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**Title:** Epigenetic alterations in gut and brain of adult rats after oral administration of miR-320-3p and miR-375-3p at mid-lactation, and preventive potential of miR-320-3p on early weaning stress.

*Running Title: Preventive potential of miR-320-3p on early weaning stress*

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26 **Abstract**

27 **Aim:** To investigate if the artificial delivery of microRNAs naturally present in the breastmilk can  
28 impact the gut and brain of young rats according to weaning.

29 **Methods:** Animals from a new transgenic rat line expressing green-fluorescent protein in the  
30 endocrine lineage (cholecystokinin expressing cells) received at Day-12, near neural diversification,  
31 a single oral bolus of mir-320-3p or miR-375-3p, embedded in DiOleyl-Succinyl-Paromomycin  
32 (DOSP), and were further early (Day-15) or regularly (Day-30) weaned. Relevant miRNA (miR-  
33 320-3p, miR-375-3p, miR-375-5p, miR-16-5p, miR-132-3p, miR-504), *polr3d*, *hspb6*,  
34 inflammation, enteroendocrine, and circadian clock-related mRNAs, chromatin complexes, and  
35 duodenal cell density were assayed at 8h post-inoculation and at Day-45.

36 **Results:** The miR-320-3p/DOSP induced immediate effects on H3K4me3 chromatin complexes  
37 with *polr3d* promoter ( $p<0.05$ ) but no long-term effects. On regular weaning, at Day-45, both miR-  
38 320-3p and 375-3p were down-regulated in the stomach, up-regulated in the hypothalamus  
39 ( $p<0.001$ ) but only miR-320-3p was up-regulated in the duodenum. After early weaning, the miR-  
40 320-3p and miR-375-3p levels were down-regulated in the stomach and the duodenum, but up-  
41 regulated in the hypothalamus and the hippocampus. Combining miR-320-3p/DOSP with early  
42 weaning enhanced miR-320-3p and chromogranin A expression in the duodenum. In the  
43 hippocampus, the miR-504 was down-regulated for both sexes, but in the brain stem, up regulated  
44 only for females, along with miR-320-3p and miR-16-5p levels. In the hypothalamus, clock levels  
45 were up regulated for both sexes. In the miR-375-3p/DOSP group, the density of enteroendocrine  
46 duodenal cells increased. The long-term effect of miR-375-3p/DOSP was more limited, according  
47 to the fourfold lower number of predicted targets than with miR-320-3p.

48 **Conclusion:** Addressing oral miRNA-320-3p loads to duodenal cell lineage is paving the way for  
49 the design of new therapeutics, manipulating long term consequences of early life stress.

## 50 Introduction

51 In recent years considerable evidence has demonstrated that the adult health status may be strongly  
52 influenced by experiences in early life modulated by epigenetic changes.<sup>1,2</sup> The inadequate  
53 interruption of lactation (Early weaning) impairs an important nutritional and maternal contact,  
54 promoting anxiety, depression, or stress in neonates, some with deleterious lifelong consequences.  
55 The miR-504 and miR-16-5p have been identified as key miRNAs, in the relationship between  
56 early life stress and the modulation of the dopaminergic and serotonergic systems.<sup>3</sup> The miR-504  
57 directly targets the 3'UTR of the dopamine D1 receptor gene (*drd1*)<sup>4</sup> and the miR-16-5p is involved  
58 in the regulation of the serotonin transporter (*sert*) in the raphe of depressive rats.<sup>5</sup> Moreover, the  
59 miR-132-3p has been described in neural cell epigenetics,<sup>6</sup> coupling circadian rhythms and the daily  
60 rhythms of neuron plasticity involved in cognition.<sup>7</sup> Although only a few changes in miRNA  
61 expression were reported after maternal separation, these studies support the notion that Early Life  
62 Stress induces susceptibility to later life stress at the epigenome level.  
63 Moreover, the absorption of miRNAs in the rat stomach has been demonstrated to be under the  
64 dependence of Systemic RNA interference-deficient transporter (SIDT1)<sup>8</sup>, opening the possibility  
65 of the natural transit of miRNAs present in breast milk from mother to offspring. Consequently,  
66 manipulating the physiopathology of rat pups through the use of extracellular miRNAs, given as  
67 supplements, will integrate new knowledge for preventing at the earliest time possible the onset of  
68 chronic pathology. The oral delivery of extracellular miRNA is of general interest<sup>9, 10</sup>, but we have  
69 little knowledge on the immediate and long-term effects on the molecular phenotype of a model  
70 organism, as well as on the consequences of combining with early-life stress. Milk contains a high  
71 amount of miRNAs which has been proposed for transfer between mother and child for immune  
72 regulation<sup>11</sup>, priming the immune system of the lactating infant when from plant origin<sup>12</sup>,  
73 transgenerational health influence<sup>13</sup>, or for trans-species effect on adult consumers through dairy  
74 products.<sup>14, 15</sup> Here, we have focused on two microRNA common to rat and human breast milk and

75 used for their delivery previously developed lipidic derivatives of natural aminoglycosides, allowed  
76 in food, shown to be efficient for intracellular delivery of siRNA, DNA, mRNA, or miRNA.<sup>16-21</sup> The  
77 miR-320-3p is associated to breast milk exosomes<sup>22</sup> and a highly conserved miR among mammals.<sup>23</sup>  
78 A *cis*-regulatory role is known for miR-320-3p which participates in a negative feedback loop at the  
79 polr3d promoter inducing transcriptional gene silencing in Human Embryonic Kidney-293 cells.<sup>24</sup>  
80 In rat pups, we have reported an immediate effect of miR-320-3p administered orally on hspb6,  
81 polr3d mRNAs and polr3d promoter in chromatin complexes.<sup>20</sup> The miR-320-3p is known for its  
82 bioactivity in various diseases from type-2 diabetes, and inflammatory bowel disease, to  
83 atherosclerosis.<sup>25-28</sup> The miR-375-3p is one of the most abundant miRNA in the gastrointestinal  
84 tract, impacting the homeostasis of the enteroendocrine lineage of mucosal cells.<sup>29</sup> It has been  
85 related to child depression<sup>30</sup>, and the differentiation of mouse neurites in the hippocampus.<sup>31</sup> This  
86 miRNA may be involved in neuroprotective mechanisms in response to stress<sup>32, 33</sup> and has further  
87 been associated with Alzheimer's Disease.<sup>34</sup>  
88  
89 In this paper, we demonstrate that force-feeding with miR-320-3p/DOSP at mid-lactation induced  
90 long-term effects on gastrointestinal epithelium and brain of young rats, deeply altering the  
91 regulation of endogenous miR-320-3p and miR-375-3p.

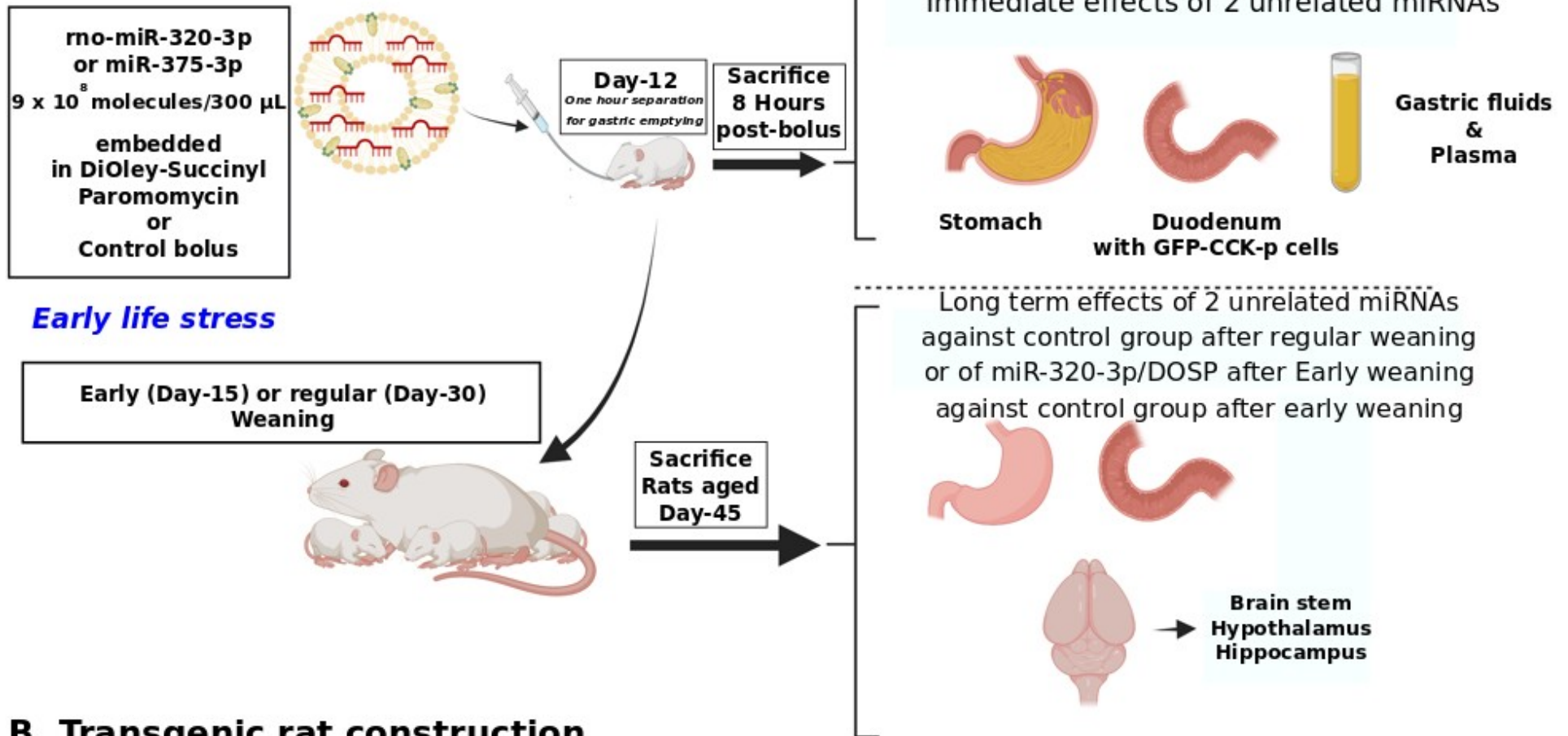
## 92 **Material and methods**

### 93 **1. Study design (Figure 1)**

94 As shown in [Figure 1A](#), we have followed the immediate effect of miRNA bolus on rat pups at  
95 Day-12 at the beginning of the dark phase and 8 hours after oral administration.<sup>20</sup> Here, we apply  
96 the miRNA supplementation from Day-12 of age because extensive changes in gene expression of  
97 neurodevelopmental processes related to cell differentiation and cytoskeleton organization, have  
98 been identified in the hypothalamus of rat pups born from low protein-fed mothers.<sup>35</sup> Rats were  
99 sacrificed at Day-45 to evaluate long-term effects on the physiology<sup>36</sup> after force-feeding with either  
100 miR-320-3p or miR-375-3p embedded in DOSP. The control groups were force-fed with the  
101 vehicle solution of DOSP. The rats pre-treated with miR-320-3p/DOSP or corresponding control  
102 groups endured or not an early weaning, before sacrifice at Day-45. The rat pre-treated with miR-  
103 375-3p/DOSP received a regular weaning. The miR-320-3p/DOSP and miR-375-3p/DOSP groups,  
104 both enduring a regular weaning, were used for evaluation of the long-term effects of 2 unrelated  
105 miRNAs. We have used 2 weaning times at Day-15 (Early) or Day-30 (Regular) applied after the  
106 single oral bolus of miR-320-3p/DOSP in order to compare consequences of the treatment  
107 combined with early life stress against stress-less weaning.<sup>37</sup> Rat pups separated from their mothers  
108 at D-15 were fed on soup made from standard chow. Litters were maintained at the UMR-1280  
109 husbandry, allocated in rooms either with a light on at 7:00 am or off at 7:00 pm. Our experimental  
110 protocol was approved by the “Comité d’éthique pour l’expérimentation animale, Pays de la Loire,  
111 France” under number #APAFIS-21917. Studies on rats were performed according to the rules of  
112 the Nantes animal experimental unit [in compliance with the European Communities Directive of  
113 2010/63/UE, 22 September 2010]. The total number of mothers was of 9. The first litter with 12 rat  
114 pups was used for immediate effect of miR-320-3p/DOSP, miR-375-3p/DOSP, or control made up  
115 of DMEM/PBS0 (4 pups per combination at random, sacrifice at 8 Hours). Eight litters were  
116 balanced at birth to 8 rat pups (4 males, 4 females). For oral inoculation, all solutions of deep-frozen

## A. Experiments

### miRNAs delivery by Force feeding



## B. Transgenic rat construction

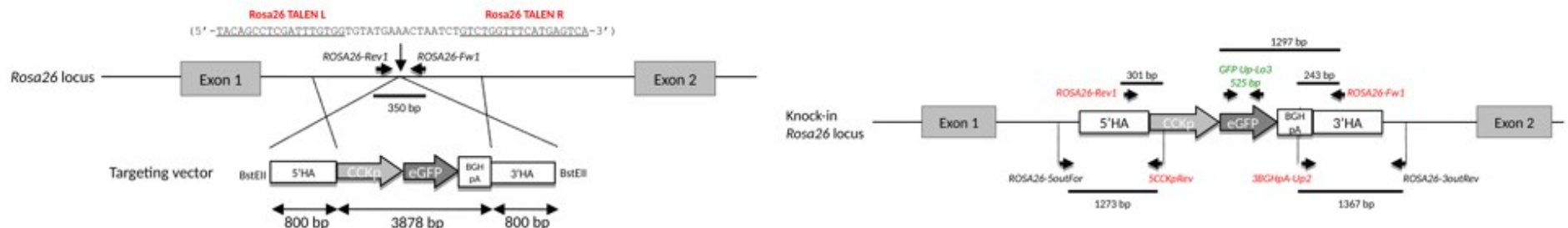


Figure 1. Study design.

