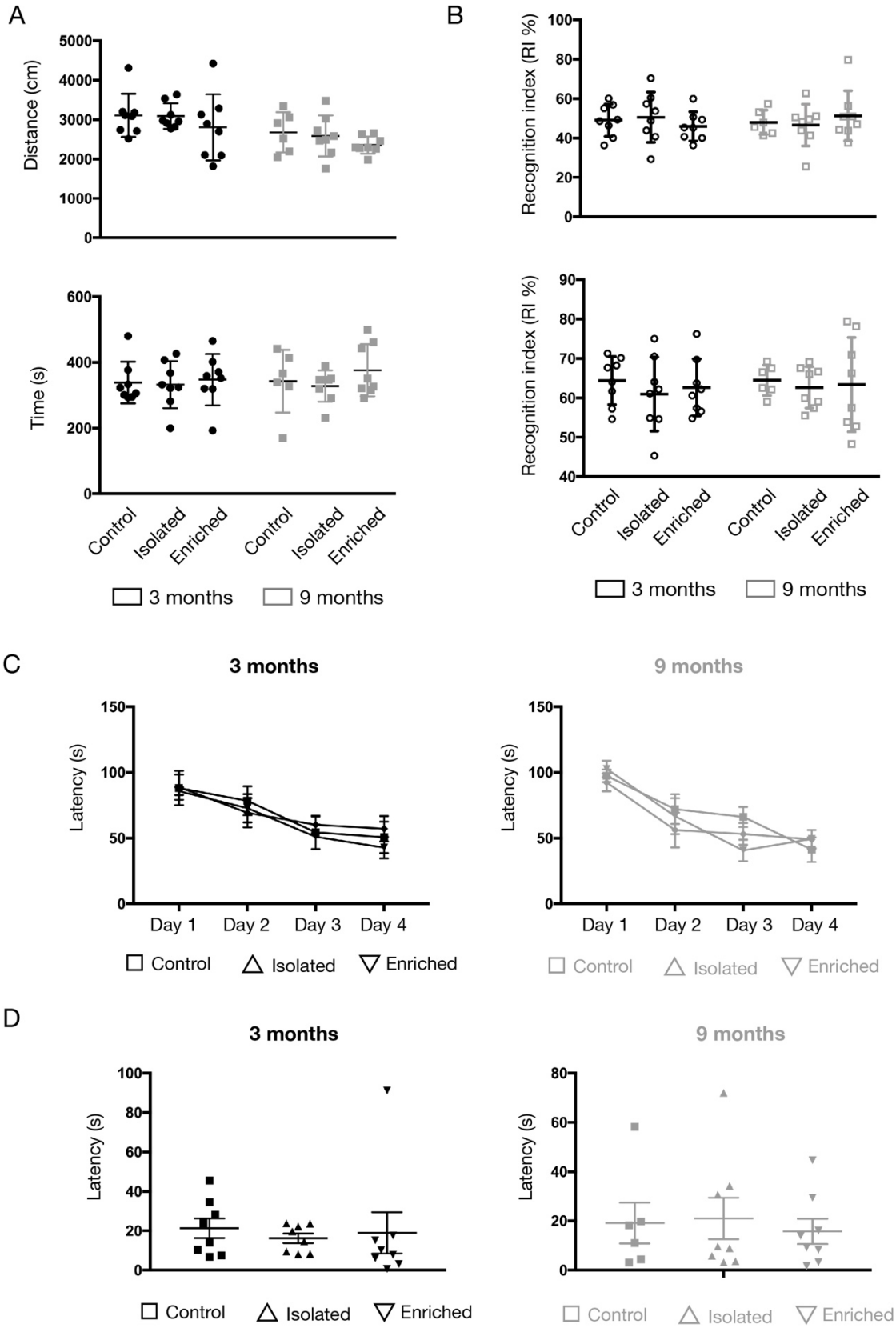
Supplemental Figures and legends

Popa et al., Suppl Figure 1

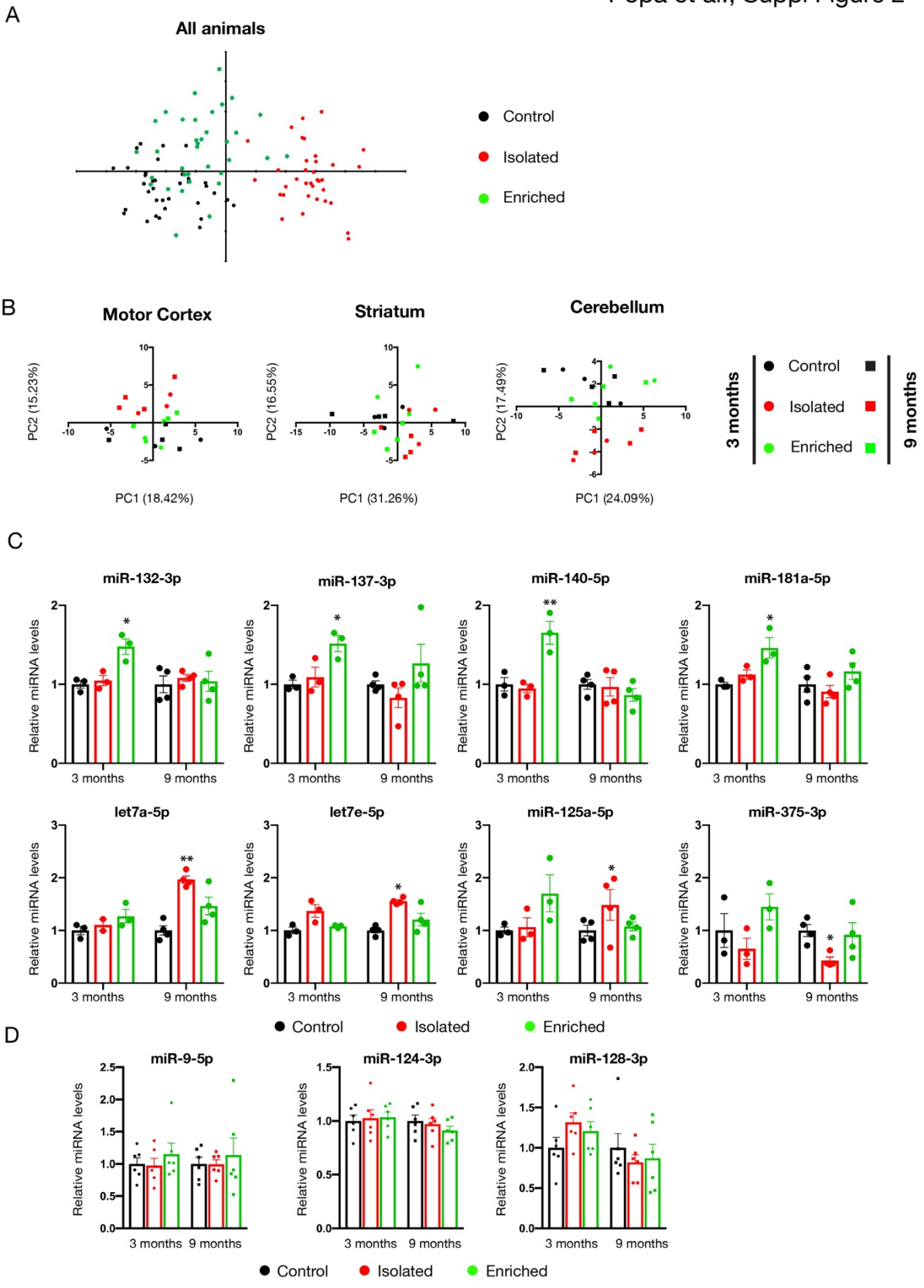


Supplementary Figure 1. Social environment does not modify exploration or cognitive performance in 3- and 9-month-old mice. Related to Figure 1

A. Open field. Neither distance traveled (upper panel) nor time spent in close proximity to the walls (lower panel) are altered by social context.
B. Novel object recognition. RI (see methods) during the exploration phase (around 50%) suggest similar interest by the two objects (upper panel). In the recognition phase (lower panel), RI increases as animals explore longer time the novel object. RI is not affected by aging or social conditions in any phase.
C. Morris Water Maze. Learning curves over 4 consecutive days for young (left) and middle-aged (right) mice show no differences among social conditions.
D. Morris Water Maze. The latency in the probe trial is not influenced by the social environment.

All values represent mean±sem

Popa et al., Suppl Figure 2



Supplementary Figure 2. Additional data on miRNA alterations associated to social environment changes. Related to Figure 2.

