during embryonic and/or early postnatal life (Muller, 2007). ASD are characterized by impaired social interaction and communication, and by restricted and repetitive behavior, features that impair normal functioning. Brain imaging and genetic studies indicate that ASD are associated with impaired connectivity at the molecular, synaptic and neuronal systems levels (Parikshak et al., 2013; van Bokhoven, 2011). Besides genetic predisposition, ASD can also be triggered during pregnancy through the maternal use of medication. The anticonvulsant and mood-stabilizing drug valproic acid (VPA) is prescribed primarily for the treatment of severe forms of epilepsy and as a mood stabilizer in affective disorders. VPA increases the risk in the development of ASD in the unborn child when used in the first trimester of the pregnancy (Markram et al., 2007; Rasalam et al., 2005). In rodents, a single intraperitoneal injection of VPA to the pregnant dam at the time of embryonic neural tube closure increases the risk for ASD-like features in the offspring. Similar to human ASD patients, affected offspring exhibit brain stem injuries, diminished cerebellar Purkinje cell number (Ingram et al., 2000), social interaction deficits, enhanced anxiety, developmental delays, lowered pain sensitivity, impaired information processing and attention, and hyperactivity with lowered exploratory pathfinding (Markram et al., 2008; Schneider and Przewlocki, 2005). The VPA animal model is considered one of the best-validated models for ASD and is frequently used to study the alterations in brain development at the level of morphology, gene expression, and neuronal functioning (Silva et al., 2009; Snow et al., 2008).

The mechanism by which VPA induces ASD is not entirely understood. One of the proposed mechanisms is that VPA acts as a histone deacetylase inhibitor thereby inducing epigenetic alterations in the developing brain (Yildirim et al., 2003). In fact, studies have shown that histone acetylation modulates the transcription not only of mRNAs but also several microRNAs (miRs) (Lee et al., 2011). Given that a specific miR can affect the expression of several mRNAs while protein synthesis from a single mRNA can be repressed by several different miRs, a vast combinatorial complexity exists that may partly account for the genetic complexity associated with ASD. Indeed, such a large family of small non-coding genes (>2800) could explain some of the missing heritability of ASD (Gogos et al., 2010). Accordingly, multiple lines of evidence suggest that altered neuronal development and aberrant synaptic plasticity and morphology, as seen in neurodevelopmental disorders such as in ASD, may result from a common post-transcriptional process that is under tight

regulation by miRs (Schouten et al., 2013). However, little is known about the pattern of expression and the functions of these small RNA molecules in different brain regions of normally developing individuals or patients that suffer from ASD (Kaplan et al., 2013).

Post-mortem neuropathology studies on ASD brains have revealed anatomical abnormalities on multiple levels in various brain regions, including the amygdala (reviewed in (Markram et al., 2007)). Behaviorally, the difficulty in relating to others, anxiety and the incapability to form appropriate social interactions has been proposed to be a consequence of amygdala dysfunction in ASD (Rodriguez Manzanares et al., 2005). Amygdala volume and growth have been reported to be increased in early life in ASD (Bellani et al., 2013; Kim et al., 2010; Nordahl et al., 2012; Schumann et al., 2004). Moreover, the marked increase in amygdala size that normally occurs between the ages of 8 to 18 years is absent in ASD individuals (Schumann et al., 2004). In adolescence and adulthood, amygdala volume is reduced in autism patients compared to controls, although contrasting evidence demonstrating enlarged amygdala volumes at this stage in life has also been reported (Aylward et al., 1999; Bellani et al., 2013). At the level of neuronal functioning, the amygdala appears extremely hyperactive and hyperplastic while inhibition by interneurons is impaired in the VPA model for ASD (Markram et al., 2008).

In the present study, we examined the consequences of prenatal VPA exposure on amygdala size and transcriptome expression. The outcome of this study suggests an increase in basolateral nucleus of the amygdala (BLA) volume in animals with an ASD-like phenotype resulting from prenatal VPA exposure. Transcriptomic analysis identified a significant upregulation of miR-181c and miR-30d in the amygdala of VPA-exposed rats. Further in vitro and in silico analyses of miR-181c suggested a role for this small regulatory RNA, in modulating neuronal outgrowth and dendritic complexity as well as spine density in amygdalar neurons, by modulating an intricate gene network associated with neuronal differentiation and synaptogenesis.

Results

Abnormal development and behavior of rats prenatally exposed to VPA

Functionally, the amygdala has been linked to autism through its involvement with socio-emotional behavior (Markram et al., 2008). To examine differential gene expression during altered synaptic plasticity and morphology as seen in ASD most studies have focused on protein-coding genes. However, recent evidence suggests an involvement of non-coding miRs in modulating gene networks involved in proper neuronal outgrowth and synaptic function (reviewed in (Olde Loohuis et al., 2012)). Here we have employed the VPA rat as a model for ASD, and examined differential miR expression in the amygdala of VPA rats (Schneider et al., 2006). To confirm successful exposure to the teratogen, several developmental and behavioral tests were performed to examine the VPA-induced phenotype. Pregnant dams injected with saline had an average nest size of 9 ($n = 11$). Dams injected with VPA had an average nest size of 8 ($n = 10$). The pregnancies of teratogen- or saline solution-administered females were otherwise healthy. Mortality rate of these teratogen-treated rats is not different from the control group.

First we evaluated whether the developmental effects of VPA exposure corresponded to previously reported features of this animal model (Schneider and Przewlocki, 2005), we compared the developmental features after VPA or saline exposure. Initial visual observation did not reveal any abnormalities in rats prenatally exposed to VPA. As shown in Supplementary Fig. 1A (Fig. S1A), mean pup weights (control $n = 36$; VPA $n = 29$) were not significantly different at several time points during development. Assessment of motor performance and co-ordination in swimming performance revealed that VPA-exposed rats displayed a significantly ($p < 0.01$) lower performance on P12 when compared to the saline controls (Fig. S1B). However, on P8 and P10 swimming performance showed no significant difference. Average eye opening was significantly slower for VPA rats ($p < 0.001$) on P15–17, while neither group showed a significant difference on P13 and P14. On P18 all rats had opened both eyes (Fig. S1C).

We also compared the social play behavior during adulthood. The VPA adolescent rats showed a decrease in the duration of pinning as compared to control group ($p < 0.05$) (Fig. S1D). In addition, the duration of displaying non-social behavior was significantly increased due to prenatal VPA exposure ($p < 0.05$). As shown in Fig. S1E, the outcome of the sensimotor-gating prepulse inhibition revealed that prepulse inhibition was significantly decreased in the VPA group in the trial with a prepulse of 5 dB above background ($p < 0.5$), while both the trials with a prepulse of 10 dB louder than background and with a prepulse of 3 dB louder than background show a trend toward statistical significance (Fig. S1E). Assessment of anxiety in the elevated plus maze demonstrated a significant difference between the VPA and saline rats in the total time spent in the open arms. Although time spent in the closed arms was slightly higher for VPA rats it did not reach significance (Fig. S1F). Open field experiments failed to show a significant difference in activity or exploratory behavior between the VPA and saline rats. There was no significant difference between the time spent in the different regions of the open field (corners, sides or middle) (Fig. S1G), neither was there a significant difference in time spent in the “home base”, an area the rat establishes as its base during an open field test which it frequents the most, as seen in another study for this animal model (Mintz et al., 2005).