A. *Experiments.* we have followed the immediate effect of miRNA bolus on rat pups at Day-12 at the beginning of the dark phase and 8 hours after oral administration.<sup>20</sup> We have sacrificed rats at Day-45 to evaluate long-term effects on the physiology<sup>36</sup> of rats force-fed with either miR-320-3p or miR-375-3p embedded in DOSP against controls force-fed with the vehicle solution of DOSP. The rats pre-treated with miR-320-3p/DOSP endured or not an early weaning, before sacrifice at Day-45. The rat pre-treated with miR-375-3p/DOSP received a regular weaning, and sacrificed at Day-45. The miR-320-3p/DOSP and miR-375-3p/DOSP were used for evaluation of the long-term effects of 2 unrelated miRNAs. We have used 2 weaning times at Day-15 (Early) or Day-30 (Regular) applied after the single oral bolus of miR-320-3p/DOSP in order to compare midterm consequences of the treatment combined with early life stress against stress-less weaning.<sup>37</sup> Rat pups separated from their mothers at D-15 were fed on soup made from standard chow. Litters were maintained at the UMR-1280 husbandry, allocated in a room either with a light on at 7:00 am or off at 7:00 pm. Our experimental protocol was approved by the “Comité d’éthique pour l’expérimentation animale, Pays de la Loire, France” under number #APAFIS-21917. Studies on rats were performed according to the rules of the Nantes animal experimental unit [in compliance with the European Communities Directive of 2010/63/UE, 22 September 2010]. The total number of mothers was of 9. The first litter with 12 rat pups was used for immediate effect of miR-320-3p/DOSP, miR-375-3p/DOSP, or control made up of DMEM/PBS0 (4 pups per combination at random, sacrifice at 8 Hours). Eight litters were balanced at birth to 8 rat pups (4 males, 4 females). For oral inoculation, all solutions of deep-frozen miRNAs and of DOSP kept at 4°C, were warmed to room temperature. Rat pups were maintained at warm (37°C) in a parallel transparent box next to the mother box during one hour for gastric emptying. Mixtures of miRNAs and DOSP were extemporally prepared. We have chosen an oral administration at the beginning of the dark phase.<sup>20</sup> Gastric fluids were collected to evaluate a putative re-export of loaded miRNA through extracellular vesicles. In the gut, we have sampled the stomach as the inoculation site and the duodenum as relevant for our transgene expression. The rationale for choosing brain area was for the hypothalamus as the main center of energy homeostasis, the hippocampus as involved in the memory of food reward and choice, the brain stem as the outcome of the vague nerve linking the intestine to the brain. We have screened the effects according to gene sets related to inflammatory and enteroendocrine status of stomach or duodenum, and on the serotonergic/dopaminergic balance of brain areas. Our transgenic GFP-CCK-p rat derived from the Sprague-Dawley strain is allowing to follow an enteroendocrine cell lineage labeled with Green Fluorescent Protein in duodenum crypts. In addition, the Green Fluorescent Protein transcripts can be followed in rat neuronal cells expressing CholeCystoKinin (CCK).

B. *Transgenic rat generation.* Targeted integration of a CCK promoter-GFP cassette into the rat Rosa26 locus (left panel). Schematic representation of the rat Rosa26 locus. TALEN cleavage (vertical arrow) in the first intron, as well as the sequences recognized by both TALENs, the targeting vector with expression cassette (3878 bp) and the 5' and 3' homology arms (HA) (800 bp each) are indicated. The PCR primers flanking the cleavage sequence used to do the first genotyping are also indicated (right panel). Schematic representation of the CCK-GFP cassette integration. To verify the integrity of the CCK-GFP cassette, genomic DNAs were PCR amplified with the primers described and the PCR amplicons were analyzed for their size and Sanger sequences.

117 miRNAs and of DOSP kept at 4°C, were warmed to room temperature. Rat pups were maintained at  
118 warm (37°C) in a parallel transparent box next to the mother box during one hour for gastric  
119 emptying. Mixtures of miRNAs and DOSP were extemporaneously prepared. Force-feeding was  
120 gently applied by trained experimenters allowing the delivery of  $9 \times 10^8$  molecules of miRNA in the  
121 stomach at the beginning of the dark phase.<sup>20</sup> Gastric fluids were collected to evaluate a putative re-  
122 export of loaded miRNA through extracellular vesicles. The stomach cell wall was sampled as the  
123 inoculation site and the duodenum was relevant for our transgene expression. All q-PCR analyses  
124 were done on 8 rats, when we did not found a sex effect. The rationale for choosing brain area was  
125 for the hypothalamus as the main center of the major center of energy homeostasis, the  
126 hippocampus as involved in the memory of food reward and choice, the brain stem as the outcome  
127 of the vague nerve linking the intestine to the brain. We had a strong sex effect in brain, so we did  
128 all q-PCR analyse on 4 rats per sex. The effects have been screened according to gene sets related to  
129 inflammatory and enteroendocrine status of stomach or duodenum, and on the  
130 serotonergic/dopaminergic balance of brain areas. Our transgenic GFP-CCK-p rat derived from  
131 the Sprague-Dawley strain is allowing to follow an enteroendocrine cell lineage labeled with Green  
132 Fluorescent Protein (GFP) in duodenum crypts. .

133 As shown in [Figure 1B](#), a Sprague-Dawley transgenic strain of rats expressing enhanced green  
134 fluorescent protein eGFP under the control of the CCK promotor, generated using the transcription  
135 activator-like effector nuclease (TALEN) methodology and knock-in in the *Rosa26* locus<sup>38</sup> was  
136 selected. This transgenic rat line allows studying CCK- eGFP+ cells, for example in duodenum  
137 crypts and villi.<sup>39</sup> In addition, the eGFP (*gfp*) transcripts can be followed in rat neuronal cells  
138 expressing CCK.<sup>40</sup> PCR for genotyping and qPCR primers are shown in [Supplementary Table 1](#).

## 139 **2. miRNAs**

140 Two miRNAs, present in breast milk, were used for oral supplementation, miR-320-3p  
141 (MIMAT0000903 AAAAGCUGGGUUGAGAGGGCGA) already described by us and others as



142 having epigenetic activity<sup>20,24</sup>, and miR-375-3p MIMAT0005307  
143 UUUGUUCGUUCGGCUCGCGUGA, known to target proliferative cells in gut and related to  
144 Vitamin-E metabolism in humans.<sup>41</sup> The rno-miR-320-3p or rno-miR-375-3p were ordered from  
145 Eurofinns, Germany and they were checked at reception by reverse transcription and q-PCR with  
146 corresponding TaqMan probes ([Supplementary Table 1](#)).

147

### 148 **3. Ribonucleic acid vector**

149 The vector was Di-Oleyl-Succinyl-Paromomycin (DOSP), used for *in vivo* short-term transfection  
150 of miRNA<sup>20</sup> and of mRNA.<sup>18</sup> The vector is non-cytotoxic and allowed in food practice. Prior to use,  
151 the quality of the vector was assessed by the size distribution of DOSP nanoparticles with a pike at  
152 200 nm on a Gold-q-Nano (Izon).

153

## 154 **4. Global analysis of miR-320-3p or miR-375-3p networks**

### 155 **4-1. miR Target enrichment analysis**

156 To identify potential target genes regulated by miR-320-3p or miR-375-3p, the miRNA-target pairs  
157 were retrieved from TargetScan 7.2 (up date: march 2018) (<http://www.targetscan.org>), miRWalk  
158 v6.0, (update: Jan 2021 [www.umm.uni-heidelberg.de/apps/zmf/mirwalk](http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk)), and miRDB v6.0 (up  
159 date: June 2019, <http://mirdb.org/>), exploring the miRNA binding sites within the complete  
160 sequence of rat genome (including 5'-UTR, 3'-UTR regions as well as coding sequences) and  
161 combining this information with a comparative analysis of predicted binding sites. The three  
162 databases were jointly mined, and overlaps of the results were generated to obtain the list of the  
163 most potential regulated transcripts.

## 164 **4-2. Pathway enrichment analysis**

165 To identify pathways in the list of the potentially regulated mRNA we used Panther v 16.0 (released  
166 2020-12-01, <http://www.pantherdb.org/>). To ensure the validity of our findings, we only considered  
167 the three pathways more relevant to both miRNAs.

168

## 169 **5. Gastric fluids and Tissue samples**

170 Briefly at sacrifice, the stomach content was collected and stored under liquid nitrogen.<sup>20</sup> Before  
171 analysis, the samples were thawed and extracellular vesicles were recovered by elution through a  
172 qEV column (Izon) and processed for analyzing the size distribution with a qNano (Izon). The  
173 stomach and the duodenum were rinsed with a Phosphate-Buffered saline solution free of calcium  
174 and magnesium ions. Pieces of the lower part of the stomach (fundus) and of the duodenum were  
175 immersed under liquid nitrogen. In addition, pieces of the duodenum were fixed in 0.1 M PBS  
176 containing 4% paraformaldehyde for 24h and then embedded in paraffin for histology. The  
177 incidence of miRNA/DOSP bolus was evaluated 8 hours after inoculation by measuring D-Glucose  
178 in blood, the contents of miR-320-3p and 375-3p in exosome fractions of gastric fluids, and in rat  
179 pup plasma. For rats on Day-45, in addition to stomach and duodenum, brain compartments were  
180 immersed directly under liquid nitrogen.

## 181 **6. Analysis of miRNA and mRNA by q-PCR**

182 Total RNA extraction was done with Qiazol (Qiagene, France), and cDNA was obtained with  
183 TaqMan miRNA kit (Thermofisher, France). All primers are listed in [Supplementary Table-1](#). We  
184 have used either TaqMan'primers, or self-designed primers with SyberGreen. At the taxonomic  
185 level, the miR-320-3p is a non-canonical miRNA<sup>42</sup> with a non-described 5p form in humans and  
186 rats. In the bioprocessing of true miRNAs like miR-375, both the 3p and 5p molecules are  
187 expressed allowing quantitative exploration by the northern blot of their ratio in human cell lines.<sup>43</sup>

188

## 189 **7. Chromatin ImmunoPrecipitation**

190 The methylation of the Histone 3 in the lysine residue 4, generally, is associated with the activation  
191 of transcription of nearby genes and we used Pierce kit methodology.<sup>20</sup> It should be underlined that  
192 little is known concerning the nuclear delivery of the non-complexed miRNA.<sup>46, 47</sup> Chromatin  
193 immunoprecipitation was performed with Pierce Chromatin Prep Module (Thermo Scientific  
194 #26158). Briefly, small tissue aliquots were cross-linked by exposition to 1% formaldehyde.  
195 Chromatin was fragmented by Micrococcus Nuclease.

196 Immunoprecipitations were performed using 1 µg of Anti-Trimethyl-Histone-3-Lys-4 (-Thermo  
197 Fisher Scientific; catalog# PA5-17420) overnight at 4 ° C. Micrococcus nuclease was used at 0.25  
198 µL/sample.

199 We collected immune complexes with agarose A/G for 2 h at 4 ° C, beads were rinsed twice by  
200 PBS0 and pelleted at 94 × g for 1 min. Immune complexes were eluted by adding  
201 100 µL of elution buffer to pelleted beads. After brief vortexing, preparations were incubated at  
202 Room Temperature for 15 min.

203 Thereafter, beads were spun down and the supernatants (eluates) carefully transferred to another  
204 tube. The elution step was repeated. Both eluates were combined.

205 We added 5 M NaCl and proteinase K allowing crosslink reversion by 1.5-h incubation at 65 ° C.  
206 Nucleic acids were recovered by Qiagen miRNA-Easy kit and analyze ChIPped chromatin using  
207 quantitative PCR. iQ SYBR Green Supermix (Bio-Rad) was used to perform real-time PCR on an  
208 iCycler iQ system (Bio-Rad) with promoter-specific primers ([Supplementary Table-1](#)).

209

## 210 **8. Density of duodenal green-labeled CCK-p enteroendocrine cells**

211 Following rehydration, thick sections (4 µm) were stained with chromogranin A (marker of total  
212 enteroendocrine cells) rabbit polyclonal antibody (diluted 1/500; 20085 Immunostar). After  
213 incubation with a secondary biotinylated goat anti-rabbit (diluted 1/1000, A24541, Life  
214 Technologies), chromogranin A was revealed with Alexa Fluor 568-conjugated streptavidin

215 (S11226, Invitrogen). Slides were mounted in Prolong Gold antifade reagent (Invitrogen) that  
216 contains DAPI to counterstain nuclei. The density of CCK-producing cells that stained in green  
217 (endogenous GFP) and positive for chromogranin A was measured by fluorescence microscopy  
218 (Zeiss, Axio Imager M2m) in the crypts of 3 sections of duodenum using a 40 X objective. The data  
219 were expressed as the percentage of chromogranin A-positive cells or CCK (GFP) positive cells per  
220 crypt.

221

## 222 **9. Sample nomenclature, selection of reference genes, statistical** 223 **analysis**

224 The nomenclature for identifying sample is: stomach “sto”; brain stem “bs”; hippocampus “hip”;  
225 hypothalamus “hy”; weaning at Day-15 “w15”; weaning at Day-30 “w30”; oral bolus with miR-  
226 320-3p/DOSP “b320”; oral bolus with miR-375-3p/DOSP “b375”; oral bolus of controls “btem”. As  
227 an example, hy-w15-btem means that the rat sample is from Hypothalamus, from a rat weaned at  
228 Day-15 receiving a control bolus. We are using 3 miRNAs as reference genes.<sup>20</sup> But the miR-146b-  
229 5p has been reported as a marker of depression.<sup>48</sup> So, we have also analyzed the datasets using  
230 either all miRNAs or all mRNAs per sample as a global normalization procedure. By applying this  
231 technique with miR-146b-5p, we did not find significant variation. Consequently, the miR-146b-5p  
232 remains under our hand, a valid reference gene ([unshown results](#)). We have decided to analyze  
233 Delta-Cq after a log10 transformation<sup>20</sup>, justifying our use of one way ANOVA for the comparison  
234 between groups, and to test multiple comparisons. Data analysis was performed with R Commander  
235 and R suite or Graph Pad software.