A. Discriminant analysis of miRNA data in all animals of both ages. The social environment enables an overall satisfying classification of the miRNA data from the 5 brain regions analyzed.
B. PCA analysis of the motor cortex, dorsal striatum and cerebellum show no or little correlation between miRNA levels in those regions and social environment.
C. miRNAs deregulated by social enrichment (upper panels) and isolation (lower panels) in the exploratory cohort of mice (n=21).
D. Highly abundant miRNAs with known functions in the brain such as miR-9-5p, miR-124-3p or miR-128-3p show no differences in the different environmental groups and ages.

All values represent mean±sem

Pathways for enrichment miRNAs (miR-140-5p & miR-181a-5p)

Pathway	# of targets	Adj p value
Neuronal system	19	0.004305
Transmission across synapses	15	0.004305
Glutamate Neurotransmitter	5	0.0061
Neurotransmitter release	6	0.0162
Linoleic acid metabolism	3	0.0162

Pathways for isolation miRNAs (let-7a-5p, let-7d-5p, let-7e-5p & miR-410-3p)

Pathway	# of targets	Adj p value
Wnt signaling (not beta-catenin)	6	0.0088
Platelet Ca homeostais	3	0.0145
Synthesis of IP3/IP4	3	0.0201
Hemostasis	10	0.0201
Wnt signaling (beta-catenin depend)	7	0.0201

Supplementary Figure 3. Pathway analysis of the target mRNAs in enriched and isolated animals using Reactome library. Related to Figure 3.

Transparent Methods

Animals

Mice were purchased from Charles Rivers Laboratories (France). Since in our experiments mice could be in physical contact, we decided to use only animals from the same sex (males) to circumvent potential confounding interactions (i.e. mating). A total of 96 C57Bl6J male mice were used in this study for the behavior and the miRNAs. Retired CD1 females (44) and juveniles (24) were also involved in the project (for housing or behavioral testing) although not included in any of the data. Half of the C57Bl6J were young adults (n=48, 10 weeks at the time of arrival) whereas the remaining animals were older adults (n=48, 9 months). Upon arrival, each male was housed with an elderly female presenting a low fertility (>12 months) for 2 weeks. This enables acclimation to the animal facility and provides a control social environment while limiting mating and fighting. The animals were kept under specific pathogen-free conditions with a regular 12-h light/dark cycle (light on at 8:00 am) and constant conditions (21 ± 1°C; 50% humidity). Food and water were supplied ad libitum. Mice also benefited from some environmental enrichment (a wooden stick and a piece of cotton with each cage change). We conducted behavioral experiments on 3 independent cohorts of 16 animals. miRNA profiling was performed on 3 independent groups of animals: i) an initial exploratory cohort of 21 animals (n=9 young and n=12 middle-aged mice); ii) a second confirmatory cohort of 15 animals (n=9 young and n=6 middle-aged mice); and) a third cohort of 12 animals for the recue experiments. This latter cohort encompasses 6 young and 6 middle-aged animals.

All procedures involving mice were approved by the local ethics committee (EU0488, #6357) and are in agreement with European regulations (Directive 2010/63/EU). A special effort was made in handling animals to minimize stress or anxiety.

Modification of social environment

Before behavioral testing or miRNA analysis, mice were randomly assigned to 3 different groups and submitted to one of these social environment conditions: i) in the control condition, animals remained in the same social context (1 aged female); ii) in the social isolation group, animals were housed individually for 1 week; and iii) in the social enriched condition, animals were exposed to novel social partner(s) every 2 days. Briefly, the first day of the enrichment the resident female was replaced by a non-familiar old female. On day 3, another old female was added to the cage. On day 5, one of the females was replaced by a novel female. Animals in isolated and enriched groups were returned to the control condition the day before starting the behavioral testing.

For rescue experiments, young mice (n=6) are first submitted to the enrichment and middle-aged mice (n=6) to isolation as described above. Then, animals are returned back with their initial home cage for an additional 1 week. After that, mice are sacrificed for miRNA quantitation.

Behavioral schedule and exclusion criteria

All behavioral experiments were conducted between 9 am and 7 pm. Animals were habituated to the behavioral room for at least 30 min before the start of the task. The 48 animals to be analyzed were divided into three cohorts that were tested

independently. For each cohort of animals, tests were conducted according to this schedule: 1) Three-chamber social task (Day 1); 2) Intruder test (Day 3); 3) Interactions in neutral arena (Day 5); 4) Openfield and Novel object recognition (Day 7); 5) Morris water maze (Day 9-13); 6) Olfactory behavior (Day 15). We randomized the order of animals on each test. All experiments were recorded using Ethovision software and with the experimenter out of the behavioral room.

