Behavioral alterations in autism model induced by valproic acid and translational analysis of circulating microRNA

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ABSTRACT

Autism spectrum disorder (ASD) is characterized by difficulties in social interaction, communication and language, and restricted repertoire of activities and interests. The etiology of ASD remains unknown and no clinical markers for diagnosis were identified. Environmental factors, including prenatal exposure to valproic acid (VPA), may contribute to increased risk of developing ASD. MicroRNA (miRNA) are small noncoding RNA that regulate gene expression and are frequently linked to biological processes affected in neurodevelopmental disorders. In this work, we analyzed the effects of resveratrol (an antioxidant and anti-inflammatory molecule) on behavioral alterations of the VPA model of autism, as well as the levels of circulating miRNA. We also evaluated the same set of miRNA in autistic patients. Rats of the VPA model of autism showed reduced total reciprocal social interaction, prevented by prenatal treatment with resveratrol (RSV). The levels of miR134-5p and miR138-5p increased in autistic patients. Interestingly, miR134-5p is also upregulated in animals of the VPA model, which is prevented by RSV. In conclusion, our findings revealed important preventive actions of RSV in the VPA model, ranging from behavior to molecular alterations. Further evaluation of preventive mechanisms of RSV can shed light in important biomarkers and etiological triggers of ASD.

Keywords:

ASD, microRNA, resveratrol, translational research, social behavior, valproate.

1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder of unknown etiology characterized by a dyad of behavioral alterations: a) social communication and social interaction impairments and b) restricted, repetitive and stereotyped patterns of behavior, interests and activities (American Psychiatric Association, 2013). In addition to these features, several other associated symptoms and comorbidities are more prevalent in individuals with ASD, including sensorial, gastrointestinal, and immune alterations, sleep disturbances and other neurological disorders such as epilepsy and TDAH (Geschwind, 2009; Grandin, 2009; Klintwall et al., 2011).

The development of complex neural circuits relies in the spatial and temporal coordination of genetic and epigenetic processes during embryogenesis. As a consequence, the neurodevelopment is especially susceptible to the influence of environmental factors (Ameis and Catani, 2015; Perera and Herbstman, 2011). The brain of autistic individuals present several structural and functional abnormalities (Hutsler and Casanova, 2016), which could be caused by environmental risk factors, including immune abnormalities during pregnancy, indicating a strong influence of the immune system in the neurodevelopment. (Gottfried et al., 2015).

Valproic acid (VPA) is a drug widely-used as anticonvulsant and mood-stabilizer. Epidemiological observations demonstrated a strong link between the prenatal exposure to VPA, and the onset of ASD in the offspring (Christianson et al., 1994; Moore et al., 2000; Rodier et al., 1997; Williams et al., 2001; Williams and Hersh, 1997). Based on these observations, a prenatal injection of VPA has been used to induce autistic-like features in animal models in rodents (Bambini-Junior et al., 2011; Schneider and Przewłocki, 2005). The VPA animal model of autism

presents face validity (strong phenomenological similarities and related pathophysiology), construct validity (the same etiology; in this case, exposure to VPA) and predictive validity (same response to treatments aiming to prevent or revert symptoms) (Mabunga et al., 2015). Therefore, this model is currently used to elucidate new pathways involved in ASD pathophysiology that can be pharmacologically targeted (Tyzio et al., 2014).

Given the importance of social behavior impairments for the diagnosis of ASD, analysis of social behavior is critical in models of autism in rodents. We previously demonstrated that rats of the VPA model present impaired social behavior in a three-chambered social approach task (Bambini-Junior et al., 2011). In addition, we showed that resveratrol (RSV), an antioxidant and anti-inflammatory molecule, prevents social impairments in the VPA model in this same test (Bambini-Junior et al., 2014). Another key component for ASD diagnosis is the presence of repetitive and stereotyped behaviors, which, in animal models, are commonly manifested by excessive and incomplete self-grooming (McFarlane et al., 2008; Schneider and Koch, 2005; Sungur et al., 2014). Animal models of autism also frequently present irregularities in sensorial processing (Dendrinos et al., 2011; Geschwind, 2009), which may interfere with the animals' ability to perceive and interact with their surroundings (Nienborg and Cumming, 2010). The social transmission of food preference (STFP) is an ethologically relevant test of hippocampus-dependent non-spatial olfactory memory (Galef-Jr and Wigmore, 1983) that is commonly used to evaluate social communication in rodents (Bessières et al., 2017; Ryan et al., 2008). Therefore, in this work we evaluated reciprocal social interaction, repetitive self-grooming and the STFP task in rats of the VPA model, as well as the influence of prenatal RSV treatment.

In addition to behavioral assessments, the investigation of common molecular targets of VPA and RSV could help to clarify pathways involved in the pathophysiology of ASD. MicroRNA (miRNA) are a set of small non-coding RNA that control protein expression by binding mainly to the 3'UTR of the messenger RNA (mRNA) and thereby mediating either RNA degradation or translation inhibition (Chandra et al., 2017). The miRNA molecules are able to modulate several cellular functions and events, including cellular proliferation and differentiation, synapse formation/maturation and general metabolism integration (Wahid et al., 2010) and were recently implicated in the pathogenesis of ASD (Wu et al., 2016). The identification of circulating miRNA may also be potentially relevant for the development of clinical diagnostic or prognostic tools (Hu et al., 2017). We hypothesized that the RSV's preventive effect of the VPA-induced impairments might involve modulation of miRNA levels. In addition, we anticipate that some of these results could be conserved inter-species and would also be relevant for patients with ASD.

Thus, the aims of the present study were to investigate possible preventive effects of RSV against VPA-induced impairments on behavior and circulating miRNA alterations in an animal model, as well as to evaluate miRNA alterations in blood from autistic patients.