Size differences in the basolateral amygdala as a consequence of prenatal VPA exposure

Since amygdala size is reportedly changed in ASD, we examined whether the same observation can be made in the basolateral amygdala (BLA) of the VPA animal model. Our investigation revealed a significant decrease in overall hemisphere size in the rats that were prenatally exposed to VPA in comparison to saline-exposed rats. As shown in Fig. 1B, in all parts of the hemispheres (ventral, medial and dorsal) the size decrease was significant. This experiment further revealed an increased BLA size in the medial parts of the amygdala (Fig. 1C). To correct for the decrease in the size of the hemispheres the BLA/hemisphere ratio was determined; this calculation revealed a significant increase in the BLA in the medial parts of the amygdala in the VPA rats as compared to saline-injected control rats (Fig. 1D).

Differential expression of miR-181c and miR-30d in the amygdala of VPA rats

Based on the anatomical characteristics in the amygdala and altered behavior in the VPA rats that were observed in our studies, and which have been previously associated with autism

(Rodier et al., 1997), we hypothesized that altered amygdala miR levels, at least in part, may explain the observed VPA-induced phenotypes. We therefore tested this postulate by conducting a microarray analysis to identify the set of miRs differentially expressed in RNA samples derived from the amygdala of either control and VPA rats (P90, $n = 5$ for both groups). Expression levels of 199 miRs were detected of which we identified miR-181c and miR-30d to be significantly upregulated in VPA-exposed rats with a fold change above 1.2 (Fig. 2A; Table S1). Individual RNA samples and a second separate set of samples from control and VPA rats were used to validate the miR microarray results by qRT-PCR. This analysis showed a significant increase in miR-181c and miR-30d expression in the amygdala of VPA rats as compared to saline-injected controls (Fig. 2B). We chose miR-15b as an additional miR with unaltered expression levels in the microarray for qRT-PCR analysis; consistent with the results generated by the microarray analysis no significant change was observed for this miR using qRT-PCR.

Enrichment of expression changes in gene involved in neuronal differentiation in the amygdala of VPA rats

To elucidate the downstream gene networks relevant for amygdala development and maintenance under the control of miR-181c and miR-30d, we applied deep sequencing to measure the mRNA abundance in the amygdala of VPA and control rats (Table S2). Using a computational-based Ingenuity Pathway Analysis (IPA), we examined the ontology of mRNAs that displayed a changed expression due to prenatal VPA exposure. The analysis revealed that the majority of the altered genes could be grouped into defined molecular pathways, such as processes involving cell morphology, development, function and maintenance. In addition, the major physiological process affected by VPA exposure is neuronal system development and function (Table S3). Since previous reports suggested that mammalian miRs have the capacity to modulate gene expression by causing the degradation of their target mRNA (Bagga et al., 2005; Lim et al., 2005; Valles et al., 2014), we examined the correlation of these two significantly altered miRs in the VPA amygdala in modulating the expression of the putative target mRNAs identified in this brain region. Notably, 101 mRNAs differentially expressed in VPA amygdala contained at least one binding site for miR-181c, whereas 84 differentially expressed mRNAs possessed a binding site for miR-30d (Fig. 3A and Table S2).

To reveal the signaling networks associated with an enhancement of miR-181c or miR-30d expression, we studied those differentially expressed mRNAs in the amygdala of VPA and control rats which contained at least one binding site for either of these two miRs. Our IPA analysis revealed differentially expressed mRNAs with either a miR-181c or a miR-30d binding site associated with different gene expression signatures and functional categories (Figs. 3B, C). miR-30d target gene analysis revealed the involvement of few downstream genes predominantly associated with nervous system and development (Fig. 3C). Similarly, many of the processes involving genes that contain at least one miR-181c binding site were uniformly conserved with the processes involving developmental processes (Fig. 3B), suggesting that both miRs' deregulation may represent an important event during the course of VPA-induced, amygdala mal-development, resulting in ASD-like features in the animal model.

Effects of miR-181c manipulation on predicted miR-181c targets involved in neuronal development

In the past, a number of studies involving animal models or human tissues have demonstrated altered levels of both miR-181 and miR-30 family members in ASD and other neurodevelopmental disorders (Abu-Elneel et al., 2008; Beveridge and Cairns, 2012; Ghahramani Seno et al., 2011; Sarachana et al., 2010). miR-181c was identified as one of the miRs most strongly altered in its levels in individuals with several neurological disorders, and in post-mortems tissues of patients with ASD (Abu-Elneel et al., 2008). Since many of the predicted miR-181c targets are enriched in neuronal outgrowth processes, such as neuritogenesis and neurite outgrowth, we hypothesized that miR-181c is involved in modulating gene expression during amygdala development, and that miR-181c-mediated regulation of neurodevelopmentally-related genes are disturbed in the amygdala of VPA rats. To gain further insight into the molecular mechanisms underlying altered neuronal development associated with VPA-induced changes of miR-181c levels, we set out to identify the target mRNAs involved in mediating the effects of miR-181c. Initially, we examined mRNA expression changes in primary neurons (DIV21) by applying a miR-181c loss-of-function in neurons in vitro from DIV8 onwards. To selectively reduce endogenous miR-181c in primary neurons in vitro, we used a sequestration vector called “sponge-miR-181c” containing four partially complementary miR-181c binding sites (Ebert et al., 2007), following the coding sequence for enhanced green fluorescent protein (eGFP). The efficacy of this sponge-miR-181c in repressing miR-181c levels was examined. Neurons expressing this sponge exhibited lower miR-181c levels as compared to GFP expressing neurons (Fig. S2D). Moreover, cotransfection of this sponge together with a miR-181c mimic to overexpressing resulted in a reduction of eGFP fluorescence, as compared to transfection with a non-targeting (NT) oligonucleotide (Fig. S2C).

Following the validation of the miR sponge efficacy, we examined mRNA expression changes in primary neurons infected with lentiviruses encoding either sponge-miR-181c or eGFP at DIV8. Two weeks after infection, we isolated total RNA, converted it into a cDNA library and performed RNA sequencing (Hafner et al., 2008). Consistent with previous findings for other small RNAs, RNA sequencing revealed that modulating miR-181c levels caused alterations in hundreds of mRNAs in neurons, as compared to mock-infected neurons. We found that sequestering endogenous miR-181c, using sponge-miR-181c, resulted in significant alterations of 1596 transcripts (716 genes upregulated, and 880 genes downregulated). We used a reads per kilobase of exon per million mapped reads (RPKM) threshold of 1 and a 1.2 fold change cut-off and quantified the number of up- or downregulated transcripts for miR-181c inhibition in neurons. Using this cut-off, miR-181c suppression enhanced the levels of 227 transcripts and decreased the levels of 530 mRNAs (Table S4). Among these transcripts, we identified 85 differentially expressed genes identified in our amygdala whole transcriptomics data set (Fig. 4A). A total of 68 differentially expressed transcripts (8.2%) contained at least one miR-181c binding site as predicted by miRWalk, among these seven transcripts were previously found altered in the amygdala of the VPA animals. Next, to select the most relevant functional gene pathways we used IPA gene ontology (GO) analysis, and examined the cellular pathways enriched upon inhibition of miR-181c. In sponge-miR-181c infected neurons, we found an enrichment of

genes involved in cellular assembly and modification, cell morphology, cell death and survival, cellular growth and proliferation and cellular development. In the physiological system, nervous system and development were the terms involving by far most dysregulated genes (Fig. 4B). Ingenuity-based subprocess analysis of the genes affected by miR-181c inhibition revealed a large number of genes involved dendritic growth and branching, and spine development, with the central genes being Akap5, ApoE, Grasp, Notch1, Ngr1 and S100b (Fig. 4C).