One 9-month-old animal in the control group showing epileptic signs, was euthanized and therefore excluded from the study. For technical reasons during the preparation of the 3-chambers test, one additional 9-month-old animal had to be excluded from the analyses.

Openfield

The open field test was used to evaluate locomotion and exploratory behavior. The open field device consisted of a non-reflective opaque plexiglass box (40x40x40 cm) and a suspended digital camera. The animals were placed in facing an open field wall and their behavior was recorded for 10 min. The total distance traveled, the time spent in the center and near the walls are calculated.

Novel object recognition

For the novel object recognition task, we used the open field device and small plastic objects. In the first phase, the animals were placed facing a wall and two identical objects (green cylinders, 3.5 cm high, 4.5 cm in diameter) were placed in front of the opposite wall of the arena (10 cm from the wall and 5.5 cm apart from each other). The mice explored the arena for 10 min and were brought back to their cages for 10 min.

In the second phase, one of the objects was replaced by a novel object (yellow triangular prism, 3.5 cm high, 4.5 cm on each side). The time spent exploring each object (nose point within 2 cm from the object) was quantified and used to calculate a recognition Index (RI) as follows: RI=(time exploring object 1)/(time exploring object 1+ time exploring object 2)

Morris water maze

The Morris Water Maze (MWM) was performed as previously described with minor modifications (LaSarge et al., 2007). Briefly, a round pool (120 cm in diameter) was filled with water (25°C) and tempera paint was added to the water until it becomes opaque. A hidden platform (10 cm in diameter) is placed about 1cm below the water surface. The spatial cues consisted of geometric figures of different colors located in privacy blinds surrounding the water tank. The subjects are monitored by a video tracking system directly above the water tank while swimming and the parameters are measured using Ethovision software in a computer.

During the training phase (4 days) each animal received 3 trials. In each trial, the animal is placed in the water tank facing to the wall and allowed to explore the maze until it reaches the platform. If the animal does not find the platform in 2 min, the experimenter guides it to the platform. In either case, the animal is left on the platform for 10 sec. Then it is dried and returned to the cage for 5 min until the next trial. Once all the animals have completed the training phase, they each perform one probe trial (2 min) on day 5, during which the platform is removed from the pool. The probe trial is performed to verify the animal's understanding of the platform location and to observe the exploratory strategy it follows when it discovers that the platform has been

removed. The latency to reach the platform and the frequency of crossings are evaluated.

Intruder test

The protocol was adapted from previously described methods (Gascon et al., 2014). To avoid any aggressive behaviors, a juvenile CD1 male (3-5 weeks) was introduced into the home cage of the test mouse and the interactions were recorded for 5 min. The time and the number of interactive and investigative behaviors (sniffing) initiated by the target mouse were quantified.

Interactions in neutral arena

In this task, the target mouse and a juvenile CD1 male (3-5 weeks) were simultaneously introduced into an open field arena (see before). The time and the number of interactive and investigative behaviors (sniffing) initiated by the target mouse during the 5 min of the test were assessed.

Three chambers social task

The three chambers apparatus is a rectangular arena (60x37.5x21 cm) made of transparent plexiglass plastic, divided into three compartments of the same size (18.5x37x21 cm). Two openings connect the center chamber with the two side chambers. In a corner of each side chamber, there is a cylindrical container (10 cm in diameter, 20 cm high). During the first trial (exploration), the target mouse is placed in the middle chamber facing a wall and allowed to explore. In the next trial (social), a stranger mouse is placed in one of the containers while the other is empty. Trials last 10 min and animals are left for 10 min in their home cages between trials. Strangers

are juvenile CD1 mice (3-5 weeks) that have been trained to be restrained in the container. For each mouse, the location of the stranger mice in the left or right side chamber is random. A social preference index is calculated as the subtraction of the time in close exploration (nose point within 2 cm of the container) from the social partner) and lthe containers. To quantify the behavior, only the first 5 min of each trial were used.

Device cleaning

Between trials or animals, behavioral devices were thoroughly cleaned with water and mild soap. After rinsing, surfaces were sprayed with a 70% ethanol solution and air-dried for 5 min.

RNA extraction

For molecular analysis, animals from each environmental group from an independent cohort of animals (n=3/group for 3 young mice, n=4/group middle-aged mice) were sacrificed by cervical dislocation. Brain was rapidly removed and dissected into ice-cold HBSS and coronally sliced (250-400 μm). Different antero-posterior levels were selected to isolate specific brain regions using a biopsy punch (500 μm diameter). Total RNA containing miRNAs and mRNAs was then extracted from these samples using NucleoSpin miRNA kit following manufacturer's protocol for RNA purification in combination with TRIzol lysis (Macherey-Nagel, Germany). RNA extraction protocol includes an on-column DNase treatment (30 min). Total RNA was quantified using a NanoDrop (Fisher Scientific).

miRNA reverse transcription and quantitative PCR

For quantification of miRNAs from specific brain regions, we first performed reverse transcription using TaqMan advanced miRNA cDNA synthesis kit and the protocol provided starting from 5 ng of total RNA (Applied Biosystems, France). Abundance of miRNAs in the samples was measured using custom TaqMan array Advanced miRNA cards (Applied Biosystems, USA) following manufacturer guidelines and a quantitative PCR thermocycler (QS7, Applied Biosystems, France). miRNAs that could be used as endogenous controls were determined as those giving the lower standard deviation of the ΔCrt values for all samples (ThermoFisher Scientific Digital Science online real-time qPCR software). From this analysis, we selected hsa-miR-16-5p and mmu-miR-101a-3p as the most stable across multiple brain regions in our experiments. These two miRNAs were therefore used as endogenous controls for the relative quantifications in all our subsequent analyses.

miRNA selection

Our array cards contained 48 miRNAs (46 target miRNAs and 2 control miRNAs, see table 1). We selected our target miRNAs according to some specific criteria:

- All miRNAs have been shown to be expressed in the mouse brain (He et al., 2012)
- We have chosen miRNAs enriched in different cell types (e.g. neurons: miR-124, miR-132; astrocytes: let-7a; oligodendrocytes: miR-219; microglia: miR-223) as well as others expressed across multiple cell types (e.g. miR-181a)
- We have selected miRNAs exhibiting different expression levels (high: miR-124, miR-9, miR-128; medium: miR-137, miR-181a; low: miR-219, miR-375) (He et al., 2012)

Although 48 miRNAs were initially included in the survey, we had to exclude 3 miRNAs from the analysis (let-7c-5p, miR-1197-3p and miR-375-5p) as they could not be consistently amplified in our samples.

Target network analysis

For the identification and analysis of downstream mRNA targets as well as the integration into functional networks, we used miRNet 2.0 (Chang et al., 2020) following the developers' guidelines. For the enrichment analysis, we used KEGG library and hypergeometric test. Network was visualized using the force atlas layout.

Data analysis and statistics

Behavioral data analysis. All behavioral tests were recorded and analyzed post-hoc. For tests involving two freely moving animals (intruder test and interactions in neutral arena), an investigator blind to the conditions manually scored the behaviors. For the other tests, automatic monitoring was carried out using dedicated software (Ethovision XT, Noldus, Netherlands). Data is presented as means ± SEM unless indicated. *Determining adequate sample size.* As we have no prior experience with the effect size relating to a postulated interaction between environment and age, we choose to conduct the behavioral experiments using small cohorts (8 mice per each of six experimental groups) arguing that, if significant, subtle changes in such small cohorts might represent bonna fide alterations. To limit the potential confounding effects of inter-lot variability, we perform our tests from three independent cohorts of 16 animals. Sample sizes used for molecular studies are smaller (n=6 animals/group) and were obtained from 3 independent cohorts.

Outlier values. We perform an automatic outlier detection in our miRNA dataset using the ROUT method (Q=5%). 19 values were identified and eliminated from subsequent analysis (representing <0.15% of the dataset).

Between-group comparisons. We perform normality tests (Anderson-Darling, D'Agostino-Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests) on all our datasets that confirmed their normal distribution. For the detection of significant differences among groups in behavioral and molecular experiments, we used therefore ANOVA analysis followed by post-hoc tests (Dunnett-adjusted pairwise comparisons). Data is presented as means ± SEM unless indicated.

The relative expression levels for each miRNA and animal are entered into a principal component analysis (PCA) and discriminant analysis (DA). In the PCA, the number of extracted components was determined by the parallel analysis with 95% percentile rule and confirmed with a scree plot. The assessment of individual miRNA influences on the obtained PCA component scores was done with general linear models.

Statistical analysis (ANOVA and post-hoc tests for multiple comparisons, outlier identification, normality tests) was performed using Prism GraphPad software (version 7) and XLStat (PCA and DA).

Supplemental references

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