236 We have used Cytoscape to create networks of miRNA, mRNA significantly deregulated in our  
237 data-set. Excel files of raw Cq data organized by tissues can be made be accessible at the UN-Cloud  
238 of the Nantes University.

239

## 240 **Results**

241

### 242 **1. In silico analysis of rno-miR-320-3p and rno-miR-375-3p** 243 **networks**

244 We have found that the sequence coding for mature miR-320-3p was identical in rat, mouse, and  
245 human. Pairwise alignment revealed that miR-320-3p is antisense, encoded in the intergenic region  
246 of chromosome 8 at approximately 200 bp upstream of the TSS of RNA polymerase III subunit D  
247 (polr3d) in the chromosome 15 in rat (rn6), the human (CRCh38.p13), and the chromosome 14 in  
248 mouse.<sup>24</sup> We have found that the sequence coding for mature miR-375-3p was identical in rat  
249 mouse, and human. The mature full sequence of miR-375-3p was located at chromosome 9 for rat,  
250 at chromosome 2 for humans, and chromosome 1 for mice. In the three genomes, it is located at the  
251 non-coding region.

252 Current miRs target prediction algorithms regularly present different numbers of potential  
253 interactions. Due to that, we have fused the results of three databases to obtain the most accurate list  
254 of target genes. For miR-320-3p, the list consisted of 111 transcripts distributed between 69  
255 pathways. For miR-375-3p, we obtained a list of 24 transcripts found in 12 pathways.

256 By target mining miRWalk on 3'-UTR, set on perfect matching between miRNA and target mRNA,  
257 we have found 126 genes common between miR-320-3p and miR-375-3p. They are corresponding  
258 to the Kegg pathway : rno01100\_Metabolic\_pathways.

259 We have found 308 genes common between, miR-320-3p and miR-132-3p, among which we are  
260 exploring PER2. We have found 112 genes common between miR-320-3p and miR-16-5p, among  
261 which we are exploring period2 (per2), circadian locomotor output cycles kaput (clock).

262 The miRNA delivered to cells with appropriate carriers or expressed in cells using suitable vectors  
263 often triggers both intended sequence-specific silencing effects and unintended sequence-non-  
264 specific immune responses.<sup>44</sup> So we have established a list of genes for exploring the inflammatory

265 status of stomach samples: Interleukin1A (*IL1A*), Interleukin6 (*IL6*), Interferon-gamma (*IFNg*),  
266 Signal transducer and activator of transcription 3 (*stat3*), Interleukin10 (*IL10*), Tumor Necrosis  
267 Factor alpha (*tnf-a*), Signal transducer and activator of transcription 1 (*stat1*), *iNOS*, *PPARg*  
268 (peptide related to food consumption), *foxa1*, Interleukin1B (*IL1B*). By data mining, these genes are  
269 related to Inflammatory status: GO:0006954\_inflammatory\_response and  
270 GO:0005125\_cytokine\_activity.

271 In brain samples, we have explored *clock* gene which is common to miR-320-3p and miR-375-3p,  
272 along with Brain And Muscle ARNT-Like 1 (*bmal1*), Period1 (*per1*), and Period2 (*per2*).  
273 Interactions between miRNA and mRNA were built using miRWalk<sup>45</sup>, and for the  
274 serotonergic/dopaminergic profiles: serotonin transporter (*sert*), 5-hydroxytryptamine receptor 1B  
275 (*5ht1b*), 5-hydroxytryptamine receptor 2C (*5ht2c*), Dopamine receptor D1 (*drd1*), Dopamine  
276 receptor D2 (*drd2*), Cholecystokinin (*cck*). They are found by data mining related to  
277 GO:0007420\_brain\_development; GO:0003676\_nucleic\_acid\_binding, and  
278 GO:0006357\_regulation\_of\_transcription\_by\_RNA\_polymerase\_II.

279 Taking advantage of our transgenic rat, we have assayed markers of enteroendocrine lineage (Paired  
280 box gene 4 (*pax4*), Paired box gene 6 (*pax6*), ghrelin (*ghrl*), Peptide YY (*pyy*), chromogranin A  
281 (*chgA*), Gastric Inhibitory Polypeptide (*gip*), Cholecystokinin (*cck*) by q-PCR.

282

283 In summary, our *in silico* analysis is showing that miR-320-3p is influencing on average a gene  
284 network four times wider than miR-375-3p.

285

## 286 **2. Immediate effects of force-feeding miRNAs/DOSP in the stomach and** 287 **duodenum of breast-fed rats at Day-12.**

288 The transgenic rat strain was checked for correct expression of the transgene by PCR using tail  
289 biopsies to check for the homozygote status. Duodenal cross-section and immunostaining were

290 realized on D12 and D45 rats to check for the expression of GFP-labelled duodenal cells according  
291 to the co-expression of *chgA*, a biomarker of total enteroendocrine cells ([unshown results](#)).  
292 As shown in [Supplementary Figure 1](#), the relative level of miR-320-3p is increased in the stomach  
293 wall of rat pups supplemented with miR-320-3p/DOSP (**B**,  $p=0.05$ ) whereas *hspb6* transcripts are  
294 decreased (**D**,  $p<0.05$ ) with a similar trend for *polr3d* mRNA (**C**). Concerning enteroendocrine  
295 markers, only *chgA* was highly up-regulated ( $p<0.0001$ ) with miR-320-3P/DOSP treatment.  
296 Likewise, the *IL1B* was down-regulated only with rat pups treated with miR-320-3P/DOSP  
297 ( $p<0.001$ ).  
298 Moreover, the treatment with miR-320-3p/DOSP induced a significant decrease in chromatin  
299 complexes harboring H3K4me3 and *polr3d* promoter in gastric cells ([Supplementary Figure 1E](#),  
300  $p<0.05$ ). Surprisingly, we did not detect any immediate effect of miR-375-3p/DOSP both on  
301 transcripts or on chromatin complexes ([Supplementary figure 1E](#)), but we infer from the long-term  
302 effects reported below that the delivery of miR-375-3p was done according to its putative  
303 cytoplasmic site of bioactivity. We did not detect leakage of both miRNAs in exosomal fractions of  
304 gastric fluids, confirming our previous demonstration that the vector delivered the miRNAs into the  
305 cytoplasm of digestive cells.<sup>20</sup> The levels of D-Glucose were not different (Group average  $\pm$   
306 standard deviation, treated with miR-320-3p: 135 mg D-Glucose/dL $\pm$ 10,9; treated with miR-375:  
307 131,2  $\pm$  15; control: 127,2  $\pm$  6,5). The levels of miR-320-3p or miR-375-3p in plasma according to  
308 treatment with miR-320-3p/DOSP (Average Cq  $\pm$  standard error: 22.79  $\pm$  0.72 or 29,57  $\pm$  0.65),  
309 miR-375-3p/DOSP (21.99  $\pm$  0.89 or 27.78  $\pm$  1.34), or control (21.83  $\pm$  1.04 or 28.38  $\pm$  0.84) were  
310 not different.

311

312 In summary, these data on the immediate effect of rno-miR-320-3p/DOSP suggest that the miRNA  
313 molecules are bioactive both in the cytoplasm and chromatin complexes, but we did not show any

314 evidence of an immediate effect of rno-miR-375-3p/DOSP at the transcript level in the stomach,  
315 even on enteroendocrine markers.

316

### 317 **3. Effects of early weaning in control groups (not** 318 **supplemented with miRNA)**

#### 319 **3.1 Stomach**

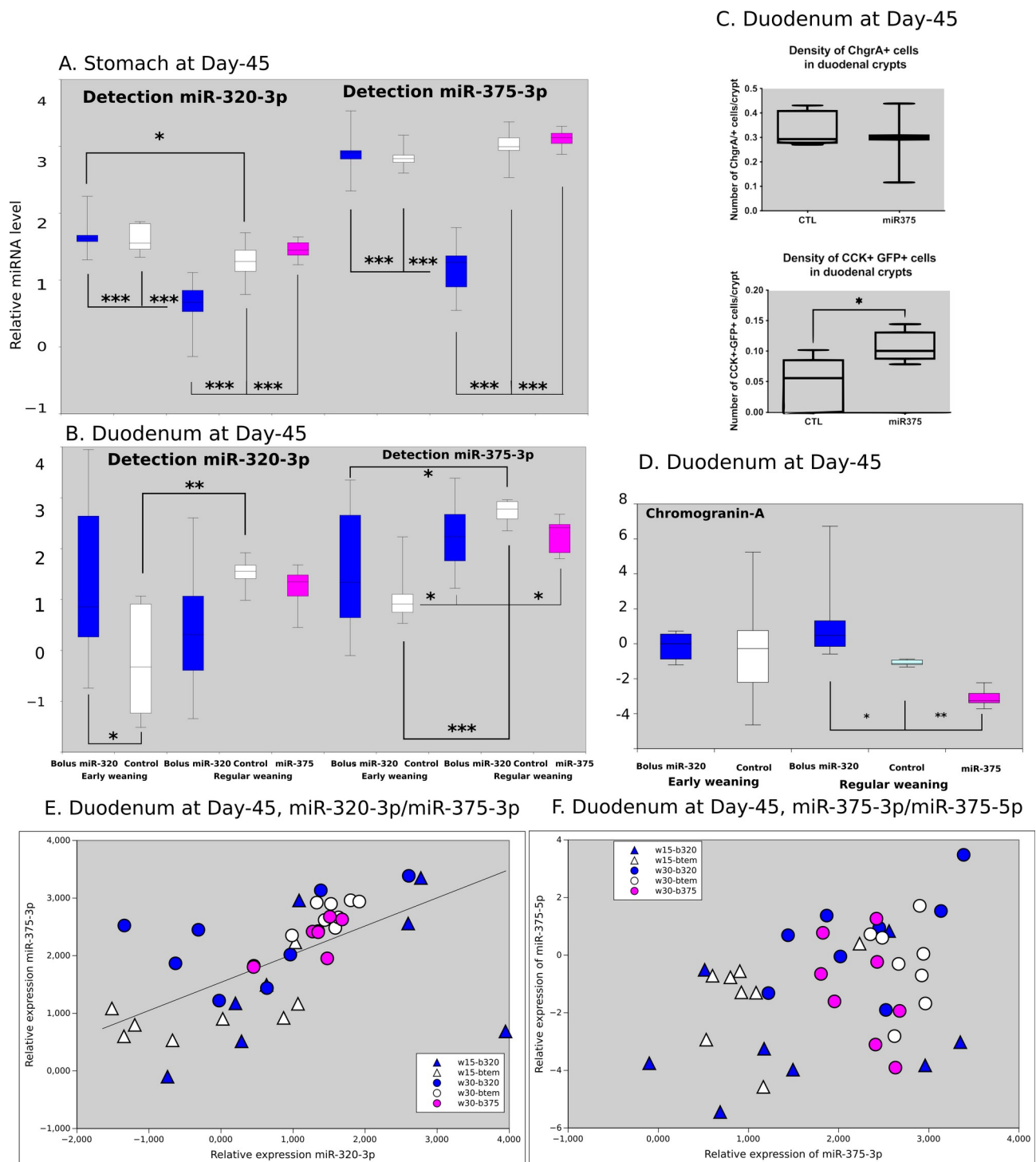
320 Gastric endogenous miR-320-3p and miR-375-3p were not significantly different between early-  
321 weaned and regularly weaned rats at Day-45 (Figure 2A; note that the miR-132-3p or miR-504  
322 were not detected in stomach samples). No difference between weaning times has been recorded for  
323 the expression of *polr3d* or *hspb6* mRNA (Supplementary Figure 2A, B). The level of *tnf-a* was  
324 down-regulated, and those of *IL6*, and *IFN-g* were up-regulated in early-weaned controls compared  
325 to the regular weaning controls (Figure 3A ; Supplementary Figure 2). The altered expressions of  
326 these cytokine transcripts suggest a long-lasting state of inflammation induced by early weaning  
327 stress. The relative levels of chromatin complexes harboring H3K4me3 were slightly lower in the  
328 early-weaned rats (Supplementary Figure 1F, G).

#### 329 **3. 2. Duodenum**

330 Endogenous miR-320-3p and miR-375-3p at Day-45 (Figure 2B,  $p < 0.05$  and  $p < 0.001$ , respectively)  
331 were down-regulated in early weaned rats as compared to regularly weaned ones. Consequently,  
332 these miRNAs could be crucial in the peripheral response to Early Life stress. We did not find  
333 strong correlation between the relative levels of miR-320-3p and miR-375-3p (Figure 2E) nor  
334 between miR-375-3p and 5p (Figure 2F). But the miR-375-5p level of w15-b320 were down-  
335 regulated in comparison with w30-b320 ( $p = 0.02$ ), and w30-btem ( $p = 0.04$ , Figure 2F).