miR-181c modulates neuronal outgrowth and synapse density in cultured amygdalar neurons

The outcome of the bioinformatics analyses of the putative dysregulated miR-181c targets following loss-of-function of this miR suggested that some of the most significantly enriched functional gene categories are directly related to the dendritic growth and branching, as well as synapse maturation and function (Fig. 4C). This finding led us to functionally evaluate the role of miR-181c in modulating dendritic growth and synaptic density. We performed a loss-of-function analysis using sponge-miR-181c in cultured primary amygdalar neurons. miR-181c-sponge virus infected amygdalar neurons exhibited decreased total dendritic length (Fig. 5B). Detailed branching studies showed that the reduction in dendritic branching is mainly arising from a decrease of the secondary, tertiary and quaternary branches (Fig. 5D). To determine whether miR-181c also affects the process of synapse formation, we measured the effects of selective miR-181c repression on the number of dendritic spines. Cultured amygdalar neurons were infected with either eGFP control, or sponge-miR-181c encoding viruses. Analysis of the images from GFP-positive 21 DIV neurons obtained using confocal laser-scanning microscopy revealed that the spine density of sponge-miR-181c infected cells was significantly decreased as compared to control virus infected neurons (Fig. 5E), indicating that miR-181c plays a positive regulatory role on synapse formation and maturation.

Materials and methods

Animals and VPA treatment

Wistar rats (Harlan laboratories, USA) were housed individually on a 12-h light cycle in a temperature-controlled (21 ± 1 °C) environment with access to food and water ad libitum. Rats were mated overnight. If a vaginal plug was found, that day was designated as embryonic day (ED) 1. Valproic acid sodium salt (Sigma Aldrich, Germany) was dissolved in 0.9% saline to a concentration of 150 mg/ml (pH = 8.3). The dosing volume was 3.3 ml/kg, and the dosage was adjusted according to the body weight of each rat on the day of injection. At ED 12.5 pregnant females received a single intraperitoneal injection (ip) of 495 mg/kg VPA sodium salt; control pregnant rats were injected with the same volume of physiological saline. The day of birth was designated postnatal day 1 (P1). Dams were allowed to raise their own young until weaning on P23. From P23 onwards animals were housed in pairs. All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands.

Growth, development, and swimming performance

Pups were weighed at P7, P14, and P26 and eye opening was checked at P12–18 (0, 1 or 2 eyes open). To measure motor development, a swimming test was performed in warm water (25–30 °C) at P8, P10, P12 and P14. Each animal was placed into the center of the aquarium and observed for 5–10 s. Swimming performance was measured as follows: 0—head and nose below surface, 1—nose below surface, 2—nose and top of head at or above water level but ears still submerged, 3—nose and top of head at or above water level waterline are at mid ear level, 4—nose and top of head at or above water level waterline are at the base of the ears.

Behavioral tests and procedures

Social play behavior—Social play experiments were conducted on rats at P30–35. Experiments were performed under dimmed light conditions (5 lx) in a clear acrylic PhenoTyper basic cage (Noldus, Wageningen, The Netherlands) measuring 45 cm × 45 cm × 60 cm of which the floor was covered with black bedding. Social play activities were recorded using an infrared camera (CPcam, Richmond, BC Canada). Preceding these tests rats were allowed a 10 min individual habituation to the test environment. On the day of the experiment, rats were socially isolated for 3.5 h prior to the test to increase social play interactions during the test (Niesink and Van Ree, 1989). Two animals were paired by treatment and gender and came from different home cages into the test cage for 15 min. Behavior was scored using the observer 5.0 software (Noldus, Wageningen, The Netherlands).

Elevated plus maze—Elevated plus maze experiments were conducted at P37–38. This experiment was performed under dimmed light conditions resulting in a light intensity of 0.8 lx in the closed and 3.0 lx in the open arms. The maze was custom made from polyvinylchloride and was elevated 65 cm above the floor and consisted of two opposite open arms (50 cm × 10 cm) and two opposite enclosed arms (50 cm × 10 cm × 40 cm). Animals were placed in the center of the maze, facing an open arm and were left to explore the maze for 5 min. Animal movement was captured using an infrared camera (Sony, Japan) positioned above the maze. Mean time spent in the different compartments was measured by scoring using The Observer 5.0 software (Noldus, Wageningen, The Netherlands).

Prepulse inhibition—Prepulse inhibition experiments were performed in rats at P39–40. The SR-LABtm startle response system (San Diego Instruments, CA, USA) was used to determine sensorimotor gating. The animals were placed in a Plexiglas cylinder that was placed in a sound-attenuating cubicle. The cylinder stood on a platform equipped with a pressure sensor measuring the startle response. The cubicle was equipped with a house light, a fan providing a background noise of 70 dB, and a speaker to deliver auditory stimuli. Each test session consisted of 25 trials and was preceded with a 5 min habituation period. Trials were presented in a pseudorandom order and consisted of no pulse (control), a 120 dB pulse without prepulse (baseline) or a 120 dB pulse with a 3, 5 or 10 dB above background prepulse. Pulses were presented for 20 ms with an interpulse interval of 80 ms. The readout for sensorimotor gating was defined as the average percentage of startle inhibition during a prepulse trial compared to the startle response during a no prepulse trial.

Open field tests—Open field experiments were performed in rats at P42–46 under light conditions. The open field consisted of a Perspex cube with open top (50 cm × 50 cm × 50 cm). Animals were placed in the center of the field and left to freely explore their environment for 30 min. Movement and location of the animal were recorded and scored real time by 2 independent automated scoring systems: Ethovision 3.1 (Noldus, Wageningen, The Netherlands), and Open Field as previously described (Cools et al., 1990).

Amygdala collection—Rats were sacrificed at P90. For RNA collection animals were sacrificed using decapitation, brains were rapidly frozen in liquid nitrogen, and stored at –80 °C until further tissue processing. Brains were processed into 200 µm slices at –15 °C using a microtome, and 1.2 mm amygdala punches were made. Brain punches were stored at –80 °C in RNAlater (Ambion, Carlsbad, CA, USA) until RNA isolation. For immunocytochemistry, animals were perfused with 4% paraformaldehyde (PFA) and brains were stored in 4% PFA at 4 °C overnight. Next day, PFA was replaced by PBS and brains were stored in PBS at 4 °C until further processing.

Amygdala volume measurements by cresylviolet staining

Perfused brains of P90 VPA rats were processed into 40 µm coronal slices using a slicing microtome. Brain slices containing the amygdala were selected for cresylviolet staining. The slices were mounted onto 0.5% gelatin pre-coated microscope glasses and dried overnight at 40 °C. The next day, a 5 min 0.5% gelatin overcoating was performed and slices were dried overnight at 40 °C. The following protocol was used for the cresylviolet staining: 10 min 96% alcohol, 2 min 90%; 80%; 70%; 50% alcohol, 2 min demi-water, 10 min 0.1% cresylviolet, 2 min 50%; 70%; 80%; 90%; 96% alcohol, 1.5 min acidified alcohol, 2 min 100% alcohol, 2 × 5 min xylene. Slices were mounted in entalan.

Imaging of the brain slices and image analysis

Images were made with a Leica stereomicroscope (Jena, Germany), using the LAS software. A 0.8× zoom was used to image the left- and right hemispheres, and 2.0× and 1.25× zooms were used to image the amygdala. FIJI analysis software was used to measure the size of the each hemisphere, as well as the basolateral nucleus of the amygdala (BLA). The measurements were divided into three categories: ventral-, medial-, and the dorsal BLA. The two-tailed Student's *t*-test was used to test for significance. Error bars represent the standard error of the mean.