2. Material and Methods

2.1. Animal model of autism induced by VPA and resveratrol treatment

The animal model of autism was induced as previously described (Bambini-Junior et al., 2011; Schneider and Przewłocki, 2005). Briefly, female Wistar rats (UFRGS-Biochemistry Department CREAL), with controlled fertility cycles, were mated overnight. The first day of gestation was determined by the presence of

spermatozoa in the vaginal smear (embryonic day 0.5). Pregnant females received a single intraperitoneal injection of 600 mg/kg VPA (sodium valproate, Sigma-Aldrich, USA) or saline solution on embryonic day 12.5 (E12.5), and daily subcutaneous injections of RSV (3.6 mg/kg) or DMSO from E6.5 to E18.5 (Bambini-Junior et al., 2014). Only males of the offspring were used in this current study. Blood samples from these animals were obtained by cardiac puncture 30 days after birth. The behavioral analyses were performed in a second cohort of animals. This project was approved by the local animal ethics committee (CEUA-UFRGS 140367/140431) and all animals were handled in accordance with the current guidelines (National Council for the Control of Animal Experimentation - CONCEA).

2.2. Behavioral tests

To investigate possible preventive effect of RSV treatment on VPA animals, we evaluated three important behavioral patterns that correspond to behavioral domains frequently altered in ASD: sociability, olfactory memory and communication, and repetitive behavior.

2.2.1. Reciprocal social behavior

The test was adapted from Schneider and Przewlocki (Schneider and Przewłocki, 2005). Rats were tested at 46 days of age in a 50x50x50 cm arena. After a 5-min habituation period in the test chamber, the interactions between the animal test and a novel rat (younger male) were recorded for 15 min. The following pro-social interactions were evaluated: nose-to-nose sniffing, anogenital inspection, flank-exploration and following behavior. The number and duration of events were scored using the Anymaze software. We also calculated the total number and time of reciprocal social interaction.

2.2.2. Social transmission of food preference (STFP)

The experiment was adapted from Wrenn *et al* protocol (Wrenn et al., 2004). Rats at 47 days of age were habituated for 72 hours to eat pelleted food made from powdered chow. In the following day, food was removed three hours before the test. Next, one animal from each cage (demonstrator) was housed alone in a separate housing box and could eat a randomly assigned flavored food for 1 hour: either cinnamon (1% w/w) or cocoa (2% w/w). Then the demonstrator rat was housed with their littermates (the observer rats) and allowed free interaction for 30 min. After this interaction period, the demonstrator animal was removed from the housing box and the observer animals were provided with two choices of powdered food in identical pellets, one with flavor of the cued food presented by demonstrator rat and the other with the alternative food. Rats could eat for one and a half hours, and the amount of cued and non-cued food eaten from each litter was weighed and recorded. The ratio of food preference for each litter was calculated, considering the amount of cued and non-cued food and the total food consumption.

2.2.3. Repetitive self-grooming behavior

Rats at 64 days of age were scored for spontaneous grooming behavior as described (Onaolapo et al., 2017). The test was performed during 10 min in a 50x50x50 cm arena. Each rat was scored individually in two parts (0-5 min and 5-10 min) and the total time (0-10 min), considering the number of body cleaning with paws and face-washing actions. No habituation was performed.

2.3. Blood samples from autistic and control subjects

Peripheral blood samples from autistic male individuals and from the control group (5-15 years-old range) were obtained at the Clinical Hospital of Porto Alegre (HCPA). Inclusion criteria were age between 5 and 15 years and clinical diagnosis of ASD according to DSM-5 confirmed using the Autism Diagnostic Observation

Schedule. Autistic individuals who presented secondary autism or autism as an associated feature of an identified genetic condition (Fragile X Syndrome, Rett Syndrome, Angelman Syndrome, Prader-Willi Syndrome, Smith-Lemli-Opitz Syndrome and Tuberous Sclerosis) were excluded from the study. This project was approved by the local ethics committee (CEUA-UFRGS 33863).

2.4. RNA Extraction and RT-qPCR Procedure

After homogenization of blood samples with Trizol® reagent (Invitrogen, USA), chloroform was added to perform phase separation, and RNA was precipitated from the upper aqueous layer using isopropanol. The precipitated RNA was washed with ethanol to remove impurities, resuspended in RNase-free water and stored at -80ºC (Chomczynski, 1993). Mature miRNA expression was evaluated by reverse transcriptase followed by quantitative polymerase chain reaction (RT-qPCR) (Chen et al., 2005). Complementary DNA (cDNA) was synthesized from mature miRNA using reverse transcriptase reaction containing 2 µg of total RNA, 1 µL of 10 mM dNTP mix (Invitrogen, USA), 3 µL of stem loop RT primer mix (Data in Brief Table 1), 4 µL M-MLV reverse transcriptase 5X reaction buffer (Invitrogen, USA), 2 µL of 0.1 M DTT (Invitrogen, USA), 1 µL of RNase inhibitor (Invitrogen, USA), 1.0 µL of M-MLV reverse transcriptase (Invitrogen, USA), and sterile distilled water to a final volume of 20 µL. The synthesis of the cDNA was completed after a sequence of three incubations at 65ºC for 5 min, 37ºC for 50 min and 70ºC for 15 min.

The quantitative PCR mix was comprised by 12 µL of cDNA (1:33), 1.0 µL of specific miRNA forward and universal reverse (10 µM) primers (as detailed in Data in Brief Table 1), 0.5 µL of 10 µM dNTP mix, 2.4 µL of 10X

PCR buffer (Invitrogen, USA), 0.8 µL of 50 mM $MgCl_2$ (Invitrogen, USA), 2.4 µL of 1X SYBR™ Green (Molecular Probes, USA), 0.1 µL of Platinum Taq DNA Polymerase (Invitrogen, USA) and sterile distilled water to a final volume of 24 µL. The fluorescence of SYBR™ Green was used to detect amplification, estimate Ct values, and to determine specificity after melting curve analysis. PCR cycling conditions were standardized to 95ºC for 5 min followed by 40 cycles at 95ºC for 10 s, 60ºC for 10 s, and 72ºC for 10 s. After the main amplification, sample fluorescence was measured from 60ºC to 95ºC, with an increasing ramp of 0.3ºC each, to obtain the denaturing curve of the amplified products and Tm estimation, to assure their homogeneity after peak detection. Data was obtained from an Applied Biosystems StepOne System (USA). The set of 16 miRNA selected for this study includes miRNA involved in immunological and/or synaptic plasticity modulation, processes altered in ASD and includes miRNA commonly altered in many neurodevelopmental disorders.