336





**Figure 2. Long term effects of miR-320-3p/DOSP or miR-375-3p/DOSP on miR-375-3p and miR-320-3p expressions in stomach (A), duodenum (B), long term effect of miR-375-3p/DOSP followed by regular weaning on the density of CCK+GFP+ duodenal cells (C) or long term effect of miR-320-3p/DOSP or miR-375-3p/DOSP followed by early or regular weaning on the level of chromogranin A transcripts in duodenum (D). Scatter plots between miR-320-3p/miR-375-3p and miR-375-3p/miR-375-5p are shown in E and F, respectively. In E, low correlation ( $R^2=0.54$ , black line) and in F, the miR-375-5p levels of w15-b320 were down-regulated in comparison with w30-b320 ( $p=0.02$ ), and w30-btem ( $p=0.04$ ). The light gray background reminds that rats were sacrificed in the dark phase. Note: \*  $p<0.05$ ; \*\*  $p<0.01$ ; \*\*\*  $p<0.001$ .**

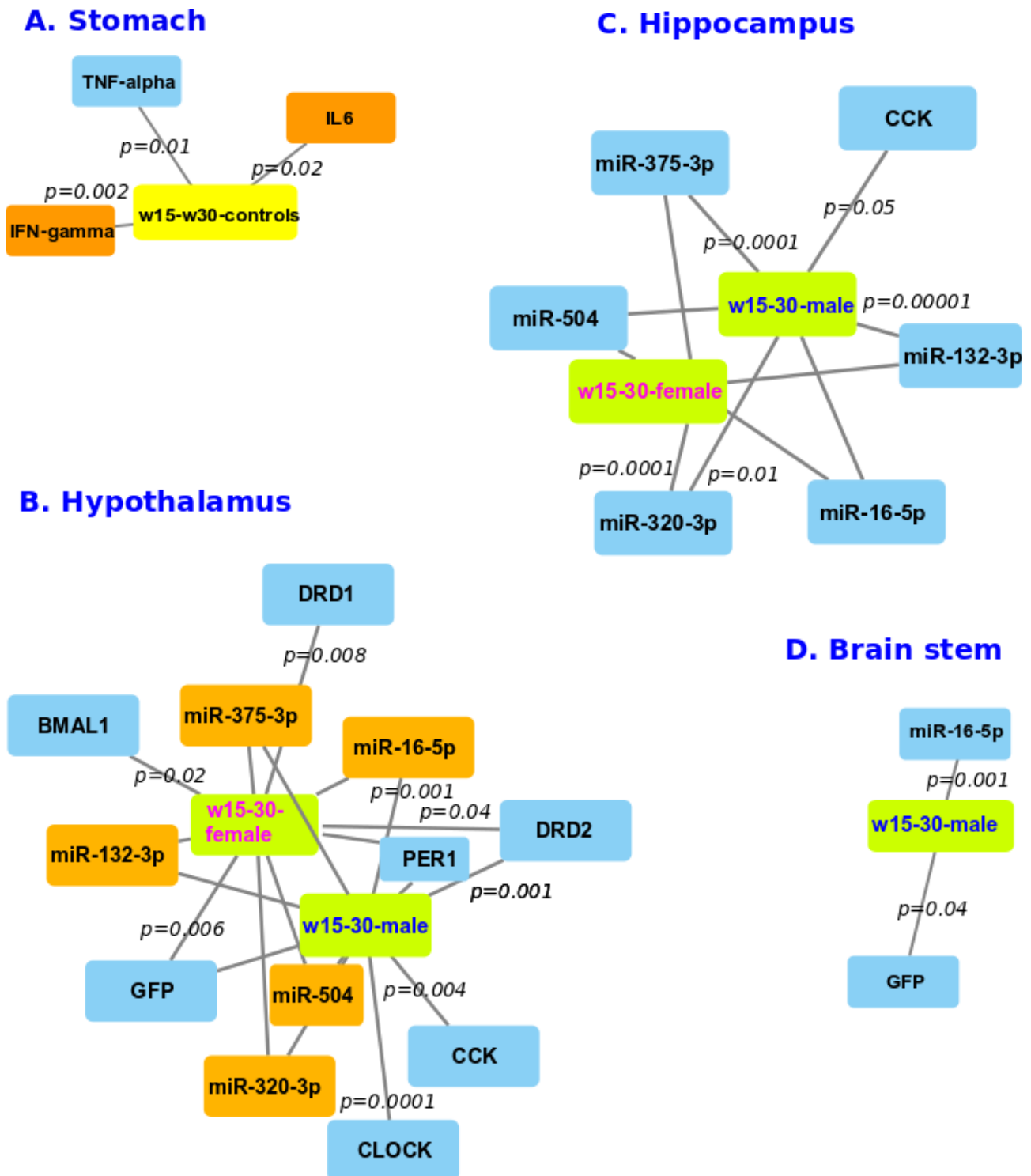


Figure 3. Comparison of early and regular weaning controls. Network of genes significantly deregulated in stomach wall (A), hypothalamus (B), hippocampus (C), and brain stem (D). Note on group nomenclature, for instance, “w15-w30” meaning comparison between the early weaned controls with the regular weaning controls. Edge length is inversely proportional to  $p$  significant threshold. Most  $p$  values are indicated in italic. Orange up-regulated, blue down-regulated.

### 337 **3. 3. Brain**

338 A comparison of controls submitted to early or regular weaning showed drastic differences between  
339 hypothalamus, hippocampus, and brain stem.

340 In the hypothalamus, all tested miRNAs (miR-320-3p, miR-375-3p, miR-16-5p, miR-132-3p, and  
341 miR-540) were up regulated in both females and males (Figure 3B, 4A, 5A). We have found a down  
342 regulation of *drd1* in the hypothalamus of early-weaned females with a higher expression of miR-  
343 504 according to Huang and Li, 2009<sup>4</sup> (Figure 6A, the correlation between *drd1* and miR-504 for 39  
344 animals was of -0.755 irrespectively of their experimental group). However, we did not find any  
345 difference in *drd1* relative level in the male hypothalamus according to weaning time, even if an up-  
346 regulation of miR-504 was recorded (Figures 3B, 5A, Supplementary Figure 3A, B). If all tested  
347 miRNAs (miR-504, miR-16-5p, miR-132-3p, miR-320-3p, miR-375-3p) were all up-regulated in  
348 the hypothalamus of early-weaned rats at Day-45 of life, only the *per1* transcripts of the circadian  
349 clock were down-regulated for male and female rats (Figure 3B; Supplementary Figure 4).  
350 However, the females had a down-regulation of *bmal1*, and the males of *clock* (Figure 3B;  
351 Supplementary Figure 4). The *cck* transcripts were down-regulated with early-weaned  
352 hypothalamus, and according to the logic of the transgene construct, a down-regulation of the *gfp*  
353 transcripts was also found in males and females. No strong correlation between *cck* and *gfp*  
354 (correlation of 0.537) was recorded, suggesting that if the promoter was driven by the same  
355 transcriptional machinery as the *cck* gene, the transgene was independently regulated from the *cck*  
356 endogenous gene promoter (Supplementary Figure 5).

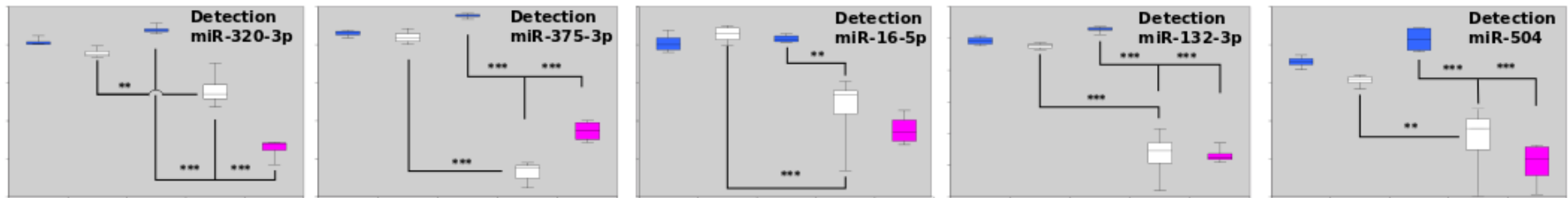
357

358 In the hippocampus, all miRNAs were down regulated (Figure 3C, 4B, 5B) like for miR-320-3p and  
359 miR-375-3p in the stomach. In Figure 3C, all tested miRNAs were down-regulated for both sexes,  
360 as well as for *cck* gene (Supplementary Figure 5).

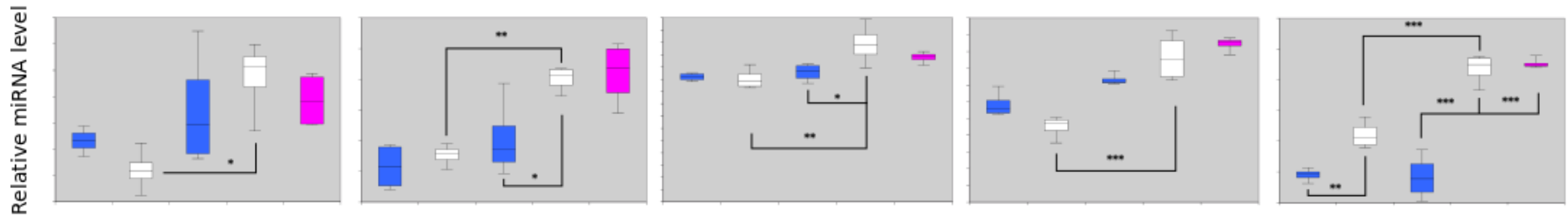
361



### A. Hypothalamus



### B. Hippocampus



### C. Brain stem

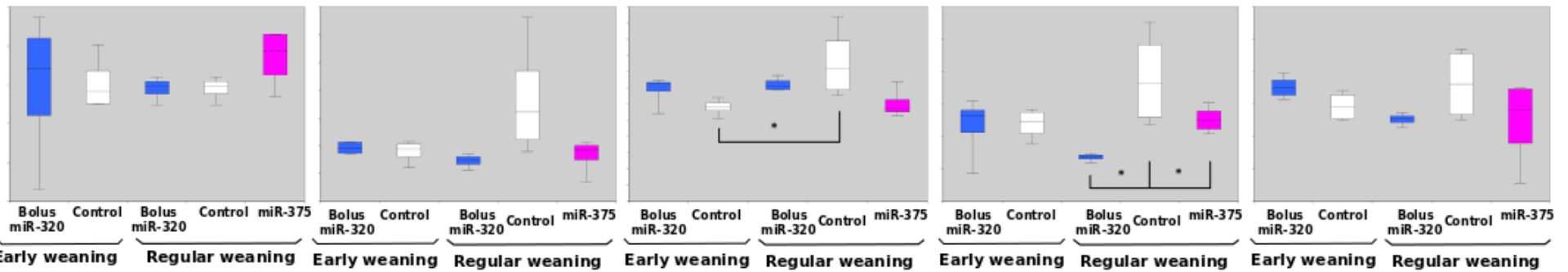


Figure 5. Evolution of miRNAs in hypothalamus (A), in hippocampus (B), and in brain stem (C) of males treated with miR-320-3p or 375-3p/DOSP according to early or regular weaning. The light gray background reminds that rats were sacrificed at ZT-20H in the dark phase. Note: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

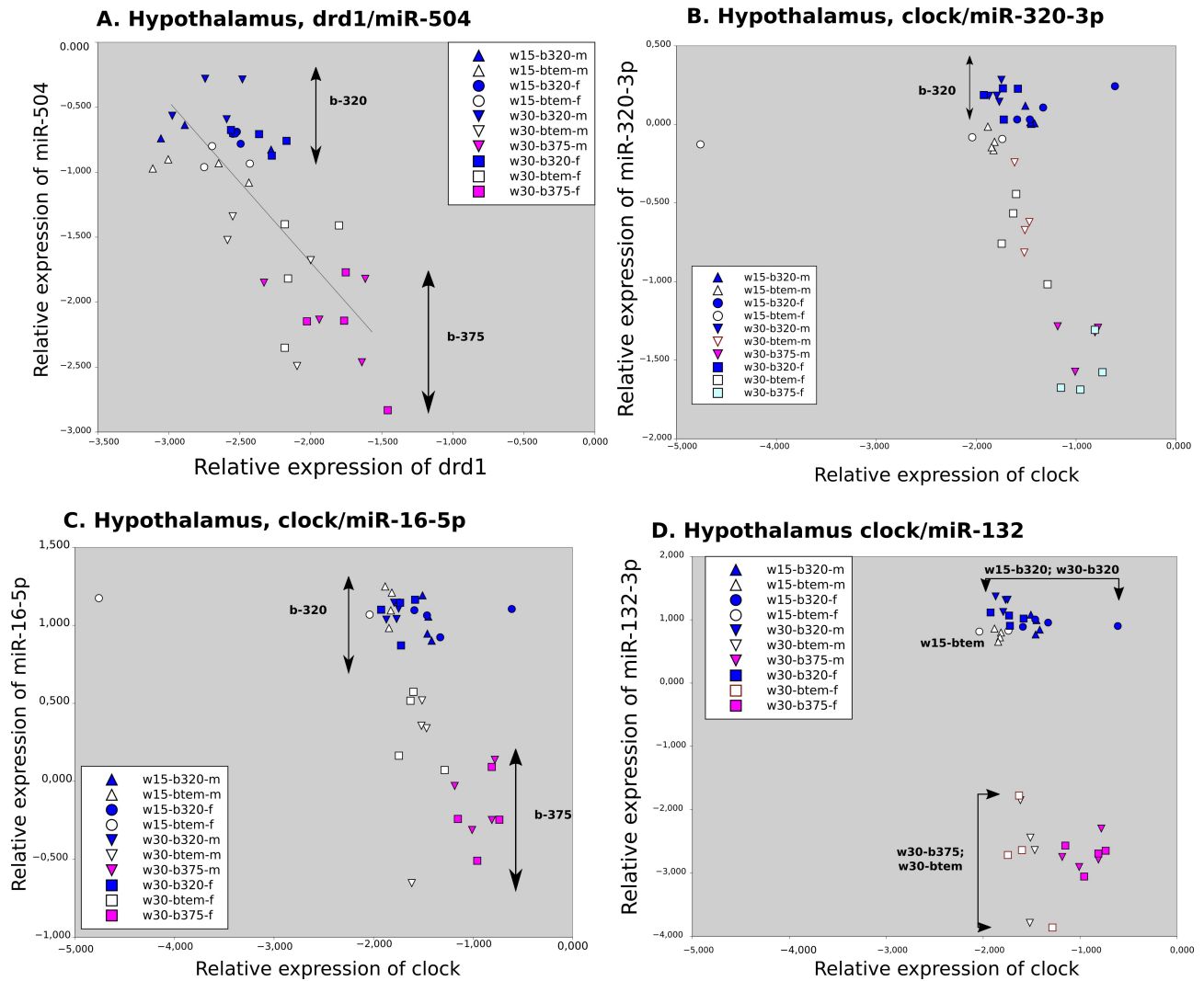


Figure 6. Long term effect of miR-320-3p/DOSP according to early or regular weaning. Note the negative correlation ( $R=-0.75$ , linear regression in black) between miR-504 and DRD1 transcripts (A), and the up-regulation of miR-320-3p (B), miR-16-5p [C] and 132-3p (D) for early weaned rats and regularly weaned rats treated by miR-320-3p/DOSP in hypothalamus cell extracts. The level of clock transcript is significantly different only in B and C. The light gray background reminds that rats were sacrificed in the dark phase. Note: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

362 In the brain stem, only miR-16-5p in males was down regulated ([Figure 3D; 4C](#)). In [Figure 3D](#),  
363 only miR-16-5p and *gfp* were down-regulated. These results obtained at Day-45 for Sprague-  
364 Dawley rats identify new molecular pathways in the follow-up of our work<sup>37</sup>, realized on Wistar rats  
365 between Day-250 and 300.