RNA isolation

Total RNA was extracted from either amygdala punches or from primary neurons using the miRNeasy Mini kit (Qiagen, Germany) according to manufacturer's guidelines. Small RNA fractions were enriched using the miRNeasy kit (Qiagen, Germany). RNA quality assessment was done using the Agilent Tapestation.

miR microarray

Total RNA containing low molecular weight RNA was labeled using the Flashtag RNA labeling kit (Genisphere, Hatfield, PA, USA) according to the manufacturer's instructions.

Briefly, for each sample, 400 ng total RNA was subjected to a tailing reaction (2.5 mM MnCl₂, ATP, Poly A Polymerase — incubation for 15 min at 37°) followed by ligation of the biotinylated signal molecule to the target RNA sample (1× Flash Tag ligation mix biotin, T4 DNA ligase — incubation for 30 min at RT (23 °C) and adding of stop solution. Each sample was hybridized to a GeneChip® miR v2 Array (Affymetrix, Santa Clara, CA, USA) at 48 °C and 60 rpm for 16 h then washed and stained on Fluidics Station 450 (Fluidics script FS450_0003) and finally scanned on a GeneChip® Scanner 3000 7G (Affymetrix). The image data were analyzed with the miR QC Tool software for quality control (www.affymetrix.com).

Rat primary neurons

All media and reagents were purchased from Life Technologies (Carlsbad, CA, USA). Dissociated amygdalar cultures were prepared from gestational day 18 fetal rats (Wistar). Pregnant rats were anesthetized with isoflurane and sacrificed by cervical dislocation. Embryonic brains were dissected in ice-cold Hank's buffer (HBSS without Ca²⁺ and Mg²⁺, 100 U/ml penicillin, 100 µg/ml streptomycin, 1× GlutaMax). Isolated amygdala was separated and incubated with 0.025% trypsin for 15 min at 37 °C as described in Lorenzo et al. (Lorenzo et al., 1992). Neurons were mechanically dissociated in NeuroBasal medium containing 10% FCS and 1× GlutaMax using fire-polished Pasteur pipettes. The cell suspension was filtered through a 70 µm cell strainer (BD Falcon) and plated on poly-D-lysine (Invitrogen, 70,000–150,000) pre-coated glass coverslips in 24-well plates at a density of 25,000 neurons per well. Medium was replaced after 6 h with Neurobasal medium containing B27 supplement, and 1× GlutaMax. Neurons were maintained in a 5% CO₂ humidified 37 °C incubator. One-third of the medium was refreshed every 3 to 4 days.

Sponge-miR-181c construct

The sponge-miR-181c sequence was designed based on the rat miR-181c-3p sequence according to the original publication describing the application of miR sponge (De Preter et al., 2002; D. Gentner et al., 2009). Briefly, the sponge-miR-181c-3p sequence contains four consecutive miR-181c-3p binding sites, each separated by four nucleotides. The miR-181c binding site contains bulged sites that are non-complementary to the miR-181c, in order to enhance sponge efficacy. Sponge-miR-181c oligonucleotides contain recognition sequences for the restriction enzymes BsrGI and EcoRI. Oligonucleotides (Sigma) were annealed in annealing buffer (containing 10 mM Tris, pH7.5, 50 mM NaCl, 1 mM EDTA) through a 5 min incubation at 90 °C followed by a slow cool down to room temperature. Annealed sponge-miR-181c oligonucleotides were ligated into the FUGW vector (Plasmid 14883, Addgene) directly following the stop codon of the eGFP cDNA.

Assessment of sponge-miR-181c function

The direct coupling of the concatemeric sponge-miR-181c sequence to the 3' site of the GFP cDNA allows efficient binding of miR-181c to the GFP-sponge-miR-181c encoding transcript, ultimately resulting in an attenuation of GFP translation. Reduced GFP fluorescent signal intensity can therefore be used as a measure of sponge-miR-181c functional efficacy (Fig. S1). For GFP quantification, the sponge-miR-181c-containing vector was transfected into HEK cells together with either a non-targeting (nt) mimic or a

mimic-miR-181c oligonucleotides using Lipo-fectamine 2000 (Life Technologies), according to manufacturer's instructions. Forty-eight hours post-transfection, cells were fixed in 4% PFA/sucrose in PBS and visualized with a Leica DFC 420C fluorescence microscope using a 20× magnification. GFP quantification was performed using the ImageJ software. We also detected significantly reduced endogenous miR-181c levels in sponge-miR-181c infected neurons as compared to GFP-infected neurons as measured by qPCR, suggesting that the sponge not only sequesters miR-181c, but also results in an enhanced turnover rate of this miR.

Virus production

Viral particles were prepared by cotransfection of HEK-293T cells with a FUGW plasmid, the HIV-1 packaging vector 8.91, and the VSVG envelope glycoprotein vector using the calcium phosphate co-precipitation method. HEK293T cells were placed overnight in a 3% CO₂ humidified 37 °C incubator. The following day, medium was replaced by fresh ultraculture (Lonza) containing 4 mM valproate (Sigma) and the CO₂ percentage was set to 10% as described (Aydin et al., 2012). Supernatants were collected 24 h and 48 h later. Viral particles were purified over a 20% sucrose cushion by ultracentrifugation for 3 h at 21,000 RPM. Viral particles were resuspended in PBS without Ca²⁺ and Mg²⁺ (Life Technologies), and stored at -80 °C until use.

Quantitative real-time PCR (qRT-PCR)

Five hundred nanograms of DNase-treated, total RNA from each sample was used for cDNA synthesis, using either the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas Inc., USA) or the miScript Reverse Transcription Kit II (Qiagen), for mRNAs and for precursor (pre-) and mature (mat-) miRs, respectively. RNA input was kept identical between cDNA synthesis reactions. Prior to qRT-PCR analysis, each cDNA sample was diluted 1/10 with MilliQ water. QRT-PCR was performed according to previously described protocols (Schmittgen et al., 2008; Schmittgen and Livak, 2008), using standard cycling conditions, and performing a melting protocol to control for product specificity. U6 and β -actin were used as housekeeping genes and normalization was performed using GeNorm taking amplification efficiency into account. The following gene-specific primers were used (all sequences in 5' → 3' direction): β -Actin forward, CCAGATCATGTT TGAGACCTTC; β -actin reverse, AGGATCTTCATGAGGTAGTCTG; U6 forward, GCTTCGGCAGCA CATATA; U6 reverse, CGCTTCACGAATTTGCGT; mature rno-miR-181c, AACATTCAACCTGTCGGTGAGT; mature rno-miR-30d forward, TGTAACATCCCCGACTGGAAG; mature rno-miR-15b, TAGCAGCACATCATGGTTTACA

RNA sequencing

Total RNA and small RNA fractions isolated as described above from either the amygdala of the VPA- and saline-treated rats at P90, or RNA isolated from primary cortical neurons (at 21 DIV) infected either with control or sponge-miR-181c lentiviruses at 8 DIV. RNA quality was assessed using TapeStation analysis, which revealed RNA quality above RIN 8.0 for all samples. RNA samples from the same condition were pooled and further processed by the HudsonAlpha Institute for Biotechnology (Huntsville, AL, USA). The RNA sequencing was

performed in three separate flowcells generating a total of 45 to 50 million 50 bp reads per sample. RNA sequencing data analysis was carried out with the GeneSifter gene expression analysis suite (Geospiza). Expression data was normalized against the number of mapped reads, and the RPKM threshold was set at 1. Significance was calculated using the Benjamini and Hochberg multiple testing correction and the level of significance was set to 0.05.

miR target prediction

The miRwalk prediction database was used to identify predicted targets of the miRs we found significantly dysregulated due to prenatal VPA exposure. miRwalk combines the prediction data of several databases from which we included TargetScan, miRanda, miRDB and miRWalk prediction lists taking into account a minimal seed length of 7 base pairs and a *p*-value cut-off of 0.05.