2.5. Calculation of miRNA relative expression

The RT-qPCR results were imported into Microsoft Excel and the geNorm program was used to assess the variance in expression levels of the miRNA analyzed (Peltier and Latham, 2008; Vandesompele et al., 2002). This program scanned the present miRNA two at a time. Then, the expression stabilities of the set of miRNA were evaluated and ranked accordingly to their stability. A pairwise variation analysis was performed by geNorm to determine the number of miRNA required for accurate normalization and to identify the most suitable miRNA to be used as normalizers.

The average value of crossing threshold (Ct) values (in triplicate) was converted to quantities for geNorm analysis by the difference between Ct value from two groups taken in each comparison. PCR efficiency was calculated from the slope of the amplification curve by exponential amplification analysis using the LinRegPCR algorithm (Ramakers et al., 2003). The relative expression was obtained using the -ΔΔCt method where Ct values of a group are subtracted from the average Ct values of the control group. The relative expression of miRNA was calculated considering the PCR efficiency and the –ΔΔCt values for each miRNA (Pfaffl, 2001) and was normalized to the normalizers identified by the geNorm software.

2.6. Statistical analysis

IBM SPSS Statistics 20.0 (IBM SPSS, Armonk, NY, USA) was used to perform the statistical analysis. Kolgomorov-Smirnov and Shapiro-Wilk tests of normality were applied to determine data distribution. For reciprocal sociability test and self-grooming behavior, we used Generalized Estimating Equations (GEE) to weight both the interventions (VPA exposure and/or RSV treatment) and the litter effect in the behavioral outcome. After a Wald Chi-Square test, we performed pairwise comparisons for the parameters that presented interaction effect between interventions (VPA-by-RSV interaction). If only main effects were observed, the individual effect of VPA or RSV was evaluated. Bonferroni's post hoc test was used as the final evaluation. Data is reported as mean ± standard error of the mean (SEM). The Poisson distribution was used for discrete variables (number of interactions), while gamma distribution was used for time variables. A two-way variance analysis and Bonferroni post hoc test were used to analyze the cued food/total food eaten percentage during the STFP test after normality tests indicated

a normal distribution of this variable. The associated data are expressed as mean ± SEM and $P<0.05$ was considered to indicate a statistically significant difference. On the other hand, the absolute consumption of each food flavor presented non-normal distribution, were compared by non-parametric Kruskal–Wallis test and the results were expressed as median ± interquartile interval.

The miRNA relative expressions of the four animal groups were compared using non-parametric Kruskal–Wallis test followed by Dunn's test for multiple comparisons. The results were expressed as median ± interquartile interval. For the analysis of the human samples, non-parametric Mann-Whitney U test was performed and the results were expressed as median± interquartile interval. All statistical analyses were supervised by the Biostatistics Unit at the Clinical Hospital of Porto Alegre.

3. Results

3.1. Behavioral tests

3.1.1. Reciprocal Sociability Test

Adult male rats prenatally exposed to VPA were tested in a sociability paradigm considering four pro-social behaviors: nose-to-nose sniffing, anogenital inspection, flank exploration and following (Figure 1). Wald chi-square test showed a significant interaction effect between VPA exposure and prenatal RSV treatment in all parameters evaluated. Bonferroni's test for multiple comparisons further revealed that animals from the VPA group presented significantly decreased number and time of nose-to-nose sniffing compared to the control group, and RSV was not able to completely prevent these alterations (Fig 1A). Anogenital inspection presented no significant difference between experimental groups (Fig 1B). There was no difference between number and time of flank exploration behavior between VPA and control

groups, although a significant difference in total time of this behavior was observed between VPA and RSV groups (Fig 1C). Rats prenatally exposed to VPA also displayed significantly reduced number and time of following behavior when interacting reciprocally with an unfamiliar rat, compared to the control group (Fig 1D). Animals exposed to VPA presented decreased total pro-social interactions and spent less time interacting with an unfamiliar conspecific rat compared to the control group (Fig 1E). When VPA animals are treated with RSV, there was a significant increase in both number and time of pro social behaviors compared to VPA animals injected only with the vehicle. Thus, the RSV treatment was able to prevent the combined social deficits of VPA rats, even though analyses of individual behaviors were not statistically significant.

3.1.2. Social transmission of food preference

Figure 2 represents the ratio of food preference of Wistar rats prenatally exposed to VPA, compared to the control animals, after treatment with RSV or vehicle. There was a significant effect of VPA exposure in food preference ($p=0.026$), with a decreased ratio of food preference. However, the RSV treatment was not able to prevent this alteration.

Regarding the absolute food consumption, animals exposed to VPA tend to eat less food with the flavor cued by the demonstrator (Data in Brief Figure 1A, $p=0.080$). On the other hand, animals presented no differences in consumption of alternative (non-cued) food across interventions (Data in Brief Figure 1B, $p=0.493$).

3.1.3. Repetitive self-grooming

The self-grooming behavior was evaluated across two testing periods (0-5 and 5-10 minutes). During the first period, no significant effect was found, indicating that the time spent self-grooming was the same for all animals tested (Fig 3B). In the

second period of test, Wald chi-square test showed that animals exposed to VPA spent more time self-grooming and this effect was not prevented by RSV (Figure 3B, p=0.005). A similar result was observed in relation to total test period, with animals exposed to VPA spending more time grooming and no preventive effect of RSV (Figure 3C, p=0.008). The latency to start a self-grooming behavior was similar across experimental groups (Figure 3D).

3.2. Analysis of miRNA expression in animal model of autism

We determined the relative expression of miRNA in blood of 30 days-old rats prenatally exposed to VPA or vehicle (saline) and treated with RSV or vehicle (DMSO). The GeNorm algorithm ranked miRNA miR181a-5p and miR181b-5p as most stable and they were used as normalizers to evaluate the relative expression of the remaining miRNA. miR134-5p was significantly increased (p=0.030) in total blood of animals prenatally exposed to VPA, and RSV was able to prevent this alteration (Figure 4). On the other hand, there were no significant differences in the 13-remaining miRNA (Data in Brief Figure 2).