366

367 In summary, after early weaning, endogenous miR-320-3p and miR-375-3p are deeply altered in the  
368 duodenum, hypothalamus, and hippocampus of young rats. These data are strongly supportive of  
369 using these milk miRNAs as supplements in lactating rat pups, even with discordant results on  
370 stomach (non-significative effects for miR-320-3p or miR-375-3p in [Figure 2A](#)) and duodenum  
371 (significant effects for miR-320-3p in [Figure 2B](#)).

372

#### 373 **4. Comparative evaluation of the long-term effects induced by** 374 **miR-320-3p/DOSP or miR-375-3p/DOSP, with subsequent early** 375 **or regular weaning**

376

##### 377 **4-1. Evaluation of transcripts on stomach or duodenum cell extracts and** 378 **of duodenal cell density by immunohistochemistry and expression of** 379 **duodenal GFP transcripts**

380

381 In the stomach, miR-320-3p and miR-375-3 transcripts were significantly down-regulated in young  
382 rats when treated with miR-320-3p/DOSP compared to controls and miR-375-3p/DOSP ([Figure 2A](#),  
383  $p < 0.001$ ). We did not observe in young rats, deregulation of miR-375-3p, nor of miR-320-3p with  
384 rat pups forced-fed with miR-375-3p/DOSP. By contrast, the miR-320-3p/DOSP treatment had  
385 significantly up regulated the endogenous miR-320-3p transcripts in the duodenum of early-weaned  
386 rats ([Figure 2B](#),  $p < 0.05$ ).

387

388 Moreover, as shown in [Figure 7A](#), the enteroendocrine markers (*pax4*, *pax6*, *chgA*) and *tnf-a* were  
389 all down regulated after treatment with miR-375-3p. By contrast, the treatment by  
390 miR-320-3p/DOSP down-regulated miR-320-3p ( $p=0.001$ ), up-regulated in parallel miR-375-3p  
391 ( $p=0.00001$ ). The *tnf-a* was up-regulated ( $p<0.0001$ ) along with a down-regulation of *stat1*  
392 ( $p=0.05$ ), of *IL10* ( $p=0.00001$ ), and of *foxa1* ( $p<0.001$ ) for rats treated with miR-320-3p/DOSP and  
393 weaned at Day-30 ([Supplementary Figure 2C, D](#)). For early-weaned rats treated with  
394 miR-320p/DOSP, these molecules were not significantly altered, suggesting that the inflammatory  
395 status was unchanged by the miRNA supplementation. The *grlh* and *pyy* transcripts were down-  
396 regulated respectively at  $p=0.04$  and  $p=0.03$ . We have found a trend to a down-regulation for the  
397 GFP-CCK-promoter transcripts and a strong correlation between *cck* and *gfp* (correlation of 0.937  
398 at Day-12) without any strong difference for the correlation (0.91 at Day-45) after  
399 miR-375-3p/DOSP or controls submitted to regular weaning.

400

401 We did not detect any difference between chromatin complexes harboring H3K4me3 and polr3d  
402 promoter in gastric cells after oral administration of synthetic miR-320-3p nor of miR-375-3p and  
403 according to the weaning periods ([Supplementary Figure 1F, G](#)). After regular weaning, no  
404 difference between polr3d, nor hspb6 transcripts at Day-45 was found both in the stomach and the  
405 hypothalamus.

406

407 In the duodenum, a long-term effect of miR-375-3p/DOSP was noted on the density of GFP-CCK-p  
408 labeled duodenal cells ([Figure 2C](#),  $p<0.05$ ). As shown in [Figure 2D](#), the miR-320-3p/DOSP  
409 treatment increased the level of *chgA* and the miR-375-3p/DOSP treatment decreased both  
410 Chromogranin A ([Figure 2D](#),  $p<0.01$ ) and Gastric Inhibitory Polypeptide ( $p<0.001$ ).

411



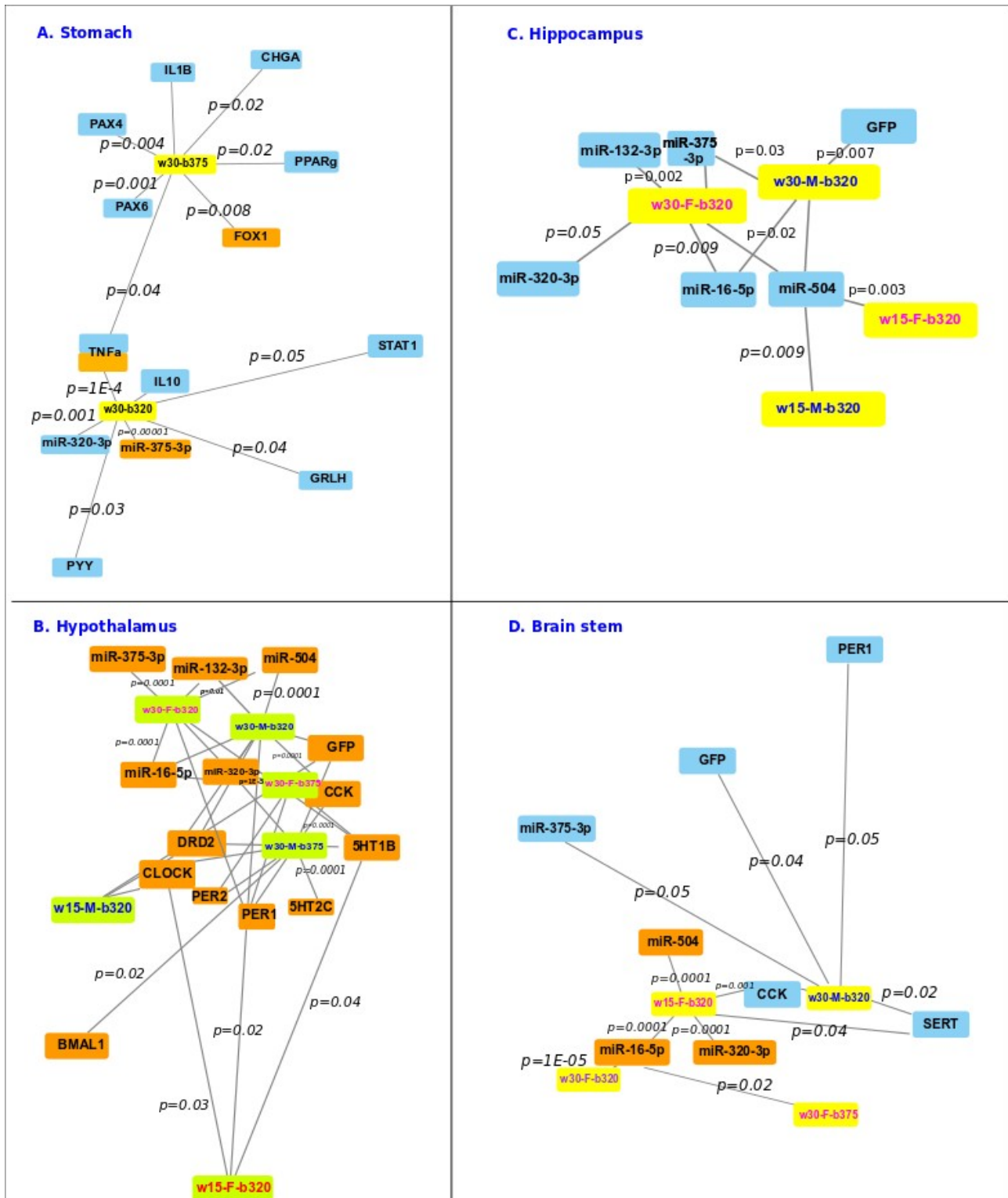


Figure 7. Long term effect of miR-320-3p/DOSP according to early or regular weaning and of miR-375-3p/DOSP with regular weaning. Network of genes significantly deregulated after treatment with miR-320-3p/DOSP or miR-375-3p/DOSP comparatively to control in the stomach wall (A), hypothalamus (B), hippocampus [C], and brain stem (D). Note on group nomenclature, for instance, “w15-w30” meaning comparison between the early weaned controls with the regular weaning controls. Edge length is inversely proportional to  $p$  significant threshold. Most  $p$  values are indicated in italic. Orange up-regulated, blue down-regulated.

412 In summary, long-term effects of an oral exposure during lactation to the high concentration of  
413 miR-320-3p were found with unforeseen consequences on the transcript levels of endogenous miR-  
414 320-3p and miR-375-3p on the group with regular weaning. The most striking result is about the up  
415 regulation of miR-320-3p in the duodenum suggesting that the stem cell compartments along the  
416 gut were differently altered by the treatment with miR-320-3p/DOSP.

417

#### 418 **4-2. Evaluation of transcripts on cell extracts of the hypothalamus,** 419 **hippocampus, and brain stem**

420

421 In the hypothalamus of females ([Figure 4A](#)) and males ([Figure 5A](#)) treated with miR-320-3p/DOSP,  
422 the endogenous miR-320-3p transcripts were up-regulated like miR-375-3p, miR-16-5p, miR-132-  
423 3p, and miR-504 (all  $p < 0.001$ ; correlation coefficients between all the miRNAs were positive and  
424 superior to 0.88). We did not detect any difference for miR-320-3p transcripts for both sexes with  
425 the groups supplemented with miR-375-3p/DOSP. By contrast, we had a strong down regulation of  
426 miR-375-3p for the males or the females treated with miR-375-3p/DOSP.

427 Interestingly, the miR-375-3p levels were altered for both sexes when treated with  
428 miR-320-3p/DOSP and weaned at Day-30. The males treated with miR-320-3p/DOSP and miR-  
429 375-3p/DOSP had an altered level of miR-320-3p, but the observation was true only for the females  
430 treated with miR-320-3p/DOSP. Surprisingly, we did not observe any strong effect of miR-320-  
431 3p/DOSP supplementation on young rat hypothalamus enduring early-weaning, as the level of  
432 endogenous miR-320-3p was already very high ([Figures 4A, 5A, 6A](#)).

433

434 All males had a deregulation of *clock* and *drd2* transcripts when supplemented with  
435 miR-320-3p/DOSP and, for the ones with regular weaning, with miR-375-3p/DOSP  
436 supplementation ([Figure 7B, Supplementary Figures 3, 4](#)). Unlike miR-504 with *drd1*, the levels of  
437 miR-320-3p, miR-16-5p or miR-132-3p were not correlated with the level of *clock* transcripts

438 (Figure 6B-D). The females having endured both stress (miR-320-3p/DOSP and early weaning),  
439 displayed alterations in 5HT1B level like females with regular weaning and in PER1, another gene  
440 of the circadian clock. Males and females treated with miR-375-3p/DOSP displayed alteration in  
441 *per2* (Figure 7B, Supplementary Figure 4).

442

443 In the hippocampus, the levels of miR-320-3, miR-375-3p, miR-16-5p, miR-132-3p, and miR-504  
444 transcripts were all down regulated for the group treated with miR-320-3p/DOSP (Figures 4B; 5B;  
445 all p at least below 0.01). By contrast, the group treated with miR-375-3p/DOSP were for all  
446 miRNAs in the range of controls. The miR-504 and the miR-132-3p were deregulated for male and  
447 female weaned at D-30 treated with miR-320-3p/DOSP, but the rats weaned at Day-15 did not  
448 shown any difference for miR-504 and miR-132 (Figures 4B; 5B; 7C).

449

450 In the brain stem of females, a significant up regulation was observed with miR-320-3p for the  
451 females treated with miR-375-3p/DOSP. An up regulation of miR-16-5p and miR-504 with the  
452 females treated by miR-320-3p/DOSP. With males, a significant down regulation of miR-132-3p  
453 transcripts was noted with the group treated by miR-320-3p/DOSP.

454 Both male and female early-weaned rats displayed a deregulation of *cck* and *sert* (Figure 7D,  
455 Supplementary Figure 5). All females had a down-expression of miR-16-5p when supplemented  
456 with miR-320-3p/DOSP and, for the ones treated by miR-375-3p/DOSP with regular weaning. The  
457 *gfp* transcripts were significantly down regulated for the males supplemented with  
458 miR-320-3p/DOSP and after regular weaning ( $p < 0.05$ ). On Figure 7D representing hippocampus  
459 data, the miR-504 was down regulated for all groups when supplemented with miR-320-3p/DOSP  
460 indicating a strong effect of this miRNA supplementation. It should be underlined that no difference  
461 was found between sex and weaning times.

462 Our transgenic rat express the *gfp* transcripts in all cells permissive for CCK promoters beside the  
463 enteroendocrine lineage of duodenum. As such, we have found a deregulation for all male young  
464 rats after early or regular weaning, treated with miR-320-3p/DOSP and miR-375-3p/DOSP, but a  
465 similar deregulation was circumscribed to the females treated with miR-320-3p and early-weaned. It  
466 should be underlined that *gfp* was altered both in hippocampus and brain stem with males  
467 supplemented with miR-320-3p/DOSP.