Functional classification of miR targets

To detect significantly enriched gene categories in the predicted miR target genes/mRNAs, we used the Ingenuity Pathway Analysis (IPA) software package (<http://www.ingenuity.com>). This software package uses the Ingenuity Knowledge Base, which is based on information from the published literature as well as many other sources, including gene expression and gene annotation databases, to assign genes to different groups and categories of functionally related genes. Each of these categories can be further divided into many subcategories. Ingenuity calculates single *p*-values for the enrichment of each gene category using the right-tailed Fisher's exact test, and taking into consideration both the total number of molecules from the analyzed data set and the total number of molecules that is linked to the same gene category according to the Ingenuity Knowledge Base. Furthermore, for each gene category, a multiple testing corrected *p*-value of enrichment, calculated using the Benjamini–Hochberg correction, is provided.

Immunocytochemistry and image analysis

Immunocytochemistry on primary neurons—For outgrowth analysis, primary neurons were infected 1 day after plating using control and sponge-miR-181c expressing viruses containing a GFP reporter gene. At DIV9 neurons were fixed in 4% PFA/sucrose in PBS for 20 min at room temperature. Following 3 washes in PBS, PFA was neutralized using 50 mM NH₄Cl for 15 min. Neurons were washed in PBS and permeabilized in 0.1% triton X-100 for 5 min, washed again, and blocked with 1% BSA in PBS for 30 min. Neurons were incubated overnight with anti-GFP (diluted 1/500, A10262 Life Technologies) and/or anti-MAP2 (diluted 1/2000, Abcam ab32454) in 1% BSA at 4 °C. Secondary antibody incubation was done the next day using Alexa-488 (Molecular Probes) diluted 1/200 in 1% BSA at room temperature for 1 h. After washing, coverslips were mounted in ProLong Gold (Life Technologies). For spine analysis, primary neurons were infected at DIV8 and allowed to grow for 2 more weeks. At DIV21, neurons were fixed and stained according to the protocol described above.

Image analysis—Images of infected neurons were captured using a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope (CLSM) at 63× magnification. For outgrowth

analysis, the ImageJ plugin NeuronJ was used with which neurons were traced and neurites were assigned with the correct branch order. For dendritic spine analysis, spines were imaged using CLSM above using an optical zoom of eight. Neuronstudio software was used to quantify spine density by determining the amount of spines per μm of dendrite.

Discussion

By means of a multidisciplinary approach, the goal of our investigation was to study the effects of prenatal administration of VPA on the anatomical and molecular properties of the amygdala of VPA-exposed rats. We hypothesized that many of the anatomical and molecular changes seen in the altered amygdala size and functionality in the VPA rats and in human possibly underlie changes in the levels of regulatory small miRs, involved in modulating critical gene networks and consequently altered neural growth throughout amygdala development. Quantitative analysis of the BLA of adolescent male VPA rats revealed an increase in the size, with a concomitant reduction of brain hemispheres in prenatally exposed animals compared to controls. The increase in the BLA size is in keeping with previous studies using magnetic resonance imaging scan in toddlers and adolescents showing that patients with autism have a larger amygdala compared to their age-matched controls (Bellani et al., 2013; Schumann et al., 2009). In addition, from animal studies it became apparent that amygdala neurons display an increased dendritic length as well as hyperarborization, possibly contributing to an increased BLA volume (Bringas et al., 2013; Sosa-Diaz et al., 2014).

In addition to altered branching, the density of neurons could lead to an increased volume of a brain region (Schumann and Amaral, 2006). In animal studies, valproate has been shown to increase levels of the neuroprotective protein Bcl-2 in the frontal cortex, as well as the levels of the anti-apoptotic Bcl-XL in neuronal progenitor cells derived from VPA-exposed rat embryos leading to a higher number of neuronal cells at later ages (Chen et al., 1999; Go et al., 2012). In agreement with this finding, hippocampi of adult rats showed increased neuronal densities as a consequence of prenatal VPA exposure (Edalatmanesh et al., 2013). Besides affecting neuronal development and survival, VPA is able to influence gene expression levels in all types of glial cells (reviewed by Dietz and Casaccia, 2010), thereby not only regulating their differentiation and survival but also indirectly influencing the neuronal circuitries of the brain (Dietz and Casaccia, 2010). VPA in addition modulates microglial responses to inflammatory insults, suggesting that VPA can regulate neuronal degenerative and regenerative processes through microglial activation (Singh et al., 2014).

In the VPA animal model for autism, the amygdala was found hyperactive, due to enhanced synaptic plasticity in combination with impaired inhibition, possibly inducing the enhanced fear memories observed in the VPA-exposed animals (Markram et al., 2008). Previously, it has been suggested that gene defects inherent to ASD result in the development of a larger and more active amygdala (Schumann and Amaral, 2006). A more active amygdala may underlie elevated levels of fear and anxiety noted in ASD, as well as a heightened and chronic stress response (Markram et al., 2007).

The concept that the amygdala may undergo an abnormal pattern of development suggested that this brain region may undergo aberrant changes in gene expression and possibly altered neural and synaptic outgrowth (Bringas et al., 2013). Interestingly, conditional Dicer KO mice which are unable to process precursor miRs into their mature form, display an array of CNS phenotypes including microcephaly, reduced dendritic branch elaboration, and large increases in dendritic spine length (Davis et al., 2008). In addition, it has been demonstrated that synaptic activity, inducing LTP or LTD, rapidly alters the level of more than 50 different miRs, among them miR-181b, in hippocampal neurons (Park and Tang, 2009). These findings, along with a previous study on altered miR levels in the hippocampus of VPA-exposed rats (Zhou et al., 2009), prompted us to examine the levels of miRs and their target mRNAs in the amygdala of VPA rats. Surprisingly, we have detected only a relatively modest increase in the expression levels of two miRs, miR-181c and miR-30d. Literature investigation suggested altered expression of members of these miR families in various neurodevelopmental disorders, including schizophrenia and ASD (Abu-Elneel et al., 2008; Beveridge and Cairns, 2012; Ghahramani Seno et al., 2011; Zhou et al., 2009). For example, an expression profiling of mammalian miRs uncovered the miR-30 family, together with a sub-set of other brain-expressed miRs with possible roles in murine and human neuronal differentiation (Sempere et al., 2004). Moreover, potential sites for miR-30 family members were found on Myc and Cyclin D1 3' UTRs suggesting that translational repression by this miR family could contribute to the downregulation of growth and cell cycle regulators during neuronal differentiation. Consistent with this finding, our IPA revealed various differentially expressed mRNAs with a miR-30d binding site associated with different gene expression signatures associated with processes involving cell division and proliferation, as well as nervous system function and development. Previous bioinformatics investigation of the downstream mRNA targets of miR-30 identified several mRNAs, including synapsin-1, which has been associated with schizophrenia and different intellectual disability disorders (ID) (Fassio et al., 2011; Tabuchi et al., 2007; Zhang et al., 2009).