3.3. Analysis of miRNA expression in autistic subjects

We also determined the relative expression of miRNA in peripheral blood of autistic subjects, comparing with controls in the same age-range (5-15 years). The GeNorm algorithm ranked miR23a-3p, miR146a-5p and miR181a-5p as most stable and they were used as normalizers to evaluate the relative expression of the remaining miRNA. There were no intra-group differences related to age on miRNA levels. The analysis of relative expression revealed significant increases in expression of miR134-5p (Figure 5A, p=0.011) and miR138-5p (Figure 5B, p=0.026) in peripheral blood of autistic children, compared to control subjects. Levels of the 11-remaining miRNA showed no differences between groups (Data in Brief Figure 3).

4. Discussion

The prenatal exposure to VPA has been used to mimic the ASD pathogenesis in animals for two decades, providing important insights about the morphological, biochemical and behavioral characteristics of ASD (Bambini-Junior et al., 2011; Rodier et al., 1997; Schneider et al., 2008; Schneider and Przewłocki, 2005). Nevertheless, the exact mechanisms by which VPA acts are still currently unknown. Social behavior impairments are a key defining feature of ASD (Dover and Le Couteur, 2007; Pascoe, 2014; Rapin and Tuchman, 2008) and are also seen in many animal models of autism, including the VPA animal model (Bambini-Junior et al., 2011; Schneider et al., 2008; Schneider and Przewłocki, 2005). However, the mechanisms that underlie these impairments are also poorly understood. Our group previously demonstrated that prenatal administration of RSV (daily subcutaneous injections from E6.5 to E18.5) prevented ASD-like social deficits induced by VPA in rats (Bambini-Junior et al., 2014). Thus, the investigation of common targets of VPA and RSV in rodents can potentially facilitate the elucidation of the mechanisms by which VPA triggers the ASD-like social impairments. Moreover, it might contribute to the discovery of new clinical markers and the development of novel therapeutic approaches for ASD.

In the present study, we extended these analyses to include other behavioral assessments: reciprocal social behavior, social transmission of food preference and repetitive self-grooming. Here, we confirm the preventive actions of prenatal exposure of RSV on social impairments of the VPA model of autism. VPA rats showed reduced total reciprocal social interaction with a conspecific (anogenital inspection, flank exploration, nose-to-nose sniffing and following behavior), which was prevented by the RSV intervention.

Whereas the reciprocal social interaction test primarily focuses on social behavior of a test animal with an unfamiliar conspecific (Schneider and Przewłocki, 2005), the STFP test allows investigating of social communication by analyzing the transmission of preferences for specific foods between conspecifics (Bessières et al., 2017). Surprisingly, RSV was not able to prevent the effects of VPA in the STFP test. Perhaps, VPA rats might have an impaired ability to detect, process or utilize socially transmitted information. Moreover, the fact that RSV is not able to prevent the deficits triggered by VPA in the SFTP, while being effective in social impairments, suggests that neural processes independent of social interaction might be important for a normal STFP behavior.

Rats exposed to VPA also spent more time self-grooming than the controls, especially in the second half of this 10-minutes test protocol, which was not prevented by RSV. Hence, RSV can prevent some but not all aspects altered by VPA. The apparent specificity of RSV for social behaviors deserves further investigation and can be used to narrow analysis down to pathways underlying the development of social impairments in ASD.

In addition to these behavioral findings, we identified miR134-5p as a miRNA altered in opposing ways by VPA and RSV. Interestingly, this same miRNA was also elevated in autistic patients compared to controls and we also identified a similar elevation on the circulating levels of miR138-5p in autistic patients. Intriguingly, both miR134-5p (Schratt et al., 2006) and miR 138-5p (Obernosterer et al., 2006) are thought to be brain-specific. miR134-5p is localized at the synapto-dendritic compartment of rat neurons and is able to negatively regulate the size of dendritic spines in postsynaptic sites by inhibiting the translation of an mRNA encoding the protein kinase Limk1, which controls spine development (Schratt et al., 2006). On

the other hand, miR138-5p may also be implicated in changes in the development of dendritic spines, since it favors the activation of the RhoA-Rock pathway (Figure 6) (Schratt, 2009; Siegel et al., 2009).Therefore, investigation of this model, especially of the molecular mechanism of VPA action, can shed light in important biomarkers and etiological triggers of ASD.

Overall, our study suggests that evaluation of miRNA expression profile may be used to identify biological pathways altered in ASD. Since miRNA can pass to bloodstream from cells, tissues and organs (Creemers et al., 2012; Ludwig et al., 2016), changes in circulating miRNA levels may reflect changes in other tissues, including nervous system and lymphoid organs. Further studies are necessary to evaluate additional miRNA and assess different developmental stages and tissues to clarify the roles of miRNA in the etiology of ASD. Nevertheless, the translational approach employed in this work could further support the potential use of miRNA as biomarkers and therapeutic targets in ASD.

Future studies exploring the molecular alterations induced by VPA during pregnancy, and how this changes lead to long-term effects in the offspring are necessary. This is particularly important because epigenetic modulation may mediate the onset of ASD phenotypes (Andrews et al., 2017; Gottfried et al., 2015; Perera and Herbstman, 2011). In addition, studies of VPA effects on mother to embryo communication can help to clarify mechanisms involved in the etiology of ASD. The anti-inflammatory and antioxidant properties of RSV might be protective for this mother-embryo relationship in animals of the VPA model of autism.

In summary, our findings revealed important preventive actions of RSV in the VPA model of autism. These effects ranged from behavior, (as seen in the evaluation of reciprocal social interaction), to molecular alterations (miR134-5p expression). We

also demonstrate for the first time that animals exposed prenatally to VPA had no preference in consuming the food presented by the demonstrator, indicating that social communication could be impaired in this model. This study reinforces the validity of the VPA model of autism and its utility to study the ASD pathophysiology.

Conflicts of interest: The authors declare that there are no conflicts of interest.

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Legends of Figures

Figure 1. Resveratrol prevents deficits in reciprocal social interaction in the VPA model of autism. Number and time of nose-to-nose sniffing (A), anogenital inspection (B), flank exploration (C), following (D) and total pro-social interactions (E). Plots show mean ± SEM. Different letters indicate statistical differences with $p<0.05$. Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. Control (n = 8), RSV (n = 9), VPA (n = 9), VPA+RSV (n = 6).