468  
469 With early weaning, the levels of miR-320-3p were up regulated in duodenum extracts of miR-320-  
470 3p/DOSP treated rats (Figure 2B). However, we did not find any difference between  
471 miR-320-3p/DOSP and controls in stomach extracts (Figure 2A), as well as for miR-16-5p (Mean  
472 Cq  $\pm$  sme of w15-b320 (19.83 $\pm$ 1.65) against w15-btem (20.16 $\pm$ 2.01)) nor in the duodenum extracts  
473 (unshown results). Likewise, in the hypothalamus of both sexes (Figures 4A, 5A) or with  
474 hippocampus , except for a down regulation of miR-504 for both sexes (Figures 4B, 5B). In the  
475 brain stem of females, a significant up regulation was found for miR-320-3p, miR-16-5p, and miR-  
476 504. A down regulation was found for the relative expression of *cck* and *sert* transcripts. A trend of  
477 up regulation was observed with miR-375-3p and miR-132-3p (Figures 4C, 5C). The significant  
478 evolution of gene expression is summarized Figure 7. In addition, we did not find any difference for  
479 *sidt1* between early weaned rats treated with miR-320-3p/DOSP and corresponding controls (a  
480 trend of down-regulation was found with *sidt1*: Average Cq  $\pm$  sme of w15-b320 (32.55  $\pm$  4.59)  
481 against w15-btem (26.19  $\pm$  7.54). We have assayed miR-375-5p finding a huge variability on self-  
482 designed primer (Supplementary Table-1): Average Cq  $\pm$  sme of w15-b320 (28.19  $\pm$  7.91) against  
483 w15-btem (28.19  $\pm$  6.21)). With TaqMan assay, the results were more homogeneous showing that  
484 supplementation with miR-320-p/DOSP indirectly silenced the miR-375-5p form (Figure 2E, F).  
485

486 In the brain stem, the early-weaned females showed a down-regulation of *sert* and *drd1*, unlike  
487 males. Both male and female early-weaned rats displayed a deregulation of *cck* and *sert* ([Figure 7](#),  
488 [Supplementary Figure 5](#)).

489 In [Figure 7C](#) representing hippocampus data, the miR-504 was down regulated for all groups when  
490 supplemented with miR-320-3p/DOSP indicating a strong effect of this miRNA supplementation. It  
491 should be underlined that no difference was found between sex and weaning times.

492

493 In summary, the supplementations with miR-320-3p/DOSP or miR-375-3p/DOSP were more potent  
494 with the young rats raised with regular weaning. Surprisingly, the early-weaned male rats were  
495 more resilient to miRNA treatment as their relative levels of miR-320-3p were already very high.  
496 The miR-504 was unchanged in hypothalamus, down regulated in hippocampus, but up regulated  
497 along with miR-320-3p and miR-16-5p in females treated by miR-320-3p/DOSP.

498

## 499 Discussion

500

501 Oral supplementation by miRNA-320-3p or miR-375-3p during lactation has long-term miRNA-  
502 specific consequences on the endogenous levels of corresponding miRNAs with a strong tissue-  
503 dependent memory. The long-term effect of miR-375-3p was more limited, according to the  
504 fourfold lower number of predicted targets than with miR-320-3p. Combining miR-320-3p/DOSP  
505 with early weaning enhanced miR-320-3p and chromogranin A expression in the duodenum. In the  
506 hippocampus, the miR-504 was down-regulated for both sexes, but in the brain stem, up regulated  
507 only for females, along with miR-320-3p and miR-16-5p levels. In the hypothalamus, clock levels  
508 were up regulated for both sexes. In the miR-375-3p/DOSP group, the density of enteroendocrine  
509 duodenal cells increased. The long-term effect of miR-375-3p/DOSP was more limited, according  
510 to the fourfold lower number of predicted targets than with miR-320-3p ([Table 1](#)).

511

512 The miRNA/DOSP complexes are delivered in the stomach, but according to the described  
513 kinetic<sup>20</sup>, they can also be delivered in proximal sites of the small intestine. Our transgenic rat  
514 model is allowing us to explore the influence of miRNA supplementation at distance from the  
515 inoculation site on the neuroendocrine cell lineage of the duodenum. Here we are using the miR-  
516 320-3p with cytoplasmic and nuclear sites of bioactivity, in parallel with miR-375-3p with  
517 bioactivity limited to the cytoplasm. Under our hand, the administration of DOSP loaded with a  
518 specific miRNA can be considered neutral for the physiological effects triggered by the miRNA.  
519 Even if, paromomycine, the polar headgroup of DOSP, has been re-evaluated as potentially  
520 targeting the mammalian ribosome machinery.<sup>49</sup> Our current vector is by-passing the physiological  
521 sidt1-adsorption of miRNA in the stomach.<sup>8</sup> Remarkably, combining force-feeding  
522 miR-320-3p/DOSP and early weaning stress did not alter sidt1 transcripts. Likewise, we did not  
523 detect the loading of miR-320-3p or miR-375-3p in gastric extracellular vesicles. The sequences of

*Table 1. List of significantly altered micro and messenger RNAs in the duodenum, brain stem, hippocampus, and hypothalamus of early-weaned transgenic rat pups treated by a single oral supplement of miR-320-3p and sacrificed at Day-45. Note that the miR-375-5p level of w15-b320 was down-regulated in comparison with w30-b320 ( $p=0.02$ ), and w30-btem ( $p=0.04$ ). No difference was found between genes assayed on the stomach.*

| Tissue              | miRNA      |         |           | mRNA |      |       |      |     |       |      |
|---------------------|------------|---------|-----------|------|------|-------|------|-----|-------|------|
|                     | miR-320-3p | miR-504 | miR-16-5p | drd2 | sert | 5ht1b | cck  | gfp | clock | per1 |
| Duodenum            | Up         |         |           |      |      |       |      |     |       |      |
| Brain stem-Female   | Up         | Up      | Up        |      | Down |       | Down |     |       |      |
| Brain stem-Male     |            |         |           |      |      |       |      |     |       |      |
| Hippocampus-Female  |            | Down    |           |      |      |       |      |     |       |      |
| Hippocampus-Male    |            | Down    |           |      |      |       |      |     |       |      |
| Hypothalamus-Female |            |         |           |      |      | Up    |      |     | Up    | Up   |
| Hypothalamus-Male   |            |         |           | Up   |      |       | Up   | Up  | Up    |      |

524 these miRNAs have no sorting sequences in exosomes<sup>50</sup>, this is in favor of the absence of re-export  
525 of these miRNAs after their cytoplasmic delivery either toward the gastric lumen or into the blood.  
526 No immediate effect of miR-320-3p/DOSP is described in plexus choroid nor cortex.<sup>20</sup> In fact, our  
527 data are indicating a very high variability of miR-320-3p detection after 8 hours, in the plasma of  
528 miR-320-3p/DOSP or miR-375-3p/DOSP groups compared to controls. It seems highly likely that  
529 DOSP complexes were able of getting through the digestive epithelium into the plasma, then  
530 reaching the brain-blood barriers. We cannot label our DOSP vector for fluorescent tracking in  
531 blood and lymph but, DOSP could be tailored for targeting specific gut cell lineages, and for taking  
532 into account the putative interaction with the ribosome machinery. Such a vector would help  
533 resolving the paradoxe of a gut delivery with consequences in the brain area on the levels of  
534 endogenous miR-320-3p or miR-375-3p.

535

536 Control rats submitted to early weaning, had deep alterations in the levels of endogenous miR-320-  
537 3p in duodenum and of miR-320-3p and miR-375-3p, and all brain compartments tested ([Figures 2,](#)  
538 [3, 4, 5](#)). These data are highly supportive of using miR-320-3p, present in breast milk, as  
539 supplements in breast-fed rat. Our data are indicating a down regulation of miR-375-3p as well as  
540 of miR-320-3p in the hippocampus of early weaned young rats in contradiction with the increased  
541 expression of miR-375-3p in the hippocampus of stressed mice.<sup>32</sup>

542



543 Our data are showing that in the brain but not in the gut, sex is playing a critical role, confirming  
544 McKibben et al (2021)<sup>58</sup> who have found that in the hypothalamus, miR-132-3p and miR-504 are  
545 responsive to Early-Life stress, with males expressing greater changes following postnatal stress.  
546  
547 Surprisingly, our supplementation by miR-320-3p/DOSP has more impact on targeted miRNAs in  
548 the young rats raised with regular weaning as compared to early-weaned rats. The profile induced  
549 by miR-320-3p/DOSP supplementation is also driving the endogenous miR-375-3p regulation. By  
550 contrast, the effect of miR-375-3p/DOSP supplementation is weaker than with miR320-3p  
551 according to the limited subset of transcripts under the regulation of this miRNA. We are showing  
552 an increased density of enteroendocrine GFP-labelled cells, suggesting that the concentration of  
553 miR-375-3p were high enough to be delivered in duodenal proliferative or stem cells with late  
554 consequences on the kinetics of the duodenum. Likewise, the chromogranin A and GIP transcripts  
555 were, relatively to control bolus, decreased in young rats subsequently to miR-375-3p/DOSP and  
556 increased after miR-320-3p/DOSP. The level of endogenous miR-320-3p at Day-45 was up  
557 regulated in the duodenum ([Figure 2B](#)) indicating that the miR-320-3p/DOSP treatment can restore  
558 this miRNA level according to its relative expression level after regular weaning. Future works  
559 could explore the effects of miRNA supplementation directly in the stem cells of duodenal epithelia.  
560 New therapeutic for preventing early life stress may take advantage of the possibility to precisely  
561 target duodenal enteroendocrine cells.

562

563 A single bolus of miRNAs before weaning has induced in young rats, long-term effect on the  
564 expression of several miRNAs and mRNA, depending on the miRNA given by force-feeding. In the  
565 stomach, the levels of endogenous miR-320-3p and miR-375-3p (Figure 2 A) were significantly  
566 lower with rat pups treated by miR-320-3p/DOSP compared to controls and miR-375-3p/DOSP-  
567 treated rat pups. In all brain compartments tested, we have found that endogenous miR-320-3p and  
568 375-3p were significantly up-regulated for rat pups treated with miR-320-3p/DOSP compared with  
569 controls and miR-375-3p/DOSP-treated rat pups both for female (Figure 4) and male (Figure 5).  
570 Force-feeding rat pups with miR-375-3p is targeting fewer genes than miR-320-3p, consequently  
571 delivering miR-375-3p is without any effect on the endogenous level of miR-320-3p. Our data are  
572 in favor of a non-described hierarchical molecular link between endogenous miR-320-3p and miR-  
573 375-3p. Force-feeding rat pups with miR-320-3p/DOSP is revealing that, like predicted with our *in*  
574 *silico* data showing a wider target range for this miRNA, the rat pups with regular weaning have  
575 deregulation in the stomach as well as in brain compartments, some impacting endogenous miR-  
576 375-3p. In addition, we have detected the expression of miR-375-5p in duodenum extracts, without  
577 any difference between early-weaned rats treated by miR-320-3p/DOSP and controls. The miR-375-  
578 3p and 5p are both described in several rat tissue.<sup>57</sup> Future research on a specific epithelial cell  
579 lineage is needed to explore the dynamic of the ratio between miR-375, 3 p and 5p molecules in  
580 single-cell. Young et al., 2022<sup>60</sup> have shown that a stoichiometry exists between miR-140-5p and  
581 140-3p with a physiological meaning for cartilage biosynthesis. Future works are needed in breast  
582 milk supplementation to take into account this risk of displacing the ratio between 5p and 3p for  
583 canonical miRNA like miR-375. To our knowledge, the incidence of a lower amount of miR-26a in  
584 mouse breast milk has been reported with physiological consequences in the adipocyte  
585 compartment (Pomar et al., 2021).<sup>59</sup>  
586 The miR-320-3p is currently explored for its bioactivity in various diseases from type-2 diabetes to  
587 atherosclerosis.<sup>26, 27, 28</sup> The *in vivo* delivery of miR-320-3p is targeting binding sites located both on

588 polr3d promoter and on polr3d 3'UTR. Polr3d is the subunit-17 of polymerase-III involved in  
589 tumorigenesis. The RNA polymerase III is now considered linked to aging and longevity through its  
590 action on TORC and insulin genes as well as its activity on genes related to telomerase activity.<sup>51, 52</sup>  
591 However, we have shown only an immediate effect on the chromatin complexes related to  
592 H3K4me3, as well as an absence of long-term effect on polr3d mRNA. The miR-320-3p has been  
593 studied for post-transcriptional gene silencing in the cytoplasm of rat endothelial and cardiac cell  
594 cultures derived from diabetes situations, on several genes among which the *heat shock protein*  
595 *family B (small) member 6*.<sup>53</sup> The *hspb6* (also *hsp20*) gene is highly expressed in several organs  
596 including the stomach.<sup>54</sup> Our data are confirming the immediate effects on *polr3d* and *hspb6*  
597 genes<sup>20</sup>, but additional works are needed to explore the long-term putative effects on polr3d  
598 complex which includes 17 subunits; as well as any effect on telomerase activity.

599

600 Early postnatal life is a critical period where stressful experiences may have the potential for long-  
601 term programming. The application of such preventive and therapeutic approaches during early-life  
602 sensitive periods is likely to be particularly promising. If one could modify the epigenetic patterns  
603 disrupted by exposure to stress through specific epigenome-targeted therapeutic interventions, then  
604 it would be possible to correct the impaired patterns of gene expression to prevent the stress-  
605 induced chronic pathologies and to improve human health and longevity. The early-weaned rats  
606 were more resilient to miR-320-3p/DOSP treatment on the expression of endogenous miR-320-3p  
607 and miR-375-3p. The innate immunity of early-weaned rats at the stomach level is also deeply  
608 altered, in part linked to the alteration in gastrointestinal permeability.<sup>55</sup> However, our treatment  
609 with miR-320-3p/DOSP did not induce significant evolution of the cytokines related to immunity.  
610 Immune dysregulation is considered to be a key pathway linking the childhood adversity to elevated  
611 rates of morbidity and mortality from a number of chronic diseases later in life. Note that we did not  
612 report up-regulation of miR-375-3p related to the double stress of miR-320-3p/DOSP and early

613 weaning. As shown in [Figures 3 and 7](#), the networks of genes significantly deregulated in the  
614 stomach or brain compartments for early weaned rats are very narrow compared to the networks  
615 obtained after a regular weaning.