Consistent with our findings of altered miR-181c levels in the amygdala of the VPA rats, it was previously reported that this miR along with miR-486 and miR-181b were strongly elevated miRs in 18 different cell lines derived from ASD families (Ghahramani Seno et al., 2011). Using the miR-181c-sponge, we have identified many gene networks associated with neuronal development and synaptic function to be under the regulatory control by miR-181c. Six of these genes were found essential for dendritic growth and dendritic branching as well as synapse functioning, namely *Akap5*, *ApoE*, *Grasp*, *Notch1*, *Nrg1* and *S100b*. Deregulated expression of a combination of these genes could lead to disturbed neuronal development at the level of synaptic plasticity as well as neurite outgrowth as seen in amygdalar neurons upon repression of miR-181c. *S100B* is a glial protein expressed in astrocytes able to modulate long-term synaptic plasticity (Nishiyama et al., 2002), as well as axonal proliferation in different types of neurons as was shown in transgenic *S100B* mice (Reeves et al., 1994). These findings support the idea that neuronal development and functioning can be affected by miR-181c inhibition through the modulation of the expression of specific genes in glial cells. *Nrg1*, *Notch1*, *Grasp*, *Akap5* and *ApoE* are neuronal factors of which the expression levels were altered as a consequence of miR-181c inhibition. The importance of *Nrg1* was previously shown in studies linking deregulation of this gene and its receptor

ErbB4 to several psychiatric disorder-like behaviors including anxiety and deficits in prepulse inhibition, both found affected in our autism model (Shamir et al., 2012). Notch signaling occurring at the level of the synapse is positively regulated by the neuronal activity-induced protein Arc (Alberi et al., 2011), and we found that its expression levels were also affected by miR-181c. *Notch1* conditional deletion disrupts both LTP and LTD and leads to deficits in memory formation (Alberi et al., 2011). Moreover, *Notch1* signaling during neuronal differentiation which involves translation of *Notch1* to the nucleus promotes dendritic branching and inhibits dendritic growth (Redmond et al., 2000). On the other hand, *ApoE* decreases dendritic complexity as well as spine density (Dumanis et al., 2009, 2011). In addition, this gene reduces glutamate receptor functioning and thereby impairs synaptic plasticity (Chen et al., 2010).

miR-181a, which shares the same seed sequence with miR-181c, has been shown to be a negative regulator of GluA2 expression, resulting in altered GluA2 surface expression, spine formation, and miniature excitatory postsynaptic current (mEPSC) frequency in hippocampal neurons (Saba et al., 2012). Notably, numerous studies suggested that synaptogenesis and normal synaptic function are dependent on the activity of a large number of proteins, and that disturbance of individual components within the network, or alterations of their activities cause synaptic dysfunction, phenotypically culminating in ASD and other IDs (Aschrafi et al., 2005; Nadif Kasri et al., 2011). In line with this, changes in spine dimensions and density have been associated with deficits in synaptic plasticity and learning, as well as with neurological disorders, including ID (Comery et al., 1997). At first glance, it may seem surprising to find that most of the dysregulated mRNAs in our sponge-miR-181c dataset do not contain a binding site for this miR. However, as was published recently, in addition to their direct targeting capacity as described above, miRs can regulate gene expression in a non-canonical manner (Helwak et al., 2013; Wang et al., 2010). This mode of action that also becomes apparent from our dataset suggests additional regulatory roles for miRs, possibly in controlling the activity of entire pathways functioning in many different cellular processes. It is also conceivable that a significant proportion of the altered transcripts were modulated by miR-181c via a secondary downstream effect. In addition, these data underline the importance of high-throughput experimental studies in addition to target prediction algorithms.

Several studies have now indicated altered levels of brain-specific small miR in ID and other neurodevelopmental disorders (Valles et al., 2014; Willemsen et al., 2011). Most recently, it has also become apparent that regulation of gene transcripts by miRs may represent a key regulatory process in the control of synaptic activity, as several studies have now indicated altered levels of brain-specific miRs in ID and other neurodevelopmental disorders (reviewed in (Olde Loohuis et al., 2012)). Our miR-181c loss-of-function experiment suggested that this small regulatory RNA has the capacity to modulate neuronal branching and synaptogenesis in vitro. Together with the results of others (Bringas et al., 2013), revealing age-dependent changes in dendritic trees and spine densities in the BLA of VPA-exposed rats, it is tempting to speculate that the behavioral and anatomical alterations observed in the VPA rats were at least in part explained by the altered robustness of miR-181c- and miR-30d-modulated gene networks involved in neuronal outgrowth and synaptic function in amygdala.

Conclusions

In conclusion, prenatal VPA exposure induced significant changes in miR and mRNA expression profiles of the rat amygdala, most likely underlying many of its anatomical and functional alterations that have also been observed in autistic individuals. In particular, dysregulation of miR-30d and miR-181c may alter the dynamics of critical gene networks involved in amygdala development and functionality, thereby contributing to the pathophysiology of ASD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Donders Center for Neuroscience fellowship award of the Radboud University Nijmegen Medical Centre and the FP7-Marie Curie International Reintegration Grant to A. Aschrafi.

References

- Abu-Elneel K, et al., 2008 Heterogeneous dysregulation of microRNAs across the autism spectrum. *Neurogenetics* 9, 153–161. [PubMed: 18563458]
- Alberi L, et al., 2011 Activity-induced Notch signaling in neurons requires Arc/Arg3.1 and is essential for synaptic plasticity in hippocampal networks. *Neuron* 69, 437–444. [PubMed: 21315255]
- Aschrafi A, et al., 2005 The fragile X mental retardation protein and group I metabotropic glutamate receptors regulate levels of mRNA granules in brain 8. *Proc. Natl. Acad. Sci. U. S. A* 102, 2180–2185. [PubMed: 15684045]
- Aydin H, et al., 2012 A convenient and general expression platform for the production of secreted proteins from human cells. *J. Vis. Exp* 65 pii: 4041.
- Aylward EH, et al., 1999 MRI volumes of amygdala and hippocampus in non-mentally retarded autistic adolescents and adults. *Neurology* 53, 2145–2150. [PubMed: 10599796]
- Bagga S, et al., 2005 Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 122, 553–563. [PubMed: 16122423]
- Bellani M, et al., 2013 Brain anatomy of autism spectrum disorders II. Focus on amygdala. *Epidemiol. Psychiatr. Sci* 22, 309–312. [PubMed: 23815810]
- Beveridge NJ, Cairns MJ, 2012 MicroRNA dysregulation in schizophrenia. *Neurobiol. Dis* 46, 263–271. [PubMed: 22207190]
- Bringas ME, et al., 2013 Rearrangement of the dendritic morphology in limbic regions and altered exploratory behavior in a rat model of autism spectrum disorder. *Neuroscience* 241, 170–187. [PubMed: 23535253]
- Chen G, et al., 1999 The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS. *J. Neurochem* 72, 879–882. [PubMed: 9930766]
- Chen Y, et al., 2010 ApoE4 reduces glutamate receptor function and synaptic plasticity by selectively impairing ApoE receptor recycling. *Proc. Natl. Acad. Sci. U. S. A* 107, 12011–12016. [PubMed: 20547867]
- Comery TA, et al., 1997 Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. *Proc. Natl. Acad. Sci. U. S. A* 94, 5401–5404. [PubMed: 9144249]
- Cools AR, et al., 1990 Search after neurobiological profile of individual-specific features of Wistar rats. *Brain Res. Bull* 24, 49–69. [PubMed: 2310946]
- Davis TH, et al., 2008 Conditional loss of Dicer disrupts cellular and tissue morphogenesis in the cortex and hippocampus. *J. Neurosci* 28, 4322–4330. [PubMed: 18434510]

- De Preter K, et al., 2002 Quantification of MYCN, DDX1, and NAG gene copy number in neuroblastoma using a real-time quantitative PCR assay. *Mod. Pathol* 15, 159–166. [PubMed: 11850545]
- Dietz KC, Casaccia P, 2010 HDAC inhibitors and neurodegeneration: at the edge between protection and damage. *Pharmacol. Res* 62, 11–17. [PubMed: 20123018]
- Dumanis SB, et al., 2009 ApoE4 decreases spine density and dendritic complexity in cortical neurons in vivo. *J. Neurosci* 29, 15317–15322. [PubMed: 19955384]
- Dumanis SB, et al., 2011 ApoE receptor 2 regulates synapse and dendritic spine formation. *PLoS One* 6, e17203. [PubMed: 21347244]
- Ebert MS, et al., 2007 MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat. Methods* 4, 721–726. [PubMed: 17694064]
- Edalatmanesh MA, et al., 2013 Increased hippocampal cell density and enhanced spatial memory in the valproic acid rat model of autism. *Brain Res* 1526, 15–25. [PubMed: 23806776]
- Fassio A, et al., 2011 SYN1 loss-of-function mutations in autism and partial epilepsy cause impaired synaptic function. *Hum. Mol. Genet* 20, 2297–2307. [PubMed: 21441247]
- Gentner B, et al., 2009a Stable knockdown of microRNA in vivo by lentiviral vectors. *Nat. Methods* 6, 63–66. [PubMed: 19043411]
- Gentner D, et al., 2009b Reviving inert knowledge: analogical abstraction supports relational retrieval of past events. *Cogn. Sci* 33, 1343–1382. [PubMed: 21585509]
- Ghahramani Seno MM, et al., 2011 Gene and miRNA expression profiles in autism spectrum disorders. *Brain Res.* 1380, 85–97. [PubMed: 20868653]
- Go HS, et al., 2012 Prenatal exposure to valproic acid increases the neural progenitor cell pool and induces macrocephaly in rat brain via a mechanism involving the GSK-3 β /beta-catenin pathway. *Neuropharmacology* 63, 1028–1041. [PubMed: 22841957]
- Gogos JA, et al., 2010 MicroRNAs in psychiatric and neurodevelopmental disorders. *Brain Res.* 1338, 78–88. [PubMed: 20388499]
- Hafner M, et al., 2008 Identification of microRNAs and other small regulatory RNAs using cDNA library sequencing. *Methods* 44, 3–12. [PubMed: 18158127]
- Helwak A, et al., 2013 Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. *Cell* 153, 654–665. [PubMed: 23622248]
- Ingram JL, et al., 2000 Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. *Neurotoxicol. Teratol* 22, 319–324. [PubMed: 10840175]
- Kaplan BB, et al., 2013 MicroRNAs in the axon and presynaptic nerve terminal. *Front. Cell. Neurosci* 7, 126. [PubMed: 23964201]
- Kim JE, et al., 2010 Laterobasal amygdalar enlargement in 6- to 7-year-old children with autism spectrum disorder. *Arch. Gen. Psychiatry* 67, 1187–1197. [PubMed: 21041620]
- Lee S, et al., 2011 Histone deacetylase regulates high mobility group A2-targeting microRNAs in human cord blood-derived multipotent stem cell aging. *Cell. Mol. Life Sci* 68, 325–336. [PubMed: 20652617]
- Lim LP, et al., 2005 Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433, 769–773. [PubMed: 15685193]
- Lorenzo A, et al., 1992 Amygdala neurons in vitro: neurite growth and effects of estradiol. *J. Neurosci. Res* 33, 418–435. [PubMed: 1469745]
- Markram H, et al., 2007 The intense world syndrome—an alternative hypothesis for autism. *Front. Neurosci* 1, 77–96. [PubMed: 18982120]
- Markram K, et al., 2008 Abnormal fear conditioning and amygdala processing in an animal model of autism. *Neuropsychopharmacology* 33, 901–912. [PubMed: 17507914]
- Mintz M, et al., 2005 Sharing of the home base: a social test in rats. *Behav. Pharmacol* 16, 227–236. [PubMed: 15961962]
- Muller RA, 2007 The study of autism as a distributed disorder. *Ment. Retard. Dev. Disabil. Res. Rev* 13, 85–95. [PubMed: 17326118]

- Nadif Kasri N, et al., 2011 Rapid synthesis of the X-linked mental retardation protein OPHN1 mediates mGluR-dependent LTD through interaction with the endocytic machinery. *Neuron* 72, 300–315. [PubMed: 22017989]
- Niesink RJ, Van Ree JM, 1989 Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. *Neuropharmacology* 28, 411–418. [PubMed: 2546087]
- Nishiyama H, et al., 2002 Glial protein S100B modulates long-term neuronal synaptic plasticity. *Proc. Natl. Acad. Sci. U. S. A* 99, 4037–4042. [PubMed: 11891290]
- Nordahl CW, et al., 2012 Increased rate of amygdala growth in children aged 2 to 4 years with autism spectrum disorders: a longitudinal study. *Arch. Gen. Psychiatry* 69, 53–61. [PubMed: 22213789]
- Olde Loohuis NF, et al., 2012 MicroRNA networks direct neuronal development and plasticity. *Cell. Mol. Life Sci* 69, 89–102. [PubMed: 21833581]
- Parikshak NN, et al., 2013 Integrative functional genomic analyses implicate specific molecular pathways and circuits in autism. *Cell* 155, 1008–1021. [PubMed: 24267887]
- Park CS, Tang SJ, 2009 Regulation of microRNA expression by induction of bidirectional synaptic plasticity. *J. Mol. Neurosci* 38, 50–56. [PubMed: 18998061]
- Rasalam AD, et al., 2005 Characteristics of fetal anticonvulsant syndrome associated autistic disorder. *Dev. Med. Child Neurol* 47, 551–555. [PubMed: 16108456]
- Redmond L, et al., 2000 Nuclear Notch1 signaling and the regulation of dendritic development. *Nat. Neurosci* 3, 30–40. [PubMed: 10607392]
- Reeves RH, et al., 1994 Astrocytosis and axonal proliferation in the hippocampus of S100b transgenic mice. *Proc. Natl. Acad. Sci. U. S. A* 91, 5359–5363. [PubMed: 8202493]
- Rodier PM, et al., 1997 Linking etiologies in humans and animal models: studies of autism. *Reprod. Toxicol* 11, 417–422. [PubMed: 9100317]
- Rodriguez Manzanares PA, et al., 2005 Previous stress facilitates fear memory, attenuates GABAergic inhibition, and increases synaptic plasticity in the rat basolateral amygdala. *J. Neurosci* 25, 8725–8734. [PubMed: 16177042]
- Saba R, et al., 2012 Dopamine-regulated microRNA MiR-181a controls GluA2 surface expression in hippocampal neurons. *Mol. Cell. Biol* 32, 619–632. [PubMed: 22144581]
- Sarachana T, et al., 2010 Investigation of post-transcriptional gene regulatory networks associated with autism spectrum disorders by microRNA expression profiling of lymphoblastoid cell lines. *Genome Med.* 2, 23. [PubMed: 20374639]
- Schmittgen TD, Livak KJ, 2008 Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc* 3, 1101–1108. [PubMed: 18546601]
- Schmittgen TD, et al., 2008 Real-time PCR quantification of precursor and mature microRNA. *Methods* 44, 31–38. [PubMed: 18158130]
- Schneider T, Przewlocki R, 2005 Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. *Neuropsychopharmacology* 30, 80–89. [PubMed: 15238991]
- Schneider T, et al., 2006 Environmental enrichment reverses behavioral alterations in rats prenatally exposed to valproic acid: issues for a therapeutic approach in autism. *Neuropsychopharmacology* 31, 36–46. [PubMed: 15920505]
- Schouten M, et al., 2013 MicroRNAs and the regulation of neuronal plasticity under stress conditions. *Neuroscience* 241, 188–205. [PubMed: 23500144]
- Schumann CM, Amaral DG, 2006 Stereological analysis of amygdala neuron number in autism. *J. Neurosci* 26, 7674–7679. [PubMed: 16855095]
- Schumann CM, et al., 2004 The amygdala is enlarged in children but not adolescents with autism; the hippocampus is enlarged at all ages. *J. Neurosci* 24, 6392–6401. [PubMed: 15254095]
- Schumann CM, et al., 2009 Amygdala enlargement in toddlers with autism related to severity of social and communication impairments. *Biol. Psychiatry* 66, 942–949. [PubMed: 19726029]
- Sempere LF, et al., 2004 Expression profiling of mammalian microRNAs uncovers a sub-set of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.* 5, R13. [PubMed: 15003116]
- Shamir A, et al., 2012 The importance of the NRG-1/ErbB4 pathway for synaptic plasticity and behaviors associated with psychiatric disorders. *J. Neurosci* 32, 2988–2997. [PubMed: 22378872]

- Silva GT, et al., 2009 Enhanced long-term microcircuit plasticity in the valproic acid animal model of autism. *Front. Synaptic Neurosci* 1, 1. [PubMed: 21423407]
- Singh V, et al., 2014 Histone deacetylase inhibitors valproic acid and sodium butyrate enhance prostaglandins release in lipopolysaccharide-activated primary microglia. *Neuroscience* 265, 147–157. [PubMed: 24480366]
- Snow WM, et al., 2008 Altered morphology of motor cortex neurons in the VPA rat model of autism. *Dev. Psychobiol* 50, 633–639. [PubMed: 18985861]
- Sosa-Diaz N, et al., 2014 Prefrontal cortex, hippocampus, and basolateral amygdala plasticity in a rat model of autism spectrum. *Synapse* 68, 468–473. [PubMed: 24985713]
- Tabuchi K, et al., 2007 A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science* 318, 71–76. [PubMed: 17823315]
- Valles A, et al., 2014 MicroRNA-137 regulates a glucocorticoid receptor-dependent signalling network: implications for the etiology of schizophrenia. *J. Psychiatry Neurosci* 39, 130269.
- van Bokhoven H, 2011 Genetic and epigenetic networks in intellectual disabilities. *Annu. Rev. Genet* 45, 81–104. [PubMed: 21910631]
- Wang WX, et al., 2010 Individual microRNAs (miRNAs) display distinct mRNA targeting “rules”. *RNA Biol.* 7, 373–380. [PubMed: 20421741]
- Willemsen MH, et al., 2011 Chromosome 1p21.3 microdeletions comprising DPYD and MIR137 are associated with intellectual disability. *J. Med. Genet* 48, 810–818. [PubMed: 22003227]
- Yildirim E, et al., 2003 Valproate administration to mice increases histone acetylation and 5-lipoxygenase content in the hippocampus. *Neurosci. Lett* 345, 141–143. [PubMed: 12821190]
- Zhang C, et al., 2009 A neuroligin-4 missense mutation associated with autism impairs neuroligin-4 folding and endoplasmic reticulum export. *J. Neurosci* 29, 10843–10854. [PubMed: 19726642]
- Zhou R, et al., 2009 Evidence for selective microRNAs and their effectors as common long-term targets for the actions of mood stabilizers. *Neuropsychopharmacology* 34, 1395–1405. [PubMed: 18704095]

Fig. 1.

Prenatal exposure to VPA causes a decrease in brain volume and an increased BLA size. A. Representative images of cresylviolet stained brain slices of saline- and VPA-exposed animals. Black dotted lines represent the surface area of the BLA. B. Quantification of the surface area of the hemispheres of saline-(white bars) and VPA-(black bars) exposed rats shows a significant decrease in the surface area of the brain of VPA-exposed animals. C. Graph representing the quantification of the BLA size in saline- and VPA-exposed rats. D. Ratio of the BLA size corrected for hemisphere surface area. Data are shown as mean $\pm$ SEM; *p*-values were determined by Student's *t*-test **p* 0.05; ***p* 0.01.

Fig. 2. miR expression analysis reveals increased expression levels of miR-181c and miR-30d in the amygdala of VPA rats. A. MicroRNA microarray analysis of the amygdala of VPA-compared to saline-exposed animals. B. qRT-PCR based quantification of miR-30d, miR-181c and miR-15b expression levels in the amygdala of VPA and saline-treated animals. Data are shown as mean $\pm$ SEM; p -values are determined by Student's t -test. * $p < 0.05$; ** $p < 0.01$.

Fig. 3.

Transcriptomics analysis of the amygdala of VPA animal model for autism. A. Overlap between predicted miR-181c and miR-30d targets sites (blue circles) compared to the differentially expressed genes in the amygdala of VPA-exposed animals (purple circle). 101 genes' expression were altered due to prenatal VPA exposure containing a miR-181c binding site. In the case of miR-30d there was an overlap of 84 genes that are altered in their expression levels by VPA and contain a target site. B. Gene ontology analysis of the 101 genes changed by VPA treatment and contained a miR-181c binding site. C. Gene ontology analysis of the 84 genes changed by VPA treatment and contained a miR-30d binding site.

Fig. 4.

Genes altered by miR-181c inhibition show overlap with VPA-induced expression changes and are involved in gene networks functionally associated with neurodevelopmental processes. A. Venn diagram comparing gene expression changes between sponge-miR-181c infected neurons (green circle), VPA-induced expression changes in the amygdala (purple circle) and genes containing a miR-181c binding site (blue circle). Sponge-miR-181c dysregulated genes show a 68 gene overlap with miRwalk target prediction genes. In addition, the overlap between the genes differentially expressed by sponge-miR-181c and the VPA-dysregulated genes is 85. B. Gene ontology analysis of the genes differentially

expressed by sponge-miR-181c reveals association of this miR to cellular development, cellular function and maintenance, cellular growth and proliferation, cell morphology and cell cycle. Within the categories of physiological system development and function category, nervous system and development were identified as the processes containing most of the dysregulated genes. C. Genes altered by sponge-miR-181c are involved in dendritic growth and branching, as well as in synaptic functioning.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5. Inhibition of miR-181c affects neuronal outgrowth. **A.** Representative images of DIV 9 amygdalar neurons infected with control and sponge-miR-181c viruses. Scale bars indicate 50 μ m. **B.** Quantification of total neurite length. **C.** Graph showing the number of branching points of control and sponge-miR-181c infected neurons. **D.** Quantification of the number of branches per branch order. **E.** Quantification of spine density of control and sponge-miR-181c infected amygdalar neurons. Data are shown as mean $\pm$ SEM; *p*-values are determined by Student's *t*-test. **p* < 0.05; #*p* > 0.05 < 0.06 (non-significant).