Figure 2. VPA exposure impairs social transmission of food preference. Percentage of cued food consumed by observer rats. Data expressed as mean ± SEM with *$p<0.05$**.** Statistical analysis: Two-way ANOVA followed by Bonferroni. Control (n = 7), RSV (n = 6), VPA (n = 8), VPA+RSV (n = 7).

Figure 3. VPA exposure increases self-grooming behavior. Time of grooming (sec): (A) 0-5 min of test, (B) 5-10 min of test, (C) total time and (D) latency to start grooming. Data expressed as mean ± SEM with *$p<0.05$ and **$p<0.01$ considered significant**.** Statistical analysis: Generalized Estimating Equations (GEE) followed by Bonferroni. Control (n = 14), RSV (n = 12), VPA (n = 12), VPA+RSV (n = 13).

Figure 4. miR134-5p expression is increased in peripheral blood of the VPA model of autism, which is prevented by RSV treatment. Plots presented as medians ± interquartile intervals with *$p<0.05$ considered significant. Statistical analysis: Independent-Samples Kruskal-Wallis followed by Dunn's test. Control (n = 6), RSV (n = 6), VPA (n = 6), VPA+RSV (n = 5).

Figure 5. mi134-5p and miR138-5p are upregulated in peripheral blood of autistic children compared to control group. Results expressed as median ± interquartile intervals with *p <0.05 considered significant. Statistical analysis: Mann-Whitney U-test. Control (n = 7) and autistic patients (n = 7).

Figure 6. Modulation of dendritic spines by miR134-5p and miR138-5p. The cytoskeleton within dendrites and spines is dynamically regulated by two antagonistic pathways: RhoA–ROCK cascade and a Rac–LIMK1 signaling. miR138-5p favors activation of the RhoA pathway and spine shrinkage by downregulation of the depalmitoylation enzyme APT1 and the resulting membrane localization and activation of the RhoA stimulatory Gα protein. On the other hand, miR134-5p inhibits LIMK1 production, thereby reducing the polymerization of filamentous actin and spine growth. Adapted from Schratt et al (Schratt, 2009).

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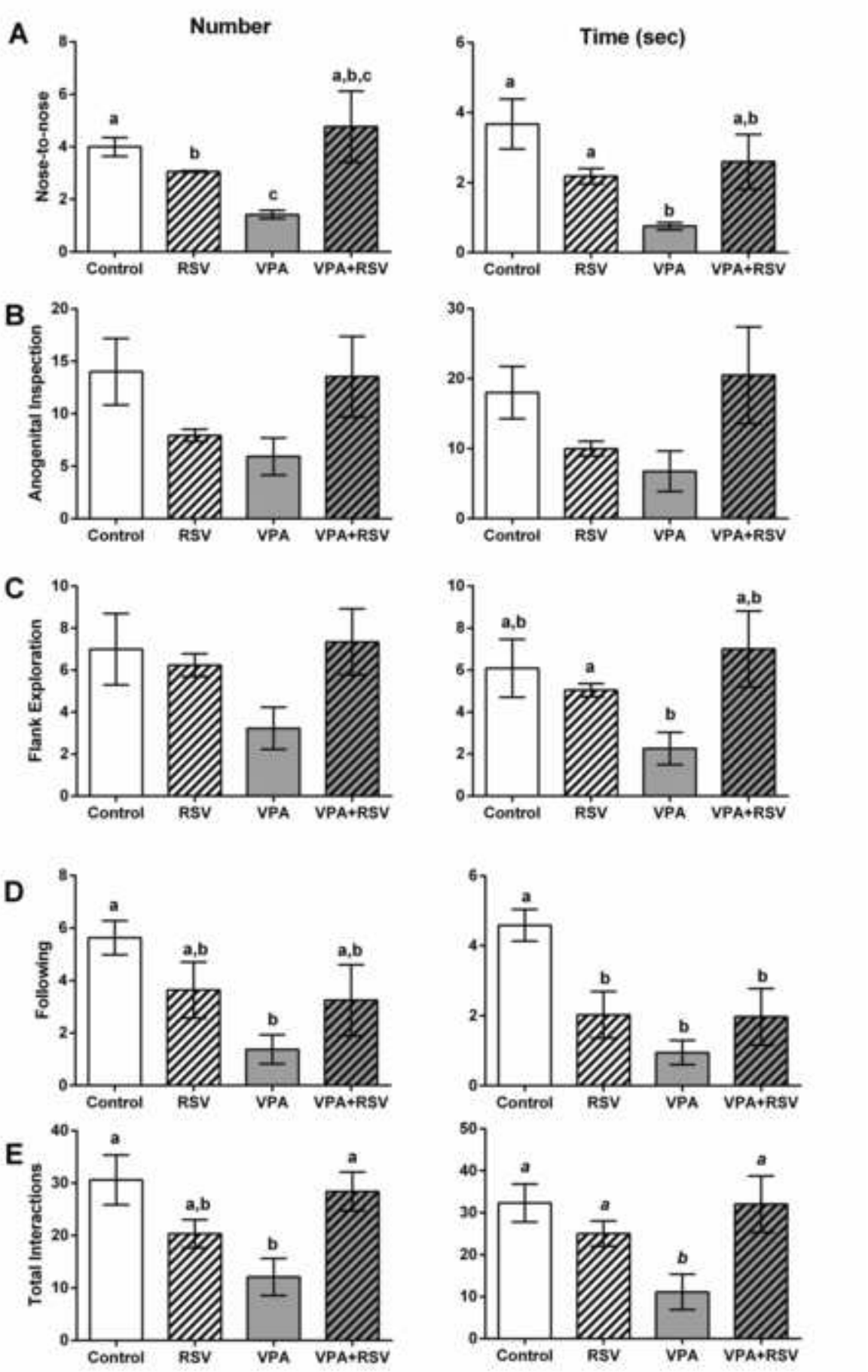