616

617 We have described the modification of clock transcripts in the hypothalamus or the liver of young  
618 Wistar rats at Day-35 showing an increased level in the nocturnal situation.<sup>36</sup> Our data obtained on  
619 transgenic Sprague-Dawley rats have been obtained with a sacrifice done in the nocturnal phase  
620 ([Figure 1](#)). However, despite *in silico* prediction, the miR-320-3p, 16-5p and 132-3p were not  
621 correlated with *clock* transcripts ([Figure 6 B-D](#)). Further experiments are needed to explore whether  
622 the effect of early-weaning stress combined or not with force-feeding miR-320-3p alters the  
623 circadian clock machinery. In [Figure 7B](#), with early-weaned rats force-fed with miR-320-3p, clock  
624 levels were high in the hypothalamus of males and period1 in females ([Table 1](#)). Future works in the  
625 developmental biology of the circadian clock could open an efficient therapeutic avenues.

626

627 In conclusion, our supplementation of lactating rat pups with extracellular miR-320-3p given before  
628 early weaning stress alters the miR-320-3p expression in duodenum, the miR-504 expression in the  
629 brain stem of female, and of clock transcripts in hypothalamus ([Table 1](#)), calling for behavioral  
630 studies. We are describing a new relationship between 2 unrelated miRNAs, miR-320-3p and miR-  
631 375-3p underlining a hierarchy between miRNA networks. The exploration of therapeutic potentials  
632 of miRNAs needs an approach in integrative physiology with a highly specific site of delivery like  
633 duodenal enteroendocrine cell lineage and articulated around the competing endogenous RNA  
634 hypothesis.<sup>61</sup> This approach would gain much momentum by the implementation of results in an  
635 international database, improving the gap between *in silico* prediction and biological observations.  
636 The development of a new milk formulation intended to manipulate the epigenetics of the baby will  
637 benefit from such preclinical models.<sup>62</sup>

638

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646 **Contribution of authors and correspondence:**

647 GT (TAVAREG@ccf.org) was in charge with brain dissection, handling, and transcript analyses. AT  
648 (madintor@yahoo.com.mx) did *in silico* analysis, part of duodenum and stomach transcript  
649 analyses. BC, LB, SR, IA, GLD (Gwenola.Ledrean@univ-nantes.fr) have constructed and  
650 maintained the transgenic rat strain. MQ and GLD realized immunohistology experiments. BP  
651 (Bruno.Pitard@univ-nantes.fr) provided DOSP and know-how. BK, MQ, GT inoculated rat pups.  
652 BK realized ChIp experiments, acting as coordinator of sacrifices, storage of samples, and  
653 centralizing analyses (Bertrand.Kaeffer@univ-nantes.fr). All authors have contributed to the  
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655

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842

### Supplementary Table 1.

Details of the primers used to construct the transgenic rats, and quantified 6 miRNA and 42 mRNA.

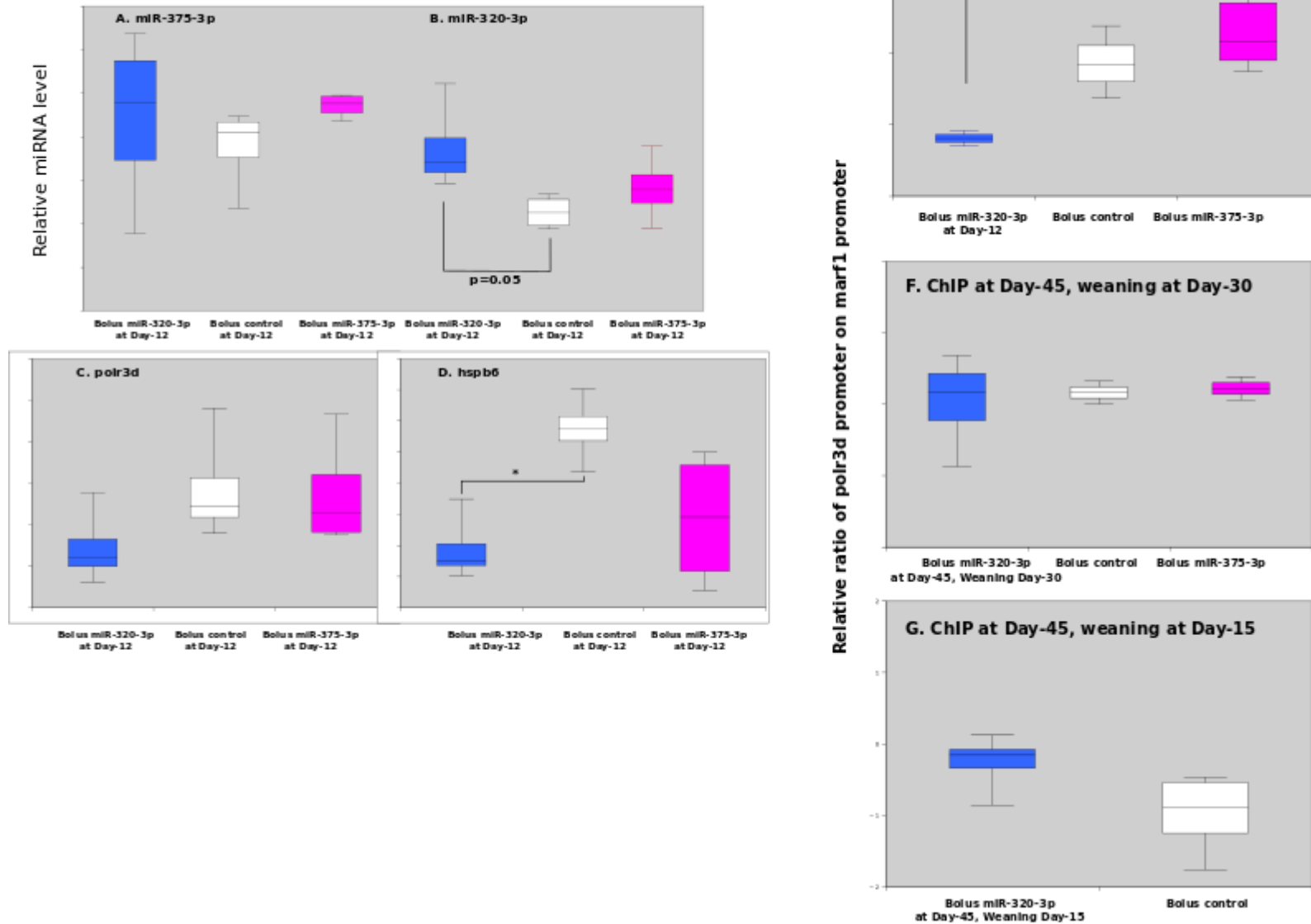
| Gene Name                  | Primer Sequence                 | Reference                          |
|----------------------------|---------------------------------|------------------------------------|
| rROSA-fw1                  | TGAACTGTGAATAGGCCCAAGTG         | Ménoret et al., 2015 <sup>38</sup> |
| rROSA-rev1                 | GCAATTTTAAAAGAGCCCAGTACTT<br>CA | Ménoret et al., 2015 <sup>38</sup> |
| GFP Up                     | CCTCGTGACCACCCTGACCT            | Ménoret et al., 2015               |
| GFP Lo3                    | TCCATGCCGAGAGTGATCCC            | Ménoret et al., 2015 <sup>38</sup> |
| rROSA26-<br>5outFor        | TCCCACCCTCCCCTTCCTCT            | Ménoret et al., 2015 <sup>38</sup> |
| 5rCCKpRev                  | TGTGACCCCGTTGCCCTGGAT           | Ménoret et al., 2015 <sup>38</sup> |
| 3BGHpA-Up2                 | CCAGATTTTTCTCCTCCTCCTG          | Ménoret et al., 2015 <sup>38</sup> |
| rROSA26-<br>3outRev        | TGGGTATCACTGGCTGTCCTAGATA       | Ménoret et al., 2015 <sup>38</sup> |
| microRNAs                  | TaqMan System                   |                                    |
| Let-7d-5p                  | Advanced-rno478439_mir          |                                    |
| Let-7g-5p                  | Advanced-rno478580_mir          |                                    |
| rno-miR-132-3p             | Advanced-rno480919_mir          |                                    |
| rno-miR-146b               | Advanced-rno480941_mir          |                                    |
| rno-miR-16-5p              | Advanced rno481312_mir          |                                    |
| rno-miR-320-3p             | Advanced-rno481048_mir          |                                    |
| rno-miR-375-3p             | Advanced-rno481142_mir          |                                    |
| rno-miR-375-5p             | Advanced-rno481142_mir          |                                    |
| rno-miR-504                | Advanced-rno481198_mir          |                                    |
| SERT                       | rno00564737_mir                 |                                    |
| microRNAs                  | SYBR-Green System               |                                    |
| miR-375-5p-fw              | GCGACGAGCCCCUCGCACAAACC         |                                    |
| Universal miRNA<br>reverse |                                 | Mei et al., 2012 <sup>56</sup>     |
| β2-microglobulin           | rno0560865_m1                   |                                    |
| β-actin                    | rno0667869_m1                   |                                    |
| usb1                       | rno01536722_m1                  |                                    |
| Polr3d                     | rno1468090_g1                   |                                    |
| Hspb6                      | rno0577590_ml                   |                                    |
|                            | MESSENGER for SYBR-Green System |                                    |
| GAPDH2-fw                  | CGG CAA GTT CAA CGG CAC AG      |                                    |
| GAPDH2-rv                  | TCC ACG ACA TAC TCA TCA GCA     |                                    |

|                         |                                    |
|-------------------------|------------------------------------|
|                         | CCA                                |
| $\beta$ -actin-fw       | CTA TCG GCA ATG AGC GGT TCC        |
| $\beta$ -actin-rv       | GCA CTG TGT TGG CAT AGA GGT C      |
| $\beta$ -2M-fw          | TGA CCG TGA TCT TTC TGG TG         |
| $\beta$ -2M-rv          | ACT TGA ATT TGG GGA GTT TTC<br>TG  |
|                         | REPORTER GENES                     |
| GFP-fw                  | AAG CTG ACC CTG AAG TTC ATC<br>TGC |
| GFP-rv                  | CTT GTA GTT GCC GTC GTC CTT<br>GAA |
| CCK-fw                  | TGC TTG GAG GAG GCG GAA TG         |
| CCK-rv                  | GCT GGG CTG AGG TGT GTG G          |
|                         | PROMOTERS                          |
| POLR3D-fw               | CAGACCAGTCACCTCATCCTTT             |
| POLR3D-rv               | AGTATTTATCAGACGGTGCCTC             |
| MARF1-fw                | GATAACCCCTATTTTGAGGTT              |
| MARF1-rv                | GCGTCTTCTCCGCGCAGGGCAT             |
|                         | FUNCTIONAL GENES                   |
| <b>Rghrl -fw</b>        | AGAGGCGCCAGCTAACAAGTAA             |
| <b>Rghrl -<br/>rev</b>  | GCAGGAGAGTGCTGGGAGTT               |
| <b>rGip-fw</b>          | CTCCTGTTCTGGCTGTC                  |
| <b>rGip-<br/>rev</b>    | GGCGATGCTGTAATCACTG                |
| <b>rPYY-fw</b>          | AGCGGTATGGGAAAAGAGAAGTC            |
| <b>rPYY-<br/>rev</b>    | ACCACTGGTCCACACCTTCTG              |
| <b>rCCK-fw</b>          | GCCGCCTGCCCTCAAC                   |
| <b>rCCK-rv</b>          | ACACACGCCGCACTTCATATC              |
| <b>rPax6-fw</b>         | ATACCTACACCCCTCCGCAC               |
| <b>rPax6-rv</b>         | TGAGTCCTGTTGAAGTGGTTCC             |
| <b>rPAX4-fw</b>         | GGATACTGGGAGCCTTGTC                |
| <b>rPAX4-rv</b>         | GGATACTGGGAGCCTTGTC                |
| <b>rFoxa1-<br/>fw</b>   | GTTCCGCACAGGGTTGGATA               |
| <b>rFoxa1-<br/>rev</b>  | CTG ACC GGG ACA GAG GAG TA         |
| <b>r Chga-fw</b>        | TACCCAATCACCAACCAGCC               |
| <b>r Chga 1<br/>rev</b> | TGAGACTCCGACTGACCATC               |

|                  |                                   |
|------------------|-----------------------------------|
| 5HT1B-fw         | AGA AGA AAC TCA TGG CCG CT        |
| 5HT1B-rv         | GGG GAG CCA GCA GAG AAT AA        |
| 5HT2C-fw         | ATT TGT GCC CCG TCT TGG ATT       |
| 5HT2C-rv         | CGC GAA TTG AAC CGG CTA TG        |
| BMAL1-fw         | GAC TTC GCC TCC ACC TGT TC        |
| BMAL1-rv         | CAT TGT CTG GTT CAC TGT CTT<br>CG |
| CLOCK-fw         | GAA CTT GGC GTT GAG GAG TCT       |
| CLOCK-rv         | GTG ATC GAA CCT TTC CCA GTG C     |
| DRD1-fw          | GTT TGT GTG GT TGG GTG GG         |
| DRD1-rv          | GCT CAT GGT GGC TGG AAA AC        |
| DRD2-fw          | GAG CCA ACC TGA AGA GAC CA        |
| DRD2-rv          | GCA TCC ATT CTC CGC CTG TT        |
| IFNg-fw          | GAT CCA GCA CAA AGC TGT CA        |
| IFNg-rv          | GAC TCC TTT TCC GCT TCC TT        |
| IL10-fw          | GCA GTA GAG CAG GTG AAG AAT<br>G  |
| IL10-rv          | CAG TAG ACG CCG GGT GGT TC        |
| IL1 $\alpha$ -fw | AAGACAAGCCTGTGTTGCTGAAGG          |
| IL1 $\alpha$ -rv | TCCCAGAAGAAAATGAGGTCGGTC          |
| IL6-fw           | TCC TAC CCC AAC TTC CAA TGC<br>TC |
| IL6-rv           | TTG GAT GGT CTT GGT CCT TAG<br>GG |
| iNOS-fw          | GAT TTT TCA CGA CAC CCT           |
| iNOS-rv          | GGT CCT CTG GTC AAC CTC           |
| PERIOD1-fw       | GCC CTG CTG CCT GCT CAT TG        |
| PERIOD1-rv       | AAC TTG GTG TGT GCC GTG GG        |
| PERIOD2-fw       | GCA CGC TGG CAA CCT TGA AG        |
| PERIOD2-rv       | GGC TGG CTC TCA CTG GAC ATT<br>AG |
| STAT1-fw         | AGG TCC GTC AGC AGC TTA AA        |
| STAT1-rv         | CGA TCG GAT AAC AAC TGC TT        |
| TGF $\beta$ -fw  | AGT GGC TGT TGC GGA GAG           |
| TGF $\beta$ -rv  | GCT GAA AGG TAT GAC ATG GAC A     |
| TNF $\alpha$ -fw | AAATGGGCTCCCTCTCATCAGTTC          |
| TNF $\alpha$ -rv | TCTGCTTGGTGGTTTGCTACC             |
| SIDT-1-fw        | CGTCATCCGGACCAAGATGT              |
| SIDT-1-rev       | AGATGTCCTGGTTGCCAGTG              |