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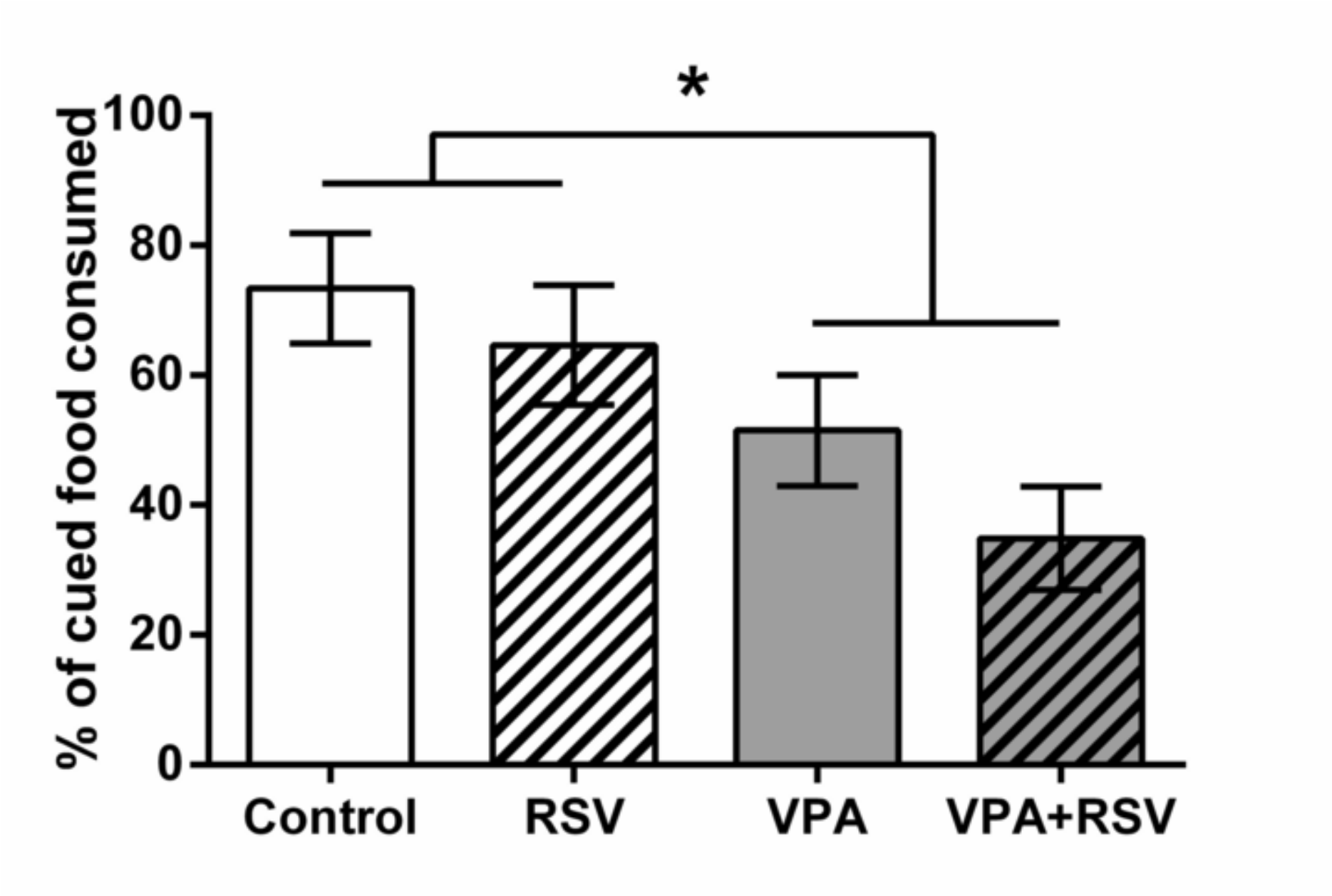

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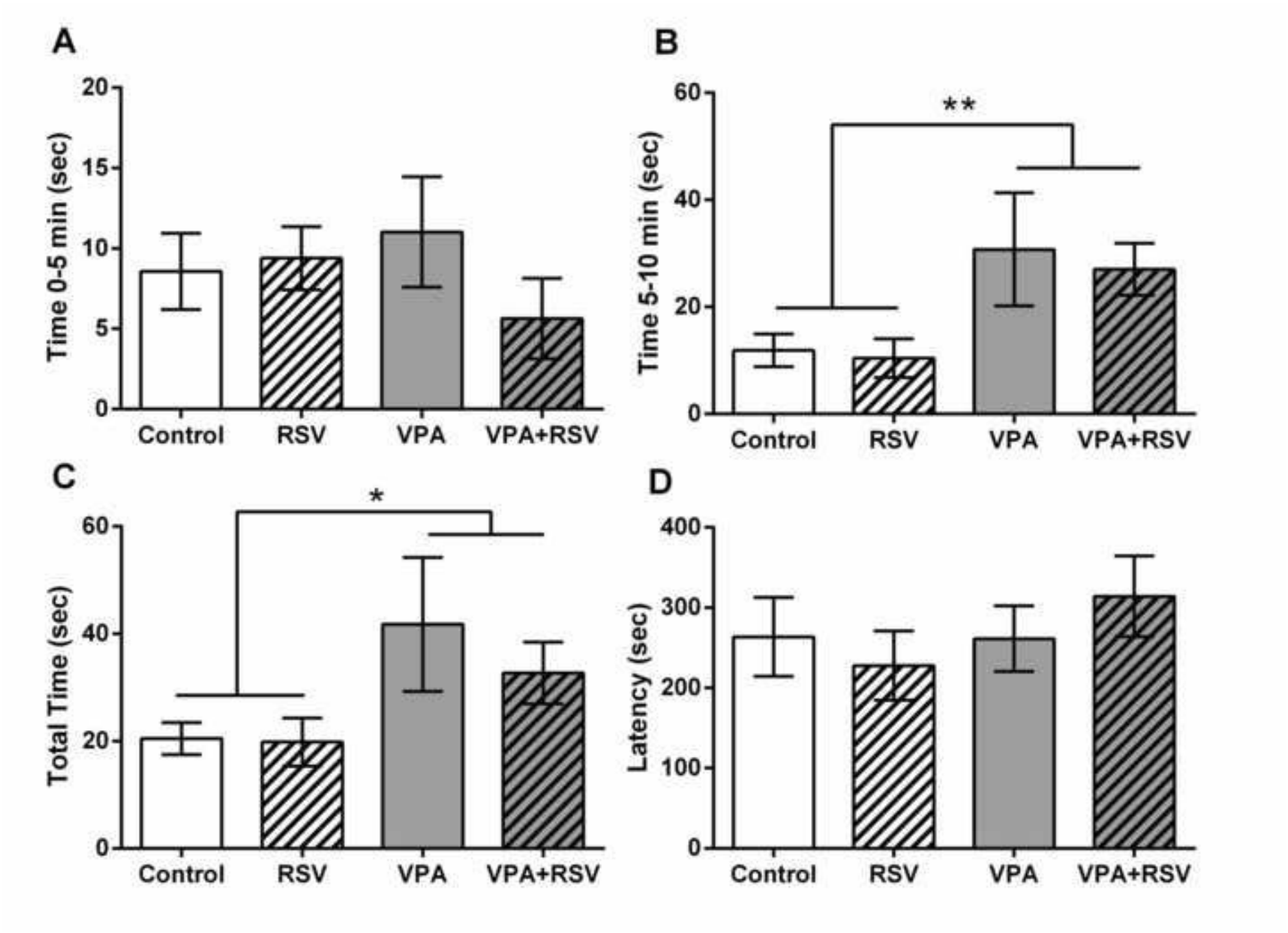

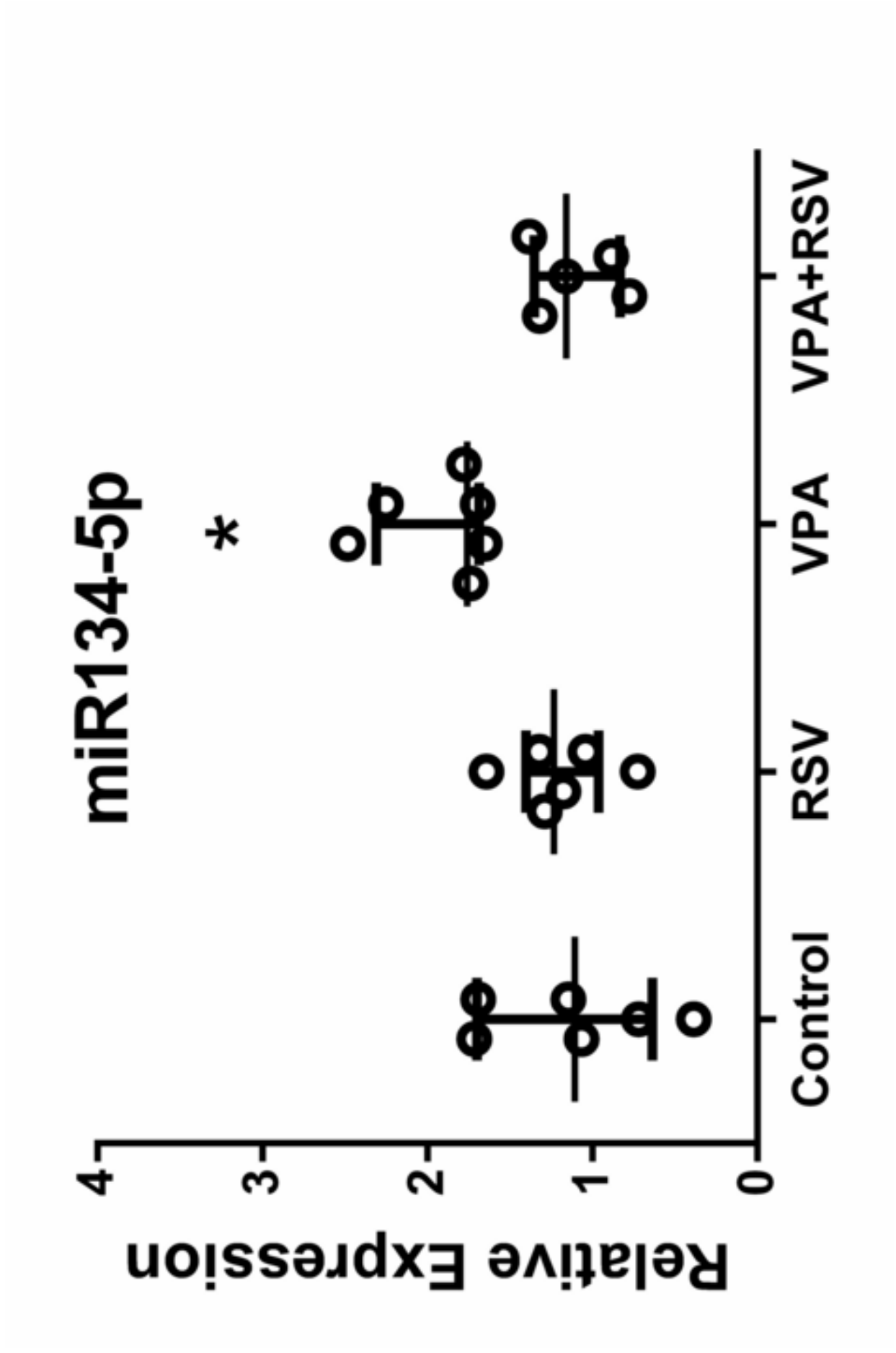

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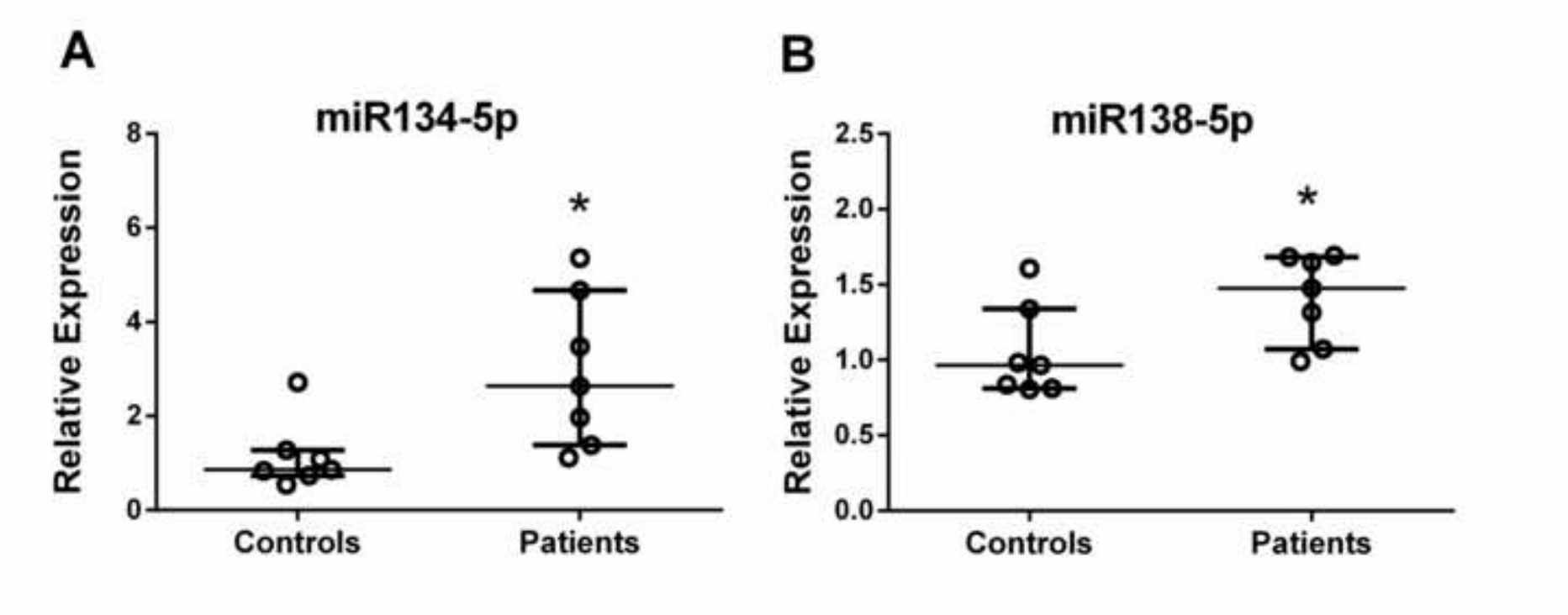

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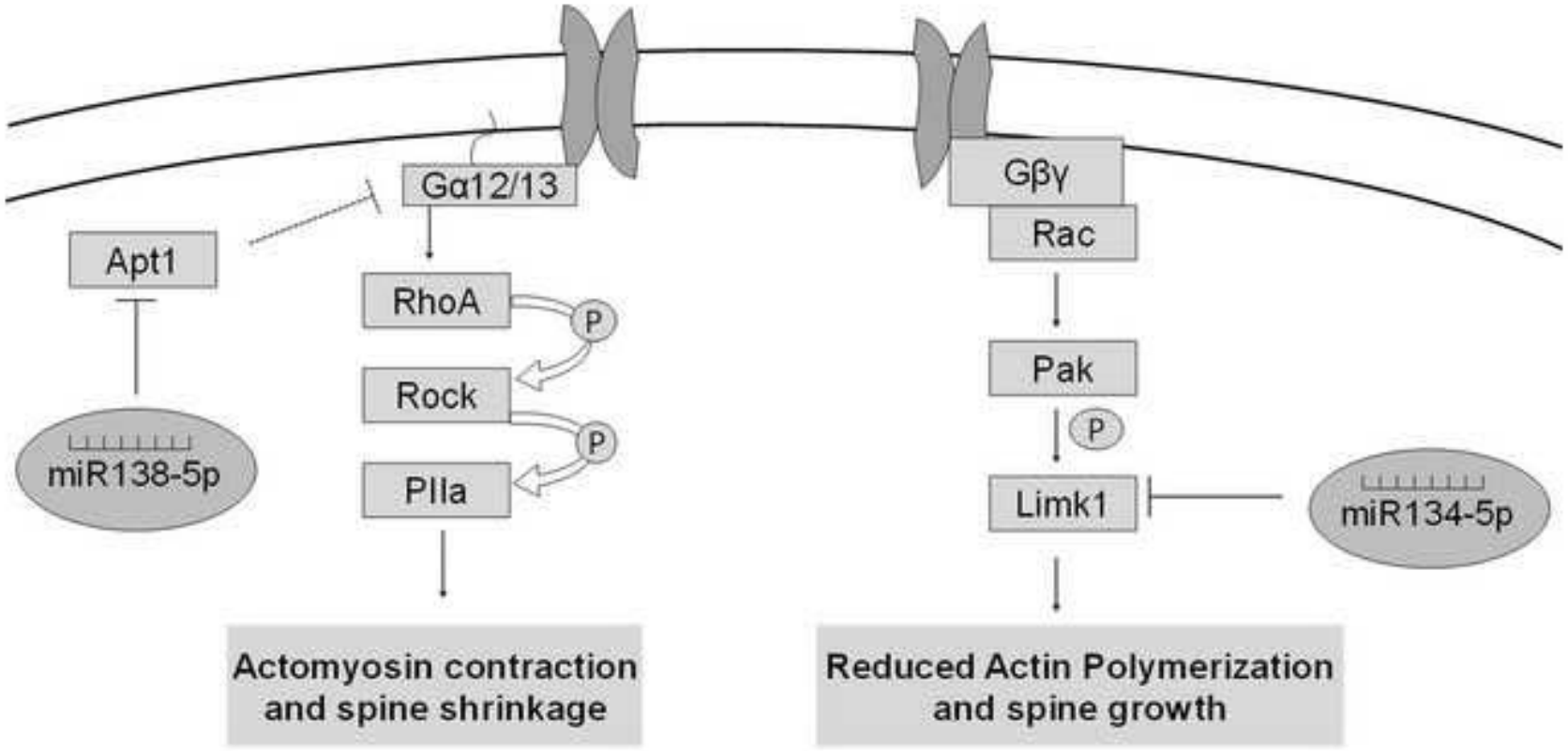

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