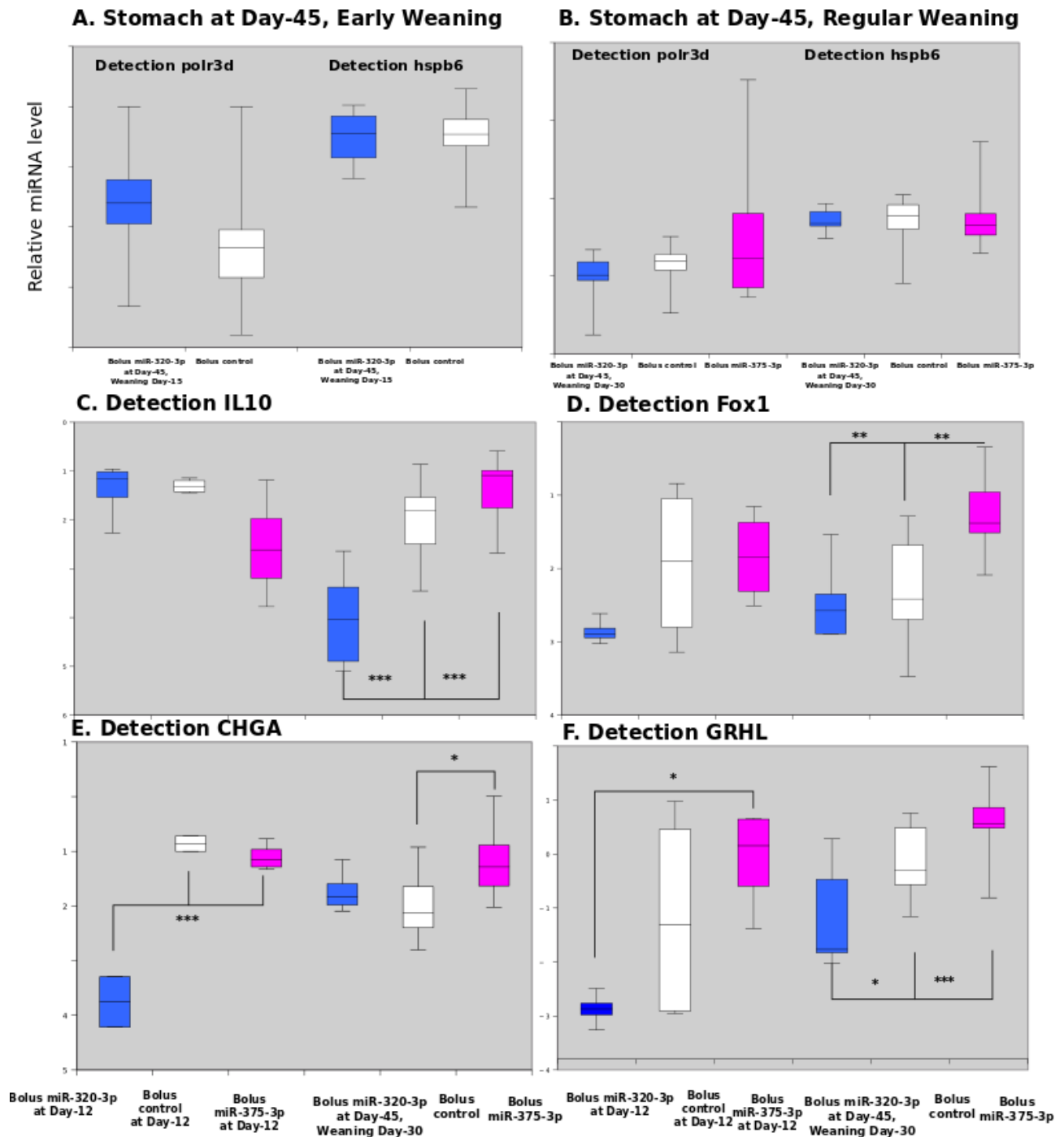
Supplementary material : Excel files of raw Cq data organized by tissues can be made accessible at the UN-Cloud of the University of Nantes.

## Supplementary Figure 1



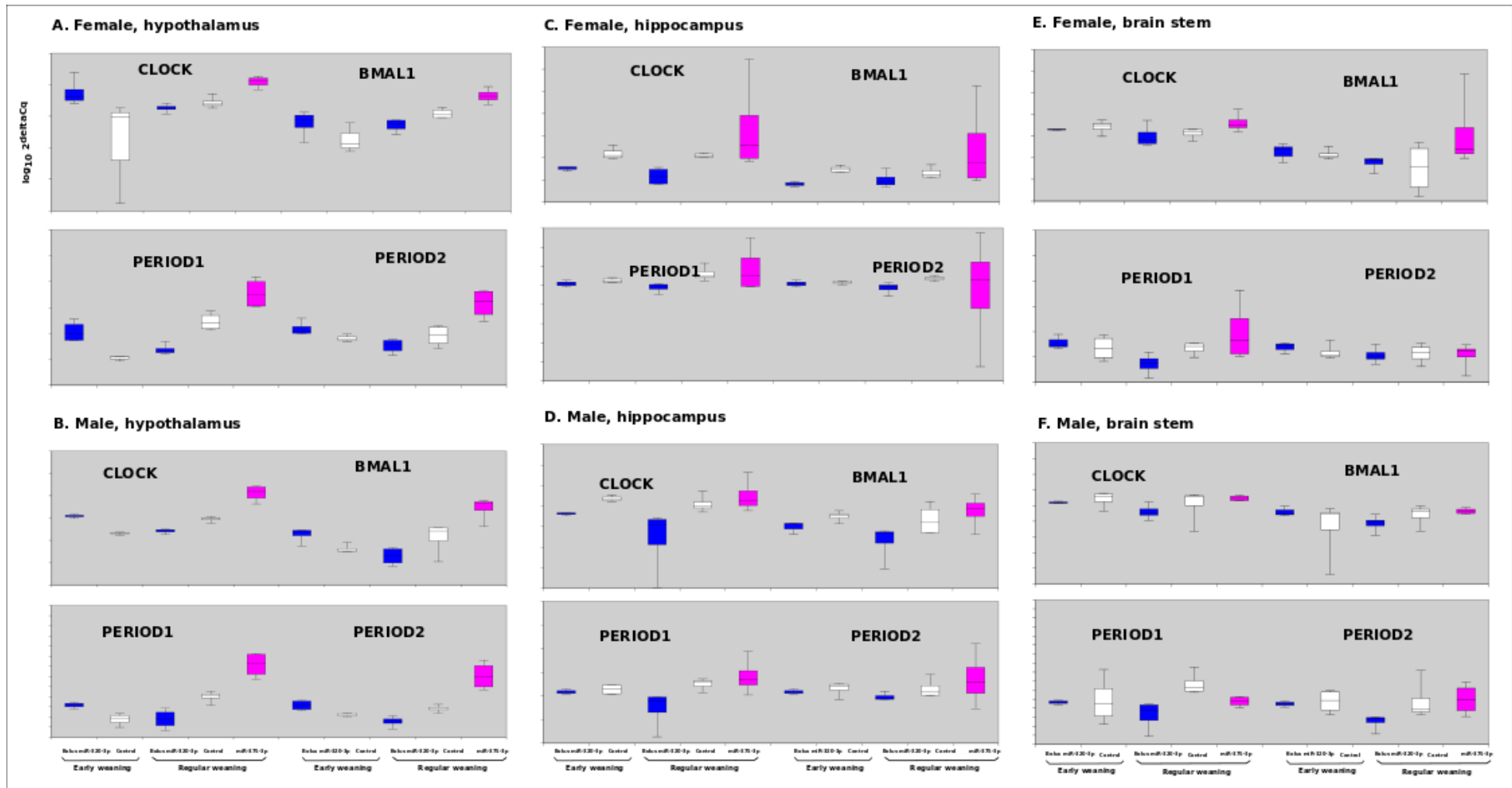
Supplementary Figure 1. Immediate effect of miRNA supplementation. Evolution of miR-375-3p (A), 320-3p (B), polr3d (C) and hspb6 (D) mRNA, 8 hours after bolus for rat pups at Day-12 in stomach wall. Chromatin-immunoprecipitation assay against H3K4me3. Note the significant alteration at 8H after a bolus with miR-320-3p in gastric cells (E) and the absence of memory effect after regular (F) or early (G) weaning. The light gray background reminds that rats were sacrificed in the dark phase. Note: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

Supplementary Figure 2.



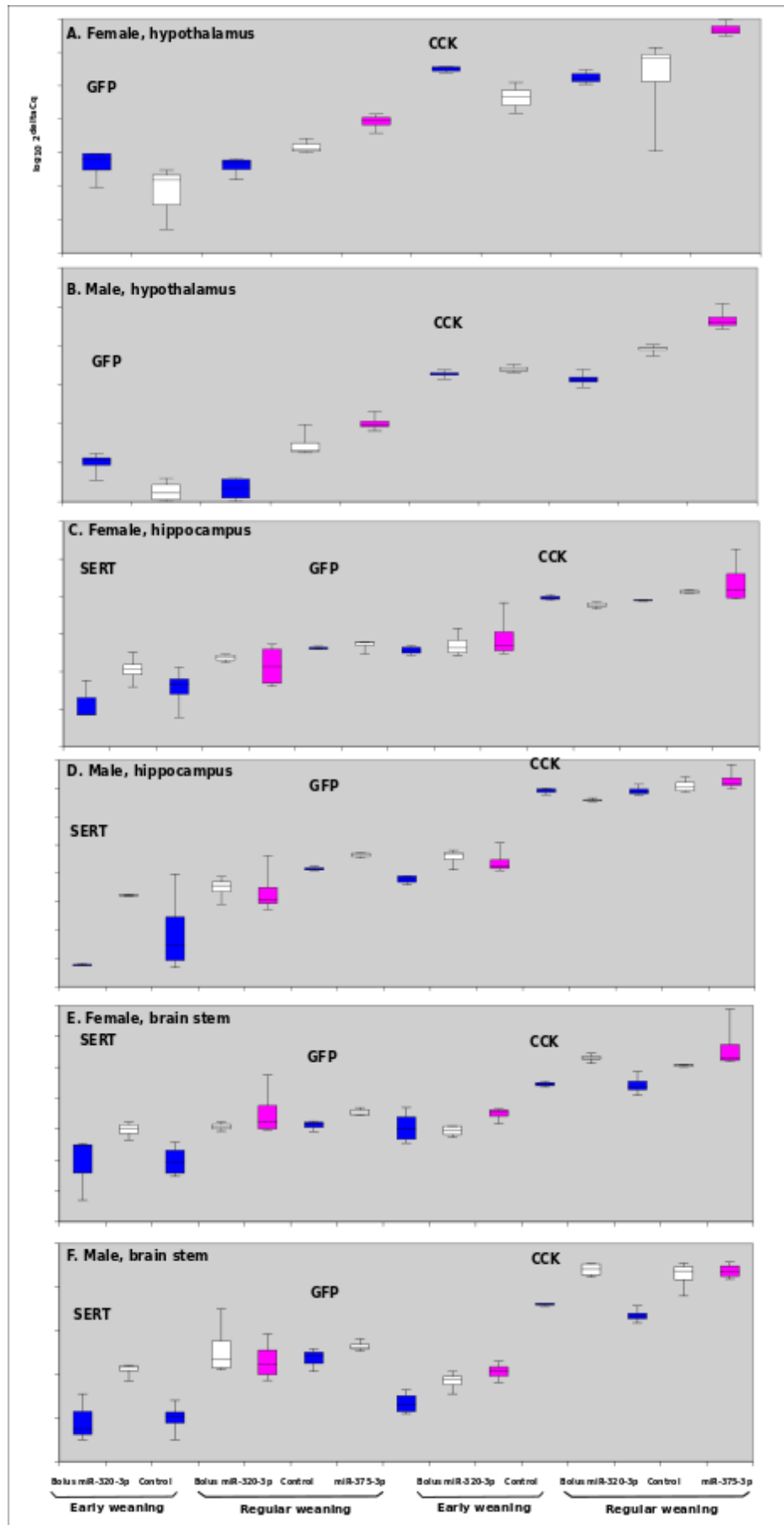
Supplementary Figure 2. Evolution of *polr3d* and *hspb6* mRNAs in stomach wall, at Day-45 after early (A) or regular (B) weaning. Concerning the inflammation status, the *IL-10* [C], *Fox1* (D), *ChGRA* (E), and *GRHL* (F) transcripts were all down-regulated at Day-45 for rat treated with miR-320-3p/DOSP. The light gray background reminds that rats were sacrificed in the dark phase. Note: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .





Supplementary Figure 4. Evolution of transcripts related to the circadian clock (*clock*, *bmal1*, *period1*, *period2*) in hypothalamus (A, female ; B, male), in hippocampus (C, female ; D, male), and in brain stem (E, female, F, male) of rat treated with miR-320-3p or 375-3p/DOSP according to early or regular weaning. The light gray background reminds that rats were sacrificed in the dark phase. Note: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .





Supplementary Figure 5. Evolution of SERT, GFP, CCK transcripts in hypothalamus (A, female ; B, male), in hippocampus (C, female ; D, male), and in brain stem (E, female, F, male) of rat treated with miR-320-3p or 375-3p/DOSP according to early or regular weaning. The light gray background reminds that rats were sacrificed in the dark phase. Note: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .