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Postnatal depression-associated breastmilk extracellular vesicles mediate intergenerational effects on offspring neurodevelopment

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Abstract

Postnatal depression (PND) increases the risk of neurodevelopmental impairments in offspring, yet the underlying mechanisms remain elusive. Here, we identify an intergenerational signaling pathway whereby maternal psychological state is encoded in breastmilk microRNAs (miRNAs) and transmitted to offspring via breastmilk small extracellular vesicles (sEVs). Using cross-fostering models, we demonstrate that maternal depression disrupts offspring hippocampal dentate gyrus (DG) newborn neuron maturation, causing cognitive deficits and depressive-like behaviors. Strikingly, gastric administration of PND-derived breastmilk sEVs to healthy pups recapitulates these neurodevelopmental phenotypes. Mechanistically, PND-induced miRNAs are packaged into breastmilk sEVs and delivered to infant brain, where they suppress mGluR8 expression in supramammillary nucleus (SuM) neurons projecting to the DG. This presynaptic mGluR8 deficiency dysregulates glutamate release, triggers aberrant synaptic transmission and calcium overload, and ultimately drives a pathological “high-frequency, low amplitude” neuronal activation pattern that compromises the functional integration of newborn neurons into hippocampal circuits. Critically, inhibiting sEV secretion from the mammary gland or sequestering the culprit miRNAs within offspring SuM neurons rescues these neurodevelopmental deficits. These findings not only uncover a novel mechanism underlying PND-related offspring neurodevelopmental impairments, but also provide a promising therapeutic target for clinical intervention of intergenerational transmission of maternal mental disorders.

Introduction

Postnatal depression (PND), also referred to as postpartum depression, is a common mental disorder affecting approximately 10-20% of women worldwide¹⁻³. It poses significant health risks to both mothers and their offspring. Affected mothers may exhibit a broad range of symptoms, including depressed mood, apathy, disruptions in appetite and sleep, cognitive impairments, and suicidal ideation^{4,5}. Children born to mothers with PND face an increased risk of adverse developmental outcomes, including delayed cognitive and language development, behavioral challenges, poorer academic achievement, and a higher prevalence of depression that can extend into adolescence⁶⁻⁹. Although the etiology of PND has been extensively studied, revealing that its pathogenesis typically arises from interactions between genetic susceptibility and environmental stressors¹⁰⁻¹⁴, the precise biological and psychological mechanisms through which maternal PND impairs offspring development remain poorly understood. It is widely believed that PND disrupts maternal-infant bonding and interferes with the mother’s transition to caregiving, thereby negatively affecting infant development¹⁵. Furthermore, neuroimaging studies have identified alterations in brain structure and functional connectivity (particularly in the hippocampus and amygdala) among infants and children exposed to maternal depression or anxiety¹⁶⁻¹⁸. However, these behavioral and neurocircuitry observations alone are insufficient to fully explain the long-term, multifaceted adverse outcomes in children’s cognitive, social, and physical development.

In the early postnatal period, breastmilk provides infants with an optimal balance of nutrients and essential bioactive components, including immunomodulatory factors, growth hormones, antimicrobials, and prebiotics, supporting their physical and neurological development¹⁹⁻²². As a result, breastfeeding positively influences offspring health, benefiting them from infancy through adolescence and into adulthood^{20,22}. Despite its well-documented health benefits and the widespread

recommendations from health organizations, breastfeeding rates remain low in many countries, with PND posing a significant barrier to achieving breastfeeding goals²³. Current evidence underscores a bidirectional relationship between PND and breastfeeding: PND reduces the likelihood of exclusive breastfeeding and often leads to early cessation, while breastfeeding, in turn, lowers the incidence of PND²⁴⁻²⁶. Given the protective role of breastfeeding against PND, clinical guidelines commonly recommend it for affected mothers, particularly those with mild symptoms. Evidence indicates that breastfeeding contributes to maternal psychological health through multiple pathways, including enhanced maternal self-efficacy and reduced activation of the hypothalamic-pituitary-adrenal (HPA) axis^{27,28}. These benefits can alleviate PND symptoms and promote a positive maternal-infant relationship, ultimately protecting infants from adverse outcomes. Conversely, PND can dysregulate multiple physiological and metabolic pathways, potentially leading to alterations in breastmilk composition²⁹⁻³². Such modifications may adversely affect infant development across cognitive, behavioral, and psychological domains. Consequently, these PND-induced abnormalities in breastmilk could offset the established benefits of breastfeeding. Given this complex interplay, it is essential to elucidate the molecular mechanisms linking maternal PND to child developmental outcomes, with a specific focus on the role of breastmilk composition.

Breastmilk is increasingly recognized not only as a source of nutrition, but as a sophisticated signaling system that actively orchestrates infant development³³. A key component of this system is the functional partnership between microRNAs (miRNAs) and small extracellular vesicles (sEVs)³⁴⁻³⁶. The majority of breastmilk miRNAs are not free-floating but encapsulated within sEVs, which are nanoscale (30-150 nm), lipid-bilayer particles secreted by mammary epithelial cells³⁷. This structural packaging shields miRNAs from degradation in the infant's gastrointestinal environment, facilitating their delivery to intestinal cells and potentially enabling systemic dissemination³⁸. The miRNA-sEV partnership constitutes a novel form of non-genetic, mother-to-infant communication that directly modulates infant's immune maturation, intestinal homeostasis, and metabolic programming³⁸. Notably, postnatal brain development is highly sensitive to dietary factors, partly due to the relative permeability of the infant blood-brain barrier (BBB)³⁹. It is thus plausible that sEV-encapsulated miRNAs from breastmilk may be absorbed, cross the BBB, and actively participate in shaping neurodevelopment. Conversely, PND can disrupt this communicative axis by altering the miRNA profile loaded into sEVs^{40,41}. Ingestion of these "PND-signature" sEVs may deliver pathological signals to the developing infant brain, establishing a molecular pathway for the intergenerational transmission of mental health risk. In this study, we elucidate the mechanistic interplay connecting maternal PND, breastmilk sEV miRNAs, and offspring neurodevelopment. Our findings support the model that maternal psychological states can be transmitted intergenerationally via breastmilk sEV miRNAs, influencing cognitive and behavioral outcomes in the offspring.

Results

A cross-fostering model to evaluate the effects of maternal PND on offspring behaviors

Perinatal stress exposure is a major risk factor for PND. We established a mouse model of PND by subjecting 6-week-old C57BL/6 female mice to chronic unpredictable mild stress (CUMS)⁴² daily for 5 weeks prior to conception, followed by continuous exposure to mild stressors throughout the 3-week gestation period (Fig. 1a). Following this paradigm, the CUMS-induced dams displayed a

typical depression-like phenotype, as demonstrated by increased immobility in the forced swim test (FST), prolonged immobility in the tail suspension test (TST), and increased latency in the novelty-suppressed feeding test (NSFT), compared to healthy control (HC) dams that experienced normal conception and gestation (Supplementary Fig. 1a-c). To evaluate the impact of depressive-like behavior on maternal care, we analyzed the frequencies of arched-back nursing (ABN) and licking/grooming (LG) behaviors. CUMS-induced dams showed a significant reduction in both ABN and LG frequencies compared to HC dams (Supplementary Fig. 1d,e). In contrast, the frequencies of passive nursing and total nursing episodes were not significantly affected (Supplementary Fig. 1f,g). We also evaluated the potential impact of CUMS on reproductive parameters. Both litter sizes and offspring sex ratios were comparable between CUMS and HC dams (Supplementary Fig. 1h,i), indicating that the stress paradigm did not markedly affect these fundamental fertility outcomes.

Given the established link between maternal PND and elevated risk of cognitive and emotional disorders in offspring⁴, we investigated whether these phenotypes could be transmitted across generations. To isolate the effects of postnatal maternal influences, a cross-fostering model was employed, allowing assessment of maternal care and breastfeeding contributions while controlling for genetic and embryonic confounding factors. In this model, the offspring of CUMS-induced dams were fostered immediately after birth by HC dams (designated as CUMS→HC, using the nomenclature of “birth mother→foster mother” to indicate the prenatal-to-postnatal fostering condition), while the offspring of HC dams were fostered by CUMS-induced dams (HC→CUMS) (Fig. 1b). Control groups consisted of offspring cross-fostered within the same maternal condition (HC→HC and CUMS→CUMS) (Fig. 1b). Behavioral assessments were conducted on all offspring at 8 weeks of age (postnatal day 56, P56).

All four groups of offspring exhibited normal growth and comparable weight gains (Supplementary Fig. 2a,b). In the Morris water maze (MWM) test, which assesses learning and memory by measuring an animal's ability to locate a hidden platform in a water tank using spatial cues, CUMS→CUMS offspring exhibited a longer latency in navigating to the hidden platform compared to HC→HC offspring during the training phase (Fig. 1c). Similarly, CUMS→CUMS offspring spent less time in the target quadrant and had a lower frequency of platform crossings during the probe trials compared to HC→HC offspring (Supplementary Fig. 2c), while their overall swimming activity remained comparable (Supplementary Fig. 2d). These results are consistent with predictions, indicating that CUMS→CUMS offspring exhibit significant learning and memory deficits compared to HC→HC offspring. Surprisingly, however, HC→CUMS offspring, despite their healthier gestational origin, exhibited longer latency in reaching the hidden platform and had fewer platform crossings compared to CUMS→HC offspring (Fig. 1c; Supplementary Fig. 2c). Furthermore, a contextual fear conditioning (CFC) test was conducted to assess hippocampus-dependent associative memory (Supplementary Fig. 2e). Although CUMS→CUMS and HC→HC offspring exhibited comparable freezing responses during baseline training phase (Supplementary Fig. 2f), CUMS→CUMS group displayed significantly reduced freezing levels during contextual memory test (Fig. 1d). Likewise, HC→CUMS offspring were impaired in contextual fear conditioning, showing less freezing time compared to CUMS→HC offspring (Fig. 1d). Moreover, the intergenerational transmission of depression-like phenotypes was assessed under baseline and

stress-challenged conditions⁴². While CUMS→CUMS and HC→HC offspring showed comparable immobility times in FST under baseline conditions (Supplementary Fig. 2g), CUMS→CUMS offspring exhibited markedly prolonged immobility following mild chronic variable stress (CVS) exposure (Supplementary Fig. 2h). A similar stress-sensitive phenotype was observed in HC→CUMS offspring, which developed greater depressive-like behavior than CUMS→HC offspring after CVS challenge (Supplementary Fig. 2g,h). Interestingly, sex-stratified analyses revealed that these behavioral abnormalities were predominantly evident in males. Specifically, only male offspring from the CUMS→CUMS and HC→CUMS groups exhibited significantly or marginally significantly longer latencies in the MWM test, reduced freezing in the CFC test, and prolonged immobility in the FST when compared to their respective male counterparts in the HC→HC and CUMS→HC groups (Supplementary Fig. 2i-k). In contrast, none of these behavioral measures showed significant differences among the female offspring. Overall, these results demonstrate that an adverse postnatal environment can override a healthy gestational origin, as HC-born but CUMS-fostered offspring developed more severe cognitive and emotional deficits than their CUMS-born but HC-fostered counterparts.

Additionally, a series of behavioral tests were conducted to assess anxiety-like behaviors, exercise capacity, and social interaction in the cross-fostered offspring. All groups of offspring exhibited comparable performance in the open field test (total distance and center time) and elevated plus maze test (open arm entries and duration) (Supplementary Fig. 2l-o). Similarly, all groups ran for similar durations and distances in the treadmill test (Supplementary Fig. 2p,q). Finally, in the three-chamber social interaction test, all groups displayed normal sociability and social novelty preference (Supplementary Fig. 2r-u). Together, these results indicate a comparable behavioral profile in anxiety-like behaviors, exercise capacity, and social interaction across all cross-fostered offspring groups.

To model the multifactorial etiology of PND, we established a complementary model using chronic social defeat stress (CSDS)⁴³, which simulates depression-like behaviors in a social context (Supplementary Fig. 3a). Ten consecutive days of CSDS were sufficient to induce a significant depressive-like phenotype in dams, as demonstrated by pronounced behavioral alterations in the FST, TST, and NSFT (Supplementary Fig. 1j-l). Similarly, CSDS-induced dams exhibited impaired maternal care quality, characterized by significantly reduced frequencies of ABN and LG behaviors compared to HC dams, while the frequencies of passive and total nursing remained unchanged (Supplementary Fig. 1m-p). Mirroring the CUMS paradigm, offspring of HC or CSDS-induced dams were cross-fostered to create four experimental groups: CSDS→HC, HC→CSDS, CSDS→CSDS, and HC→HC (Supplementary Fig. 3b). Compared to their respective controls (CSDS→HC and HC→HC), both HC→CSDS and CSDS→CSDS offspring exhibited impaired spatial memory in the MWM test and deficient associative memory in the CFC test (Supplementary Fig. 3c-g). Furthermore, HC→CSDS and CSDS→CSDS offspring recapitulated the depressive-like phenotypes observed in their stressed dams after exposure to CVS (Supplementary Fig. 3h,i). These results indicate again that maternal care and breastfeeding outweigh genetic predisposition in shaping offspring development and behavior in the context of maternal depression.

Evaluation of the effects of maternal PND on neurogenesis in the dentate gyrus of offspring

To investigate the mechanisms underlying cognitive and emotional abnormalities in offspring raised by stressed dams, we systematically mapped neuronal activation in key brain regions (hippocampus, medial prefrontal cortex (mPFC), lateral habenula (LHb), amygdala (AMY), and thalamus) across lactation using c-Fos immunocytochemistry⁴⁴. These regions were chosen for their established roles in cognition, emotion, and neuropsychiatric disorders⁴⁵⁻⁴⁹. To avoid confounding effects from feeding-related neuronal activation and to specifically measure baseline neural activity, we examined c-Fos activation in offspring brain regions one hour after the cessation of nursing, with real-time video monitoring used to exclude any active suckling episodes. At P14 and P21, corresponding to 2 and 3 weeks of lactation, offspring raised by HC dams (CUMS→HC and HC→HC) exhibited predominant c-Fos localization within the granule cell layer (GCL) of the hippocampal dentate gyrus (DG), whereas those raised by stressed dams (HC→CUMS and CUMS→CUMS) showed a marked reduction in c-Fos immunoreactivity in the same region (Fig. 1e; Supplementary Fig. 4a-c). Notably, this effect was specific to the DG, as no significant differences in c-Fos activation were observed in other brain regions (Fig. 1e; Supplementary Fig. 4a-c). To further quantify the DG-specific alterations identified in our initial screening, we measured the absolute density of c-Fos⁺ cells (number per mm²). This quantitative analysis confirmed that offspring raised by CUMS-induced dams exhibited a significantly lower density of c-Fos⁺ neurons in the GCL of hippocampal DG compared to those raised by HC dams (Fig. 1f,g).

The hippocampus is a brain region critical for cognition and highly vulnerable to stress, with strong implications for the pathophysiology of depression^{50,51}. Given the pronounced reduction in hippocampal neuronal activation in offspring raised by stressed dams, we performed transcriptomic profiling of hippocampal tissues from the four offspring groups at P21 and P56 using RNA deep sequencing. A panel of differentially expressed genes (DEGs; fold change > 1.5, adjusted $p < 0.05$) were identified in CUMS→CUMS and HC→CUMS offspring compared to HC→HC and CUMS→HC offspring. Hierarchical clustering of these DEGs revealed similar hippocampal gene expression profiles in CUMS→CUMS and HC→CUMS offspring, which formed a distinct cluster separate from HC→HC and CUMS→HC offspring (Supplementary Fig. 5a). Gene Ontology (GO) enrichment analysis revealed that the most significantly enriched biological processes were related to neuronal development and function (Supplementary Fig. 5b). These results indicate that the offspring raised by stressed dams exhibit convergent molecular alterations in the hippocampus.

A defining feature of the hippocampus is its capacity for adult neurogenesis, the continuous generation of functionally integrated newborn neurons throughout life⁵². This process occurs specifically in the DG region, where newborn neurons are critical for hippocampus-dependent learning and memory⁵³. We hypothesized that maternal PND disrupts hippocampal neurogenesis in offspring during the lactation period. To test this, we stereotactically injected a retrovirus (RV) encoding EGFP into the DG of cross-fostered offspring at P14. Given that low-titer RV selectively infects dividing progenitor cells⁵⁴, this approach enabled us to label and subsequently analyze the morphology of individual newborn neurons. When examined at P21, CUMS→HC offspring showed a substantial overlap between c-Fos signals and EGFP-labeled newborn neurons, whereas HC→CUMS offspring exhibited significantly less colocalization (Fig. 1h,i). These results indicate that maternal PND significantly influences the activation of newborn neurons in the offspring hippocampus.

To determine the cause of the loss of activated newborn neurons, we examined hippocampal neurogenesis at P21, coinciding with the end of the lactation period. We labeled mitotically dividing cells in cross-fostered offspring with bromodeoxyuridine (BrdU), a thymidine analog⁵⁵, and quantified the proliferation and generation of new neurons from neural stem cells (NSCs) by co-immunostaining for BrdU and the neural stem cell marker Nestin (Supplementary Fig. 5c). Comparisons between groups (HC→CUMS versus CUMS→HC and CUMS→CUMS versus HC→HC) showed no significant differences in the number of BrdU⁺Nestin⁺ cells in the DG (Supplementary Fig. 5d,e). Consistent results were obtained using endogenous proliferation markers, proliferating cell nuclear antigen (PCNA) and minichromosome maintenance protein 2 (MCM2) (Supplementary Fig. 5f-i). These results demonstrate that maternal PND does not impair the proliferation of NSCs in offspring.

Next, we examined the differentiation and development of newborn immature neurons at P21 by immunostaining for doublecortin (DCX), a marker for immature neurons⁵⁶. Our analysis revealed a significant reduction in DCX⁺ cells in HC→CUMS offspring compared to CUMS→HC offspring, as well as in CUMS→CUMS offspring relative to HC→HC offspring (Fig. 1j,k). To further assess the maturation of these newborn neurons, we intraperitoneally administered BrdU to cross-fostered offspring at P21, with injections repeated at 24-hour intervals for a total of three doses. The maturation of these BrdU-labeled, adult-born neurons was then evaluated at P56 by co-immunostaining for BrdU and the neuronal marker NeuN (Supplementary Fig. 5j). A decrease in the number of BrdU⁺NeuN⁺ cells was found in HC→CUMS offspring compared to CUMS→HC offspring (Supplementary Fig. 5k,l). To validate these results, we stereotactically injected RV-EGFP into the DG of cross-fostered offspring at P21 to label newborn neurons and quantified the number of RV-EGFP⁺ cells at P56. Both CUMS→CUMS and HC→CUMS offspring exhibited a reduced number of RV-EGFP⁺ neurons at P56 compared to their respective controls (Supplementary Fig. 5m,n). Collectively, these findings suggest that maternal PND specifically disrupts the maturation and development of newborn neurons without affecting NSC proliferation.

To investigate the dynamic impact of maternal PND on the maturation and integration of newborn neurons in offspring, we stereotactically injected RV-EGFP into the DG of cross-fostered offspring at P0 to label newborn neurons and tracked their developmental trajectory at multiple time points (P7, P14, P21, and P56) (Fig. 1l). Morphological analysis from P7 to P56 revealed that newborn neurons in CUMS→HC offspring progressively developed complex dendritic arbors, reaching a maturity level by P21 that was comparable to that observed at P56. In contrast, HC→CUMS offspring exhibited significantly reduced total dendritic length and fewer dendritic branches in newborn DG neurons compared to CUMS→HC offspring, with these deficits emerging as early as two weeks after birth (Fig. 1m-o). When these offspring reached adulthood at P56, we identified RV-EGFP-labeled newborn neurons and assessed their synaptic activities using whole-cell patch-clamp recordings (Supplementary Fig. 4d). Notably, newborn neurons in HC→CUMS offspring showed a significant increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs) compared to those in CUMS→HC offspring, while the amplitude remained unaltered (Fig. 1p-r). Together, these morphological and electrophysiological results demonstrate that maternal PND impairs the maturation and synaptic integration of a specific population of newborn

neurons in the offspring DG, with deficits initiating during lactation and persisting into adulthood.

Assessment of altered miRNA profiles in breastmilk of PND

Our previous research demonstrated that diet-derived plant miRNAs can survive digestion and regulate gene expression in consumers, a process known as cross-kingdom regulation⁵⁷. This discovery positions maternal breastmilk miRNAs as potential signaling molecules transmitted to the offspring during lactation. We hypothesized that this maternal-to-offspring transfer of breastmilk miRNAs represents a significant yet underexplored mechanism of intergenerational communication. Consequently, alterations in breastmilk miRNA profiles induced by maternal stress may constitute a key molecular link in the developmental programming of offspring mental health.

To test this hypothesis, we performed small RNA (sRNA) deep sequencing to compare sRNA profiles in breastmilk from PND patients versus HC mothers. sRNA composition analysis revealed that miRNAs, tRNA-derived small RNAs (tsRNAs), and rRNA-derived small RNAs (rsRNAs) were the most abundant sRNA species in breastmilk (Supplementary Fig. 6a). Length distribution analysis showed a predominant peak for miRNAs at 20-23 bp, whereas tsRNAs were broadly distributed across 23-34 bp and rsRNAs across 18-42 bp (Supplementary Fig. 6b). We subsequently focused on miRNAs due to their well-established gene regulatory functions, high degree of conservation, and experimental tractability for mechanistic studies⁵⁸. Using thresholds of $|\text{fold change}| > 2$ and $p < 0.05$, we identified 18 miRNAs (14 upregulated and 4 downregulated) that were significantly altered in the breastmilk of PND patients compared to HC mothers (Fig. 2a). Hierarchical clustering analysis further distinguished PND patients from HC mothers based on their breastmilk miRNA profiles (Supplementary Fig. 6c). To validate the sRNA sequencing results, we quantified the expression of these 18 miRNAs in breastmilk samples from 40 PND patients and 51 HC mothers using quantitative RT-PCR. We confirmed consistent and differential expression of several miRNAs, with miR-15a-5p, miR-15b-5p, and miR-205-5p showing the most pronounced upregulation (Fig. 2b). Similarly, in the mouse model, the levels of miR-15a-5p, miR-15b-5p, and miR-205-5p were also significantly upregulated in the breastmilk of CUMS-induced dams compared to HC dams (Supplementary Fig. 6d). These results indicate that breastmilk miRNAs are highly sensitive to maternal experience and are differentially expressed in response to PND-related stress.

Tracking the *in vivo* delivery of breastmilk miRNAs to the offspring brain

To directly assess whether breastmilk miRNAs are delivered to the offspring brain and functionally repress target genes, we constructed an adeno-associated virus (AAV)-based mCherry reporter containing a 3'-UTR with a perfect binding site for the human-specific miRNA hsa-miR-302f, which has no murine homologs⁵⁹. We selected the hippocampus as a representative brain region and stereotactically injected the AAV-mCherry reporter into this area in normal pups at P7. The following day, we administered AAVs expressing either hsa_miR-302f (AAV-CMV-hsa_miR-302f) or a scrambled control RNA (AAV-CMV-NC) into the mammary glands of foster dams. Following two weeks of breastfeeding, we quantified mCherry expression in the pup hippocampus to evaluate hsa_miR-302f-mediated repression (Fig. 2c). Dams injected with AAV-CMV-hsa_miR-302f exhibited significantly higher levels of hsa_miR-302f in their breastmilk compared to those injected with AAV-CMV-NC (Supplementary Fig. 6e). Consistently, pups fostered by AAV-CMV-

hsa_miR-302f-injected dams showed elevated hippocampal hsa_miR-302f levels, while the levels of the internal control U6 small nuclear RNA (U6 snRNA) remained unchanged (Supplementary Fig. 6f). Consequently, these pups displayed markedly attenuated mCherry fluorescence in the hippocampus compared to those fostered by AAV-CMV-NC-injected dams (Fig. 2d; Supplementary Fig. 6g). These findings demonstrate that maternal breastmilk miRNAs are efficiently delivered to the offspring's hippocampus via lactation, where they enable intergenerational regulation of offspring gene expression.

To further investigate the delivery and accumulation of endogenous PND-associated miRNAs in the offspring brain, we measured the levels of mature and primary forms of miR-15a-5p, miR-15b-5p, and miR-205-5p in offspring hippocampus following the lactation period. Offspring fostered by CUMS-induced dams exhibited significantly higher hippocampal levels of the mature miR-15a-5p, miR-15b-5p, and miR-205-5p compared to those fostered by HC dams (Supplementary Fig. 6h). In contrast, no differences were observed in the levels of their primary transcripts (pri-miR-15a-5p, pri-miR-15b-5p, and pri-miR-205-5p) (Supplementary Fig. 6i). These results suggest that the elevated levels of these specific miRNAs in the offspring hippocampus originate primarily from exogenous transfer via breastmilk, rather than from transcriptional upregulation of their endogenous counterparts.

Manipulation of breastmilk miRNA composition significantly influences offspring neurodevelopment

miR-15a-5p and miR-15b-5p are frequently implicated in mental illnesses such as major depressive disorder and schizophrenia, where they are known to target genes critical for neuronal plasticity and neurogenesis^{60,61}. Similarly, miR-205-5p has been strongly associated with the regulation of neuronal development and synaptic function^{62,63}. Given the established roles of these miRNAs in neurodevelopment and neuropsychiatric disorders, we selected them as candidate PND-associated breastmilk miRNAs and specifically overexpressed them in the mammary glands of healthy foster dams. This strategy allowed us to obtain breastmilk enriched with these miRNAs and evaluate their effects on offspring behavior and hippocampal neuronal maturation.

Since breastmilk miRNAs are predominantly secreted by mammary epithelial cells³⁷, we employed an AAV2/9 serotype, which has been shown to efficiently infect mouse mammary epithelium, under the control of the mammary-specific MMTV promoter to achieve cell-type-restricted miRNA overexpression^{64,65}. A mixture of AAV-MMTV-miRNA constructs was delivered via intraductal injection into the mammary glands, and the injected dams subsequently nursed healthy P0 offspring until adulthood (Fig. 2e). Successful viral transduction in mammary alveolar epithelial cells was confirmed (Supplementary Fig. 7a), and quantitative RT-PCR analysis of breastmilk revealed significantly elevated levels of miR-15a-5p, miR-15b-5p, and miR-205-5p (Supplementary Fig. 7b). Morphological assessment of newborn neurons in offspring at P56 revealed that those fostered by AAV-MMTV-miRNA-injected dams exhibited significantly reduced total dendritic length and branching complexity compared to controls fostered by AAV-MMTV-NC- or PBS-injected dams (Fig. 2f-h). Furthermore, whole-cell patch-clamp recordings of RV-EGFP-labeled newborn neurons in the DG at P56 (Supplementary Fig. 7c) revealed a significant increase in mEPSC frequency in the AAV-MMTV-miRNA group relative to controls, with no change in amplitude⁴ (Fig. 2i-k).

Conversely, to determine whether silencing these miRNAs in breastmilk could rescue the adverse phenotypic outcomes in offspring nursed by CUMS-induced dams, we designed miRNA sponges, which bind and sequester specific miRNAs, thereby preventing their interaction with endogenous targets⁶⁶. We constructed three AAV-MMTV-driven vectors, each containing tandemly arrayed, bulged binding sites complementary to miR-15a-5p, miR-15b-5p, or miR-205-5p, respectively (AAV-MMTV-SP(miRNA)). These vectors were pooled in equal proportions and administered via intraductal injection into the mammary glands of CUMS-induced dams, which continued to nurse healthy P0 offspring until adulthood (Fig. 2l). Using this strategy, we neutralized the miRNAs within the mammary glands of CUMS-induced dams and evaluated the functional consequences of miRNA loss-of-function in breastmilk on offspring development. Successful viral transduction in mammary alveolar epithelial cells was confirmed (Supplementary Fig. 7d), and quantitative RT-PCR analysis demonstrated that sponge-mediated sequestration significantly attenuated the elevated levels of miR-15a-5p, miR-15b-5p, and miR-205-5p in the breastmilk of CUMS dams (Supplementary Fig. 7e). Consequently, offspring fostered by CUMS dams injected with the control sponge exhibited significant cognitive deficits in the MWM and CFC tests compared to those fostered by HC dams; these behavioral impairments were largely rescued in offspring fostered by CUMS dams injected with the miRNA sponges (Fig. 2m,n). At the cellular level, the offspring fostered by CUMS dams injected with the control sponge displayed impaired dendritic arborization and reduced branching complexity (Fig. 2o-q) and showed an elevated mEPSC frequency (Fig. 2r-u). Strikingly, sponge-mediated miRNA sequestration significantly rescued these structural and functional alterations (Fig. 2o-u). Collectively, these results establish a causal role for breastmilk miRNAs in shaping offspring neurodevelopment, demonstrating that aberrant miRNA levels disrupt the structural and functional maturation of newborn hippocampal neurons, whereas their removal can reverse these deficits.

Evaluation of sEV-mediated transfer of breastmilk miRNAs and its intergenerational impact on offspring neurodevelopment

sEVs possess a unique lipid bilayer structure that is essential for the intercellular transfer of miRNAs. This architecture protects encapsulated miRNAs from enzymatic degradation in the extracellular environment and facilitates their delivery to recipient cells through membrane fusion and cargo release³⁶. Thus, sEVs serve not merely as passive carriers but as active and indispensable vehicles for functional miRNA transmission. We therefore hypothesize that the sEV-mediated transfer of breastmilk miRNAs constitutes a key biological pathway through which maternal adversity is transmitted to the offspring.

Using a Cre-dependent EV reporter mouse model, we first tracked the *in vivo* delivery of breastmilk sEVs to the offspring brain (Fig. 3a). This model uses Rosa26-CD63^{Flag}EGFP-mCherry knock-in mouse, in which a CD63^{Flag}EGFP-mCherry^{f/f} reporter system was inserted into the Rosa26 locus⁶⁷. The reporter consists of a loxP-flanked stop cassette upstream of a modified CD63 construct, which is fused at its extracellular domain to a FLAG tag and at its C-terminus to EGFP, and is co-expressed with an mCherry protein. When crossed with CAG-Cre mice, the offspring ubiquitously express CD63^{Flag}EGFP-mCherry, enabling fluorescent tagging of sEVs via the tetraspanin CD63, while mCherry identifies tissues expressing the reporter⁶⁷. As expected, prominent mCherry fluorescence was observed in breast tissue sections from female reporter mice (Supplementary Fig. 8a). Nano-

flow cytometry (NanoFCM) confirmed successful CD63-EGFP labeling of sEVs, revealing that breastmilk sEVs from these mice exhibited significantly higher EGFP fluorescence (Supplementary Fig. 8b,c), with EGFP⁺ sEVs constituting ~30% of the total breastmilk sEV population (Supplementary Fig. 8d). When normal pups were fostered by these reporter dams, strong EGFP signals were detected in the hippocampus, cortex, and hypothalamus after three days of nursing (Fig. 3b; Supplementary Fig. 8e). Weaker but discernible fluorescence was also present in the thalamus, amygdala, striatum, midbrain, and cerebellum (Fig. 3b; Supplementary Fig. 8e). Importantly, EGFP fluorescence was observed to colocalize with the neuronal marker NeuN in the hippocampus (Fig. 3c), suggesting that breastmilk-derived sEVs are taken up by neurons in breastfed offspring.

Next, we established a standardized protocol to purify sEVs from breastmilk. Briefly, breastmilk samples were initially processed by differential ultracentrifugation (dUC) to obtain a crude sEV pellet, which was subsequently refined by size-exclusion chromatography (SEC), yielding 26 fractions⁶⁸⁻⁷⁰. Each fraction was systematically characterized using a BCA assay to determine total protein content and nanoparticle tracking analysis (NTA) to quantify particle concentration. The distribution of protein and particle counts across the fractions was calculated. While protein content was broadly distributed across multiple fractions, a pronounced peak in particle concentration was observed specifically in fractions 6-8 (Supplementary Fig. 9a,b), indicating strong enrichment of sEVs in this range, a distribution profile consistent with previous reports⁷⁰. Immunoblotting further confirmed the presence of canonical sEV markers, including TSG101 and CD63, predominantly in fractions 6-8, with negligible expression in other fractions (Supplementary Fig. 9c). In contrast, lactoferrin (LTF), a highly abundant breastmilk whey protein included as a negative control, was mainly detected in other fractions and was present at lower levels in fractions 6-8 (Supplementary Fig. 9c)^{68,69,71}. We further characterized the isolated sEV fractions using NanoFCM. Since sEVs possess a phospholipid bilayer membrane that is susceptible to disruption by detergents such as Triton X-100, we compared particle counts before and after Triton X-100 treatment to indirectly evaluate sEV purity⁷². The NanoFCM results confirmed that fractions 6-8 exhibited the highest particle counts across all fractions, and this signal was markedly diminished upon Triton X-100 exposure (Supplementary Fig. 9d,e). These results demonstrate that fractions 6-8 obtained via the dUC-SEC workflow represent sEV-enriched fractions. Interestingly, quantification of miR-15a-5p, miR-15b-5p, and miR-205-5p across the fractions revealed a distinct enrichment peak specifically in fractions 6-8, with only minimal levels detected elsewhere (Supplementary Fig. 9f-h). This distribution pattern strongly supports the established view that breastmilk miRNAs are predominantly packaged and secreted within sEVs⁷³, a mechanism that ensures their extracellular stability and preserves their biological activity.

We next characterized the sEVs (SEC fractions 6-8) isolated from the breastmilk of PND patients and HC mothers (designated as PND-sEVs and HC-sEVs, respectively). NTA showed that sEVs from both groups had similar particle concentrations ($\sim 1.0 \times 10^{11}$ particles/mL) and size distributions, with a peak around 100 nm (Supplementary Fig. 9i,j). Transmission electron microscopy (TEM) confirmed that particles within both groups exhibited the characteristic morphology and size of sEVs (Supplementary Fig. 9k). After unit conversion, the calculated particle concentrations in the breastmilk of PND patients and HC mothers were found to be several orders of magnitude higher than those in an equivalent volume of serum (Fig. 3d), highlighting the natural

abundance of sEVs in breastmilk and supporting their potential for infant uptake and neurodevelopmental regulation.

Following the purification of PND-sEVs and HC-sEVs (SEC fractions 6-8), we characterized their sRNA profiles by deep sequencing. Composition analysis confirmed a significant enrichment of miRNAs, tsRNAs, and rsRNAs within breastmilk sEVs (Supplementary Fig. 10a). Notably, the proportional abundance of miRNAs was higher in the sEV fraction than in whole breastmilk (Supplementary Fig. 6a,10a), consistent with previous reports indicating the selective sorting and secretion of miRNAs into sEVs⁷⁴. Length distribution analysis further revealed that miRNAs exhibited a relatively uniform profile, predominantly within the 20-23 bp range, whereas tsRNAs and rsRNAs displayed a broader size distribution (Supplementary Fig. 10b). Comparative analysis revealed a more pronounced alteration in miRNA levels in PND-sEVs relative to HC-sEVs (57 upregulated and 26 downregulated) than in whole breastmilk, and hierarchical clustering analysis successfully distinguished PND patients from HC mothers based on their breastmilk sEV miRNA profiles (Supplementary Fig. 10c). Specifically, sRNA sequencing identified a significant upregulation of miR-15a-5p, miR-15b-5p, and miR-205-5p in PND-sEVs compared to HC-sEVs (Fig. 3e). This finding was subsequently validated by quantitative RT-PCR, which confirmed the elevated expression levels of these specific miRNAs in PND-sEVs (Supplementary Fig. 10d). Consistently, AAV-MMTV-mediated overexpression of these miRNAs in the mammary glands of HC dams also resulted in their significant enrichment in breastmilk sEVs (Supplementary Fig. 10e). Conversely, AAV-MMTV-SP(miRNA)-mediated miRNA sequestration significantly restored the levels of these miRNAs in the breastmilk sEVs of CUMS-induced dams (Supplementary Fig. 10f).

To establish breastmilk sEVs as an independent causative factor linking maternal PND to adverse offspring outcomes, we designed a cross-species experiment. Given the minimal species specificity of sEVs and the high conservation of miRNA sequences and functions between humans and rodents^{75,76}, we hypothesized that administering human breastmilk sEVs to mouse pups could provide mechanistic insights relevant to human physiology. Accordingly, equal amounts of isolated PND-sEVs and HC-sEVs (SEC fractions 6-8) were administered to normal mouse pups via daily intragastric injection throughout the lactation period (Fig. 3f). As a negative control, sEV-free fractions (SEC fractions 1-5 and 9-26) were administered to another group of normal pups. To minimize the potential confounding influence of maternal care, all pups were fostered by the same cohort of healthy dams prior to phenotypic analyses.

We first confirmed that human breastmilk sEVs did not elicit detectable immune responses in recipient pups, as levels of key immune factors remained unchanged in both HC-sEV- and PND-sEV-treated groups compared to sEV-free control groups (Supplementary Fig. 11a,b). This finding is consistent with previous reports on the low immunogenicity of sEVs across species and supports the physiological relevance of our cross-species model⁷⁶. In behavioral tests, pups treated with PND-sEVs showed significant cognitive deficits compared to those receiving HC-sEVs. In the MWM test, PND-sEV-treated pups exhibited prolonged escape latencies, reduced time spent in the target quadrant, and fewer platform crossings, despite comparable swimming speed activity (Supplementary Fig. 11c-e). Similarly, in the CFC test, PND-sEV-treated pups displayed impaired context-evoked freezing responses, despite normal freezing behavior during training (Fig. 3g;

Supplementary Fig. 11f). In contrast, pups administered sEV-free fractions performed normally in both the MWM and CFC tests (Fig. 3g; Supplementary Fig. 11c-f), indicating that non-sEV components in breastmilk do not share the same capacity to influence offspring phenotypes. At the molecular level, levels of mature miR-15a-5p, miR-15b-5p, and miR-205-5p were significantly elevated in the hippocampus of recipient pups following PND-sEV treatment (Supplementary Fig. 11g), whereas their primary transcripts remained unchanged (Supplementary Fig. 11h). To directly visualize the neuronal uptake of breastmilk miRNAs, we performed RNAscope *in situ* hybridization combined with NeuN immunofluorescence. Consistent with the brain distribution pattern of exogenous sEVs, pups treated with PND-sEVs exhibited markedly stronger hybridization signals for miR-15a-5p, miR-15b-5p, and miR-205-5p in the hippocampus and hypothalamus compared to HC-sEV-treated controls, with the majority of these signals co-localizing with NeuN⁺ neurons (Fig. 3h; Supplementary Fig. 11i). At the neurological level, administration of PND-sEVs resulted in a decreased number of DCX⁺ cells in the DG region (Supplementary Fig. 11j-k). Furthermore, EGFP-labeled newborn neurons in PND-sEV-treated pups exhibited impaired maturation, as evidenced by reduced total dendritic length, lower dendritic complexity, and decreased spine density compared to those in HC-sEV-treated controls (Fig. 3i,j; Supplementary Fig. 11l). Whole-cell patch-clamp recordings demonstrated that newborn neurons from PND-sEV-treated pups exhibited a significantly increased mEPSC frequency, with no corresponding change in amplitude, when compared to controls (Fig. 3k-m; Supplementary Fig. 11m). Collectively, these results demonstrate that sEVs from the breastmilk of PND mothers can traverse species barriers and reprogram neurodevelopmental trajectories in recipient pups, leading to cognitive deficits and impaired maturation of newborn neurons.

A reduction in breastmilk sEV content mitigates the adverse effects of PND-associated breastfeeding on offspring

To definitively establish the role of sEV-mediated miRNA transfer in PND-associated neurodevelopmental impairments, we specifically reduced sEV secretion into the breastmilk of PND dams and assessed the phenotypic consequences in their offspring. We employed a conditional knockout (cKO) strategy targeting Ras-associated protein 27a (Rab27a), a key regulator of sEV release⁷⁷. A Rab27a cKO mouse model (C57BL/6JGpt-Rab27a^{em1Cfloxed}/Gpt) was generated using CRISPR/Cas9 technology, with successful genotyping confirmed by PCR (Supplementary Fig. 12a). Since breastmilk sEVs are primarily secreted by mammary epithelial cells, we injected an AAV2/9 vector encoding a MMTV promoter-driven Cre recombinase (AAV2/9-MMTV-EGFP-IRES-Cre) into the mammary glands of Rab27a cKO females to achieve mammary-specific suppression of sEV secretion³⁷. Immunoblotting analysis verified the effective downregulation of Rab27a (Supplementary Fig. 12b). As anticipated, mammary gland-specific Rab27a cKO significantly suppressed sEV secretion into breastmilk, reducing the particle count by 57.53% compared to that in an equivalent volume of natural breastmilk (Supplementary Fig. 12c). This reduction was further confirmed by immunoblot analysis, which demonstrated markedly decreased levels of the sEV markers CD63 and TSG101 in sEVs isolated from the breastmilk of cKO females (Supplementary Fig. 12d). Importantly, the morphological integrity of the remaining sEVs was preserved (Supplementary Fig. 12e), and the fundamental nutritional composition of the breastmilk remained largely unchanged (Supplementary Fig. 12f). Consistent with this profile, despite the substantial reduction in sEV content, the offspring fostered by Rab27a cKO females developed normally

(Supplementary Fig. 12g,h) and exhibited behavioral performance comparable to wild-type (WT) females-fostered controls across the MWM, CFC, and FST tests (Supplementary Fig. 12i-k).

Subsequently, Rab27a cKO or WT females were subjected to the CUMS paradigm to induce PND, and normal pups were fostered with these dams to monitor phenotypic changes (Fig. 3n). At the molecular level, while CUMS-induced WT dams showed elevated levels of miR-15a-5p, miR-15b-5p, and miR-205-5p in their breastmilk compared to HC dams, Rab27a cKO significantly attenuated the enrichment of these miRNAs in breastmilk (Supplementary Fig. 12l). At the behavioral level, while offspring fostered by CUMS-induced WT dams exhibited significant learning and memory deficits in both the MWM and CFC tests, those fostered by CUMS-induced Rab27a cKO dams showed a substantial rescue of these cognitive impairments (Fig. 3o; Supplementary Fig. 12m,n). At the neurological level, while the offspring fostered by CUMS-induced WT dams exhibited delayed maturation of newborn neurons in the DG region, those fostered by CUMS-induced Rab27a cKO dams showed significant recovery, evidenced by an increased number of DCX⁺ cells (Fig. 3p; Supplementary Fig. 12o) and enhanced dendritic complexity of newborn neurons in the DG (Fig. 3q,r; Supplementary Fig. 12p). Whole-cell patch-clamp recordings further showed a significantly increased mEPSC frequency in offspring fostered by CUMS-induced WT dams; however, this synaptic alteration was substantially restored in offspring fostered by CUMS-induced Rab27a cKO dams (Fig. 3s-u; Supplementary Fig. 12q). These findings underscore the unique role of breastmilk sEVs in the intergenerational transmission of PND-associated deficits.

Single-cell RNA sequencing identifies a distinct excitatory neuron subpopulation with a SuM molecular signature

We performed single-cell RNA sequencing (scRNA-seq) on whole-brain samples from 3-week-old pups continuously exposed to either HC-sEVs or PND-sEVs throughout lactation. After rigorous quality control and doublet removal, 18,572 high-quality cells were retained for downstream analysis (Supplementary Fig. 13a). Unsupervised clustering and cell-type annotation identified eight major brain cell populations, including excitatory neurons, inhibitory neurons, astrocytes, oligodendrocytes, oligodendrocyte precursor cells, endothelial cells, microglia, and neural stem cells, based on the expression of well-established marker genes (Fig. 4a; Supplementary Fig. 13b). Comparative analysis showed no significant differences in the relative abundance of these major cell types between PND-sEV- and HC-sEV-treated pups (Fig. 4b), indicating that global brain cell composition was not substantially altered by maternal sEV exposure.

Notably, neurons constituted the majority (> 70%) of all captured cells, with excitatory and inhibitory neurons clearly distinguished by canonical markers: *Snap25* and *Syt1* for excitatory neurons, and *Gad1* and *Gad2* for inhibitory neurons (Supplementary Fig. 13c). In the hippocampal DG, excitatory neurons, primarily granule cells, represented the predominant neuronal population⁷⁸. These neurons, especially the newborn granule cells generated during postnatal neurogenesis, play critical roles in learning and memory and are also implicated in the pathogenesis of depression^{51,79,80}. To further resolve the transcriptional heterogeneity within this population, we performed subclustering analysis on excitatory neurons. Unsupervised clustering identified eight distinct excitatory subpopulations (E1-E8) (Fig. 4c). While the relative proportions of these subpopulations did not differ significantly between PND-sEV- and HC-sEV-treated pups (Supplementary Fig. 13d),

the E1 subpopulation exhibited a unique gene expression signature compared to other clusters (Supplementary Fig. 13e), suggesting potential functional specialization or distinct molecular states. To explore the developmental dynamics of hippocampal excitatory neurons, we conducted pseudotime trajectory analysis on excitatory neurons using the Monocle2 algorithm. This approach orders cells along a continuous path based on transcriptional similarity, thereby reconstructing cellular maturation and lineage progression⁸¹. With neural stem cells set as the trajectory root, the analysis revealed three distinct cell states (S1-S3) and two major differentiation branches (Fig. 4d). Notably, the E1 subpopulation was distinctly segregated at the terminal end of Trajectory 2 within state S1 (Fig. 4d), indicating that E1 likely represents a terminally differentiated or functionally specialized neuronal state. Furthermore, we examined gene expression patterns in the E1 subpopulation of excitatory neurons. While the expression of representative neuronal marker genes showed no significant differences between the E1 and other excitatory subpopulations (Supplementary Fig. 13f), a substantial downregulation of neuronal genes was observed in the E1 subpopulation, with 473 genes downregulated and 110 upregulated compared to other excitatory clusters (Fig. 4e).

To determine whether the transcriptional signature observed in the E1 subpopulation could be linked to PND-associated breastmilk miRNAs, we predicted the target genes of miR-15a-5p, miR-15b-5p, and miR-205-5p and compared them with the 583-gene signature (473 downregulated + 110 upregulated) characteristic of the E1 subpopulation. Strikingly, 256 (43.91%) of these genes were predicted as direct targets of these miRNAs, a proportion significantly higher than expected by chance, as confirmed by random permutation analysis (Fig. 4f). GO enrichment analysis indicated that these 256 miRNA-targeted genes were primarily enriched in key neurodevelopmental processes such as “synapse organization,” “axon guidance,” and “axonogenesis” (Supplementary Fig. 13g). These findings suggest that the E1 excitatory subpopulation, as a transcriptionally distinct group, may be influenced by PND-associated miRNAs, potentially contributing to alterations in hippocampal maturation and synaptic development.

To identify direct targets of PND-associated breastmilk miRNAs, we focused on the top 50 downregulated genes in the E1 subpopulation, with particular attention to those potentially regulated by miR-15a-5p, miR-15b-5p, and miR-205-5p. A heatmap visualizes the expression patterns of these genes across excitatory neuron subpopulations and their predicted targeting by these miRNAs (Fig. 4g). Notably, among the genes specifically downregulated in E1 subpopulation and computationally predicted to be co-targeted by all three miRNAs, we identified a significant enrichment of group III metabotropic glutamate receptors (mGluRs), mGluR7 and mGluR8. mGluR7 and mGluR8 are presynaptic receptors known to modulate glutamatergic synaptic transmission⁸²⁻⁸⁴. In particular, mGluR8 regulates glutamate release from neuronal terminals in the DG, and its knockout animal models display pronounced learning and memory deficits^{85,86}. These observations point to mGluR7 and mGluR8, especially mGluR8, as potential candidates through which PND-associated miRNAs may disrupt DG circuit maturation.

To further elucidate the identity and regional relevance of the E1 subpopulation, we examined its transcriptional similarity to cells originating from distinct brain regions. After integrating the datasets using Harmony to correct for potential technical variations, we calculated the Euclidean

nearest-neighbor distances between each E1 cell and cells originating from the hippocampus (HIP), amygdala (AMY), thalamus (TH), and hypothalamus (HY) in the corrected low-dimensional embedding space. For every E1 cell, we calculated the average distance to its five nearest neighbors from each reference region using the R package RANN⁸⁷. The resulting distances were then averaged across all E1 cells, with smaller average distances indicating greater transcriptional similarity between populations. Quantitative comparison revealed that the E1 subpopulation showed the strongest transcriptional affinity to hypothalamic cells, followed by thalamic and amygdalar cells, while exhibiting relatively lower similarity to hippocampal cells (Fig. 4h). These results suggest that the E1 subpopulation shares closer molecular features with hypothalamic neurons.

To further define the identity of the E1 subpopulation, we performed reference-based mapping using a published single-cell dataset⁸⁸. Our analysis revealed that most cells from Cluster 4 in the reference dataset were confidently mapped to the E1 subpopulation (Fig. 4i,j). Notably, Cluster 4 was originally annotated as consisting primarily of neurons from the supramammillary nucleus (SuM). The SuM is a specialized hypothalamic structure that serves as a functional interface between the hypothalamus and the hippocampus. Given that the SuM is known to send dense axonal projections to the DG and modulate learning and memory in mice^{51,89,90}, we sought to confirm the existence of direct structural projections from the SuM to the GCL of the DG. We injected the anterograde tracer rAAV-hSyn-mCherry into the SuM and RV-EGFP into the DG to anatomically trace the SuM→DG circuit and to visualize contacts between SuM axonal terminals and RV-EGFP-labeled newborn neurons in the DG. Histological analysis revealed abundant mCherry fluorescence specifically localized within the GCL across multiple anterior - posterior levels of the DG (Fig. 4k), supporting a robust anatomical projection from the SuM to DG granule cells. These results provide crucial insight into the spatial specificity and potential physiological role of the E1 subpopulation, suggesting that the E1 subpopulation likely represents a SuM-like neuronal population projecting to the DG.

Identification of mGluR8 as a direct target of PND-associated breastmilk miRNAs

Given the spatial overlap between the E1 subpopulation and SuM neurons, together with the established role of mGluR8 in modulating glutamate release in the DG, we hypothesized that SuM projections form presynaptic mGluR8-containing terminals onto newborn DG granule cells under normal conditions. In the context of PND, however, aberrant breastmilk miRNAs may disrupt mGluR8 function within this SuM→DG circuit, impairing presynaptic signaling and hindering the functional integration of newborn neurons into learning and memory circuits.

To determine whether PND breastmilk miRNAs affect the expression of group III mGluRs, we first examined the developmental expression patterns of mGluR7 and mGluR8 in the DG, using DCX as a marker for immature neurons and PROX1 (Prospero Homeobox 1) for mature DG granule cells⁹¹. Under normal conditions, DCX⁺ newborn neurons exhibited significantly fewer mGluR8⁺ puncta than PROX1⁺ mature neurons (Supplementary Fig. 14a,b), and mGluR8 expression was markedly higher than mGluR7 in the DG (Supplementary Fig. 14c). Following PND-sEV administration during lactation, pups showed a significant reduction in mGluR8 expression in both DCX⁺ and PROX1⁺ neurons (Supplementary Fig. 14d-g), with a similar downregulation observed for mGluR7 (Supplementary Fig. 15a-d). We next monitored mGluR8 expression levels in the SuM from birth

to 8 weeks of age. In HC-sEV-treated controls, mGluR8 expression increased progressively during lactation and reached adult-like levels by P21 (Fig. 4l). In contrast, PND-sEV treatment significantly delayed this developmental upregulation of mGluR8 (Fig. 4l). Consistent with these observations, offspring fostered by control dams injected with AAV-MMTV-NC or PBS exhibited a gradual increase in mGluR8 expression, whereas those fostered by AAV-MMTV-miRNA-injected dams showed significantly reduced mGluR8 expression levels (Fig. 4m). Conversely, while offspring fostered by CUMS dams injected with the control sponge displayed decreased mGluR8 expression, sponge-mediated miRNA sequestration effectively rescued this deficit, restoring mGluR8 to near-normal levels (Fig. 4n). Together, these results demonstrate that the normal developmental upregulation of mGluR8 during the lactation period (P0-P21) is markedly suppressed by PND breastmilk miRNAs. Notably, this suppression extended beyond weaning, as mGluR8 levels failed to recover even after cessation of miRNA exposure, remaining significantly lower than controls through P56.

Next, we validated the direct downregulation of mGluR8 by miR-15a-5p, miR-15b-5p, and miR-205-5p *in vitro*. Bioinformatic analysis revealed that miR-15a-5p, miR-15b-5p, and miR-205-5p bind to non-overlapping sites within the 3'-UTR of mGluR8 following canonical base-pairing rules (Supplementary Fig. 16a). Primary neurons infected with AAV vectors overexpressing each miRNA (Supplementary Fig. 16b) showed substantially increased levels of miR-15a-5p, miR-15b-5p, and miR-205-5p (Supplementary Fig. 16c-e), accompanied by a significant reduction in mGluR8 protein expression (Supplementary Fig. 16f,g). Direct binding of these miRNAs to the mGluR8 3'-UTR was confirmed by luciferase reporter assays: co-transfection of miRNA mimics with a wild-type mGluR8 3'-UTR reporter significantly reduced luciferase activity, whereas this inhibitory effect was completely abolished when the 3'-UTR contained mutant binding sites (Supplementary Fig. 16h). To determine whether breastmilk sEVs from PND patients exert similar effects, we treated primary neurons with equal doses of HC-sEVs or PND-sEVs. Efficient neuronal uptake of breastmilk sEVs was confirmed by prominent mCLING⁹² labeling in co-cultured neurons (Supplementary Fig. 16i). Consistent with the miRNA overexpression results, PND-sEV treatment substantially suppressed mGluR8 expression (Supplementary Fig. 16j-l).

Presynaptic mGluR8 dysfunction in the SuM→DG circuit underlies neurodevelopmental impairments induced by PND breastfeeding

To investigate whether the SuM→DG projection influences the maturation of newborn neurons and to define the role of presynaptic mGluR8 at DG synapses, we performed stereotaxic viral injections in P0 pups. A Cre-inducible mGluR8 knockdown virus (AAV-hSyn-DIO-RNAi(mGluR8)-mCherry) was delivered into the SuM, while a retrograde Cre virus (AAVRetro-hSyn-Cre-EGFP) was injected into the DG. This retrograde virus is transported to SuM neurons that project to the DG, where Cre expression excises the DIO cassette, enabling selective presynaptic mGluR8 knockdown specifically in DG-projecting SuM neurons (Fig. 5a). Pups were then fostered by HC dams until weaning. Successful viral transduction was confirmed at P21, with EGFP-labeled Cre expression observed in the DG and retrograde tracing showing EGFP signal in the SuM, where it colocalized with mCherry signal from the DIO virus (Fig. 5b). At P56, RNAi(mGluR8)-treated pups exhibited significant learning and memory impairments in the MWM and CFC tests (Fig. 5c,d). Morphologically, these pups displayed shorter dendritic length and reduced complexity of dendritic

arborization (Fig. 5e-g). To assess synaptic properties, we recorded mEPSCs from newborn neurons that had been labeled with RV-EGFP (Fig. 5h). Presynaptic mGluR8 knockdown in SuM neurons projecting to the DG resulted in a significant increase in mEPSC frequency without changes in amplitude (Fig. 5i-k). These behavioral and electrophysiological phenotypes closely resembled those previously observed in pups exposed to PND breastmilk miRNAs, suggesting that presynaptic mGluR8 deficiency in DG-projecting SuM neurons may be a key mechanism underlying the observed developmental and functional deficits.

Conversely, we investigated whether presynaptic mGluR8 overexpression could rescue the maturation deficits and behavioral abnormalities caused by breastfeeding with CUMS dams. A Cre-inducible mGluR8 overexpression virus (AAV-hSyn-DIO-mGluR8-mCherry) was injected into the SuM of P0 pups, together with AAVRetro-hSyn-Cre-EGFP into the DG, to achieve selective presynaptic mGluR8 overexpression (Fig. 5l). Pups were then fostered by CUMS dams until weaning. Immunofluorescence at P21 confirmed EGFP-labeled Cre expression in the DG and its retrograde transport to the SuM, where it colocalized with the DIO virus-derived mCherry signal (Fig. 5m). At P56, this intervention rescued the learning and memory deficits observed in CUMS-fostered pups in behavioral tests (Fig. 5n,o). Moreover, the reductions in dendritic length and complexity seen in CUMS-fostered pups were significantly ameliorated by presynaptic mGluR8 overexpression (Fig. 5p-r). Electrophysiological recordings showed that the elevated mEPSC frequency characteristic of CUMS-fostered pups was normalized to control levels by presynaptic mGluR8 overexpression (Fig. 5s-u; Supplementary Fig. 16m). These results demonstrate that presynaptic mGluR8 overexpression effectively rescues the functional, morphological, and behavioral deficits in offspring resulting from CUMS dam rearing.

***In vivo* calcium imaging reveals a “high-frequency, low-amplitude” activation profile**

To investigate how mGluR8 knockdown modulates the activity of individual newborn neurons in the hippocampal DG via the SuM→DG projections, we used the aforementioned AAV-hSyn-DIO-RNAi(mGluR8)-mCherry and AAVretro-hSyn-Cre-EGFP to achieve mGluR8 knockdown in the presynaptic terminals adjacent to DG neurons at P0. Adult mice were then co-injected with a genetically encoded calcium indicator (GCaMP6f) and RV-tdTomato to label the newborn neurons. Single-neuron calcium imaging was subsequently performed in freely moving male mice using a miniature head-mounted microscope⁹³⁻⁹⁵. Neuronal activity was recorded over a two-day experimental epoch under a CFC paradigm (Fig. 6a). A “calcium event” was defined as a transient fluorescence fluctuation resulting from rapid intracellular calcium elevation, expressed as a Z-score relative to the cell’s mean fluorescence over the entire recording period, thereby directly reflecting neuronal activation. Event amplitude was used to quantify the intensity of neuronal activation. Time-series average intensity projection verified the co-expression of GCaMP6f and RV-tdTomato, along with the spatial localization of the DG region through immunofluorescence staining (Fig. 6b,c). Neuronal activity tracings and calcium signal heatmaps revealed that DG neurons in the mGluR8 knockdown group displayed more calcium events than those in the control group (Fig. 6d,e). During Day 1 (tone stimulation phase), neither the frequency nor the amplitude of tone-evoked calcium events differed significantly between the mGluR8 knockdown and control groups across individual stages (Fig. 6f,g) or when all stages were combined (Fig. 6h,i). These results indicate that mGluR8 knockdown does not impair the immediate processing of auditory inputs by DG neurons, ruling out

nonsensory deficits as a confounder for subsequent memory-related phenotypes. In the subsequent interval period (no stimulation), the mGluR8 knockdown group exhibited a significantly higher frequency of calcium events compared to control group, although event amplitude remained unchanged (Fig. 6j-m). This result suggests that mGluR8 deficiency enhances the intrinsic excitability of DG neurons during the post-stimulation interval period. To further assess whether mGluR8-dependent dysregulation of excitability persists across memory-related behavioral phases, we recorded neuronal activity during the memory retrieval phase on Day 2. During the exploration period, mGluR8-knockdown mice again showed a significant increase in calcium event frequency, with no change in amplitude (Fig. 6n,o), indicating persistently elevated baseline excitability in DG neurons. These results are consistent with our electrophysiological data showing increased mEPSC frequency in PND-affected offspring, collectively demonstrating that mGluR8 knockdown compromises synaptic transmission efficiency and leads to hyperexcitability in DG neurons.

To assess the stability of functional properties in memory-related (context-associated) neurons across days, we performed cross-session registration of neurons activated during the Day 1 interval period and the Day 2 exploration phase. This approach enabled us to track and associate the same neurons across distinct behavioral stages. Although neuron reactivation rates did not differ significantly between groups (Fig. 6p,q), the calcium event frequency of context-associated neurons was markedly reduced in the mGluR8 knockdown group (Fig. 6r). These findings point to two interrelated deficits: diminished activity in context-associated neurons (critical for fear memory encoding) and aberrant excitation in non-context neurons. This functional defect represents a core mechanism underlying the disruption of fear memory encoding.

Mice formed a contextual fear memory after the Day 1 foot shock, a training phase that corresponds to the critical period for initial sensory input and memory encoding⁹³. To investigate if mGluR8 knockdown induces memory impairments via calcium overload, a potential mechanism of neuronal dysfunction⁹⁶⁻⁹⁸, we analyzed calcium event dynamics during the Day 1 interval by constructing a frequency-amplitude distribution model. Neuronal activity patterns were fitted using an exponential model to characterize population-level dynamics. The mGluR8 knockdown group exhibited a distinct “high-frequency, low-amplitude” activation profile compared to controls (Fig. 6s). This pattern reflects a state of “inefficient high-frequency activation”, wherein neurons generate excessive non-specific excitation while failing to encode contextual information precisely. These findings offer direct *in vivo* evidence that mGluR8 deficiency leads to dysregulated neuronal coding patterns. This mechanistic insight explains the impaired fear memory retrieval previously observed in offspring exposed to PND-associated breastmilk, thereby bridging aberrant neural dynamics with specific behavioral deficits.

To determine whether administering PND-associated breastmilk sEVs to healthy pups during lactation could recapitulate the “high-frequency, low-amplitude” neuronal activation pattern observed in mGluR8 knockdown models, we performed calcium imaging of DG neurons in mice exposed to either PND-sEVs or HC-sEVs under a similar behavioral paradigm (Supplementary Fig. 17a). Co-expression of GCaMP6f and RV-tdTomato was confirmed by time-series average projection and immunofluorescence staining (Supplementary Fig. 17b,c). Consistent with the knockdown model, neuronal activity tracings and calcium signal heatmaps revealed distinct firing

patterns between the two groups, with the PND-sEV group exhibiting a significantly higher number of calcium events than the HC-sEV group (Supplementary Fig. 17d,e). Similarly, during the Day 2 exploration phase, the PND-sEV group showed a significant increase in calcium event frequency, but no change in amplitude, compared to the HC-sEV group (Supplementary Fig. 17f,g). Notably, although the frequency of co-activated cells displayed a decreasing trend in the HC-sEV group, both the proportion and magnitude of this decrease were more pronounced in the PND-sEV group (Supplementary Fig. 17h). These neurons also exhibited the same “high-frequency, low-amplitude” activation pattern in the fitted dynamic model (Supplementary Fig. 17i). These results demonstrate that PND-associated breastmilk sEVs are sufficient to induce functional deficits in DG neurons, mirroring those caused by direct mGluR8 knockdown, thereby establishing a causal role for PND-sEVs in disrupting the encoding and retrieval of fear memory.

Overexpression of PND-associated miRNAs induces neurodevelopmental deficits in offspring

To determine whether miR-15a-5p, miR-15b-5p, and miR-205-5p are sufficient to recapitulate the effects of PND breastmilk miRNAs, we overexpressed these miRNAs directly within SuM→DG projection neurons. Self-complementary AAV vectors (scAAV-hSyn-miRNA) encoding each miRNA were pooled and injected into the SuM of healthy neonatal pups at P0. These pups were subsequently raised by HC dams throughout lactation and assessed in adulthood (Fig. 7a). Behaviorally, scAAV-hSyn-miRNA-treated pups exhibited significant cognitive deficits in both the CFC and MWM tests (Fig. 7b; Supplementary Fig. 18a). At the cellular level, miRNA overexpression impaired dendritic elongation and reduced branching complexity in newborn neurons (Fig. 7c,d; Supplementary Fig. 18b). RV-EGFP-based labeling of newborn neurons (Supplementary Fig. 18c), combined with electrophysiological recordings, revealed a significant elevation in mEPSC frequency in treated pups (Fig. 7e-g). These results demonstrate that direct overexpression of these miRNAs in the SuM is sufficient to recapitulate the neurodevelopmental abnormalities observed in pups exposed to PND breastmilk miRNAs.

Buffering the activity of miR-15a-5p, miR-15b-5p, and miR-205-5p mitigates the adverse effects of maladaptive breastfeeding on offspring

To determine whether buffering the activity of miR-15a-5p, miR-15b-5p, and miR-205-5p could counteract their aberrant elevation and alleviate neurodevelopmental deficits in offspring fostered by CUMS-induced dams, we constructed three self-complementary AAVs (scAAVs) expressing miRNA sponges targeting these miRNAs (scAAV-hSyn-SP(miRNA)). These scAAVs were pooled in equal proportions and administered into the SuM of healthy pups on P0. These pups were subsequently fostered by CUMS-induced dams throughout the lactation period and evaluated in adulthood (Fig. 7h). In behavioral tests, scAAV-hSyn-SP(miRNA)-treated pups raised by CUMS dams outperformed those receiving control scAAV in both the CFC and MWM tests, reaching performance levels similar to those of control pups raised by HC dams (Fig. 7i; Supplementary Fig. 18d). Furthermore, scAAV-hSyn-SP(miRNA) treatment robustly increased the number of DCX⁺ newborn neurons in the hippocampal DG (Fig. 7j; Supplementary Fig. 18e) and significantly improved the dendritic length and branching complexity of these neurons (Fig. 7k,l; Supplementary Fig. 18f). When newborn neurons were labeled using RV-EGFP and analyzed by electrophysiological recording (Supplementary Fig. 18g), control scAAV-treated pups nursed by CUMS-induced dams exhibited a marked increase in mEPSC frequency, whereas this

electrophysiological alteration was reversed by injection of scAAV-hSyn-SP(miRNA) (Fig. 7m-o). These results demonstrate that sponge-mediated buffering of miR-15a-5p, miR-15b-5p, and miR-205-5p effectively blocks the adverse effects of maladaptive breastfeeding on offspring neurodevelopment.

Discussion

The mechanisms by which maternal PND affects offspring are complex, involving various factors such as diminished maternal care and breastmilk nutritional imbalances^{4,99,100}. To ensure the robustness of our conclusions, we adopted a systematic experimental strategy to minimize confounding influences: First, using a cross-fostering paradigm, we isolated the effects of postnatal maternal care and breastfeeding from genetic and in utero influences. Second, to dissociate breastmilk components from maternal behavior, we directly modulated miRNA levels in breastmilk or administered PND-derived sEVs to pups raised by HC mothers, thereby establishing a causal role for sEV-encapsulated miRNAs in the intergenerational transmission of maternal psychological traits. Third, using transgenic mice expressing EGFP-tagged sEVs, we visually confirmed the transport of mammary gland-derived sEVs to the offspring hippocampus via breastfeeding. Fourth, through mammary gland-specific overexpression of a human-specific miRNA, we demonstrated that breastmilk miRNAs are not only delivered to but also functionally regulate gene expression in the offspring brain. Fifth, using a cKO approach targeting Rab27a to specifically suppress sEV secretion in the mammary gland of PND dams, we observed significant neurodevelopmental alterations in offspring, thereby confirming sEV-mediated miRNA transfer as a critical mechanism governing offspring phenotypes. Finally, by overexpressing specific miRNAs in the offspring brain or using miRNA sponges to sequester miRNA activity, we established a direct link between altered miRNA levels and the detrimental effects of PND-associated breastfeeding. Collectively, these lines of evidence demonstrate that maternal PND conveys pathological traits to offspring through breastmilk sEVs and their miRNA cargo. Specifically, abnormally elevated miRNAs in the breastmilk of PND dams downregulate mGluR8 expression in offspring's SuM→DG circuit during lactation. We propose that impaired presynaptic mGluR8 signaling leads to excessive glutamatergic transmission, which triggers neuronal hyperexcitability, calcium overload, and excitotoxicity during early development. This excitotoxic cell death may explain the reduction in c-Fos⁺ cells observed in the DG at P21, whereas the disrupted integration of newborn neurons into learning- and memory-related circuits likely underlies the cognitive and emotional deficits manifest in adulthood by P56.

The biological significance of this study is as follows: (1) Identification of a novel mother-infant communication pathway. While breastmilk has traditionally been regarded as a source of nutritional and immunological support^{101,102}, our findings expand this perspective by demonstrating that sEV-encapsulated miRNAs in breastmilk mediate epigenetic programming of offspring neurodevelopment. This reveals a previously unrecognized route through which maternal psychological states or environmental exposures may be transmitted to the infant brain, with potential long-term physiological and neurological consequences. (2) Discovery of a miRNA-based mechanism of maternal-offspring inheritance. Extracellular miRNAs are remarkably stable and facilitate intercellular and interspecies communication³⁸. Recently, sperm-derived miRNAs have been shown to transmit paternal environmental signals across generations⁴². Here, we establish that stress-responsive breastmilk miRNAs act as signal carriers, forming a biological interface that

transduces maternal experience into phenotypic outcomes in the offspring. (3) Emphasizing the critical role of maternal mental health in shaping offspring development. Approximately 10-20% of mothers worldwide experience PND². Although breastfeeding is widely recognized for supporting neurocognitive development, this study indicates that PND-associated breastfeeding may exert a double-edged effect on offspring health. Elucidating the molecular mechanisms linking maternal PND to offspring neurological outcomes holds considerable public health value and underscores the urgent need for targeted maternal mental health interventions during lactation to prevent transgenerational neurodevelopmental disorders.

In summary, this study establishes maternal mental health as a critical factor defining breastmilk sEV composition and, consequently, offspring neurodevelopment and cognition. The “maternal mood – breastmilk sEV miRNA – offspring neurodevelopment” axis emerges as a key pathway through which early-life environment exposures influence long-term brain health, providing a meaningful intervention target to promote the well-being of both mother and child.

Materials and Methods

Human subjects

Breastmilk miRNA expression profiles were analyzed in an independent cohort comprising 91 postpartum mothers, including 40 diagnosed with PND (age: 30.37 ± 0.70 , gestational time: 38.50 ± 0.33 weeks) and 51 HC mothers (age: 30.84 ± 0.79 years, gestational time: 38.38 ± 0.38 weeks). None of them had given birth before. The demographic information of HC mothers and PND patients is provided in Table S2. The nutrient levels in breastmilk from HC mothers and PND patients are summarized in Table S3. Fresh breastmilk samples were collected using an electric breast pump.

The diagnosis of PND was based on the Edinburgh Postnatal Depression Scale (EPDS). Inclusion criteria were: age ≥ 18 years, singleton pregnancy, vaginal delivery, clinical diagnosis of depression. Exclusion criteria included: assisted reproduction, conversion from vaginal delivery to cesarean section, requirement of pharmacological treatment for PND, presence of other pregnancy complications or comorbidities (including cardiac, malignant tumor, respiratory failure, or renal diseases), and inability to comply with study procedures and follow-up requirements. This study was approved by the Ethics Review Committee of Nanjing Maternity and Child Health Care Hospital. Informed consent was obtained from all participants at the time of enrollment, in accordance with ethical standards.

Mouse models

All animal experiments were approved by the Animal Ethical and Welfare Committee of Nanjing University and conducted in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. Wild-type C57BL/6J mice, C57BL/6JGpt-Rab27a^{em1CfloX}/Gpt mice, and CAG-Cre mice (on a C57BL/6J background) were obtained from GemPharmatech (Nanjing, China). Rosa26-CD63^{Flag}EGFP-mCherry knock-in mice were generated via CRISPR/Cas9-mediated gene editing. Mice were group-housed (3–5 per cage) under a 12-hour light/dark cycle (lights on at 8:00 AM, off at 8:00 PM) with ad libitum access to food and water.

In this study, female C57BL/6J mice were used to establish maternal depression models. To evaluate the effects of maternal depression on offspring, the behavioral performances of both male and female offspring were analyzed. However, all other experiments, including neuronal morphology assessments, immunofluorescence staining, electrophysiological recordings, transcriptome sequencing, and single-cell sequencing, were conducted exclusively on male offspring.

Cross-fostering protocol

A cross-fostering protocol was conducted 24 hours after birth. In this design, pups born to CUMS/CSDS-exposed dams were fostered by HC dams, and reciprocally, pups from HC dams were fostered by CUMS/CSDS-exposed dams. Additionally, to control for the fostering procedure itself, pups from CUMS/CSDS-exposed dams were fostered by other CUMS/CSDS-exposed dams, while pups from HC dams were fostered by other HC dams. All offspring remained with their assigned foster mothers from P2 until weaning. Each animal was designated using a “birth mother→foster mother” nomenclature to specify its rearing condition.

Chronic unpredictable mild stress paradigm

The chronic unpredictable mild stress (CUMS) paradigm was applied to female C57BL/6J mice (6 weeks old) for five weeks prior to conception⁴². Each day, the mice were exposed to a random combination of three distinct stressors selected from a pool that included wet bedding, food deprivation, restraint, stroboscopic illumination (150 flashes per minute), light-dark cycle reversal, cage tilting (45°), and loud noise (90–105 dB). The timing and sequence of these stressors were varied daily to prevent habituation and ensure unpredictability.

After confirmed conception, the mice continued to receive a milder stress regimen throughout the three-week gestation period, involving stressors such as light-dark cycle reversal and cage tilting (45°). This protocol ensured the development of a stable depression-like phenotype upon completion of the stress induction protocol.

Chronic social defeat stress paradigm

The chronic social defeat stress (CSDS) model was established based on a resident-intruder paradigm, following previously described methods⁴³. Briefly, female CD-1 mice were housed individually for 7 days to establish territorial aggression, while female C57BL/6J mice were group-housed (4–5 per cage) for acclimation. CD-1 mice were screened for aggressive behavior by introducing a C57BL/6J intruder into their cage for 3 minutes. Those initiating more than five attacks with a latency under 1 minute and displaying this aggressive phenotype on at least two out of three screening days were selected as aggressors for the CSDS model.

Female C57BL/6J mice (8 weeks old) underwent a 10-day CSDS protocol. Each day, the mice were placed into the cage of a novel CD-1 aggressor for 5–10 minutes to experience direct social defeat. After the defeat session, the C57BL/6J mice were separated from the aggressor using a perforated transparent divider, allowing for sensory exposure over a 24-hour period. The aggressor was replaced daily to prevent habituation. Upon completion of the 10-day protocol, a depression-like phenotype was consistently observed in the female C57BL/6J mice.

General behavioral testing procedures

Prior to testing, all mice were handled by investigators for three consecutive days to reduce stress from human contact. On each test day, animals were acclimated to the testing room for at least 30 minutes before the start of the procedure. All behavioral assessments were conducted between 10:00 AM and 5:00 PM to control for circadian influences. To prevent bias, investigators performing the tests and analyzing the data were blinded to the group assignments throughout the study.

Morris water maze test

The Morris water maze (MWM) test was conducted using a circular pool filled with water (50 cm deep, $23 \pm 2^\circ\text{C}$). A round transparent platform (10 cm in diameter) was submerged 1 cm below the water surface in the center of the target quadrant. Distinct visual cues were placed around the pool to facilitate spatial learning. White dye was added to the water to enhance contrast for tracking the black-colored mice.

During the training phase, mice underwent four trials per day for five consecutive days, with each trial starting from a different point. During each trial, mice were placed gently into the water and allowed to explore for 1 minute to locate the submerged platform. If a mouse failed to find the platform, it was guided to the rescue platform. After resting on the platform for 10 seconds, the mouse was retrained from a different starting position using the same procedure. The latency for each mouse to find the platform (defined as at least 3 seconds of continuous stay) was recorded for each trial. On day 6, a probe trial was conducted with the platform removed. The mice were released from the opposite quadrant and allowed to explore freely for 1 minute. The number of platform crossings, time spent in the target quadrant, and latency to first reach the former platform location were recorded. Mice exhibiting an average swimming speed below 10 cm/s were excluded from analysis to control for potential motor deficits.

To ensure experimental rigor and reproducibility, environmental conditions, including water temperature, lighting, humidity, and noise levels, were maintained constant throughout the study. All procedures were performed by the same investigators to minimize handling-related variability.

Contextual fear conditioning test

In contextual fear conditioning (CFC) test, mice were trained to associate the fear conditioning chamber with an aversive stimulus (a mild foot shock, the unconditioned stimulus) to assess contextual fear conditioning. They were also conditioned to associate tone cues (the conditioned stimulus) with mild foot shocks to evaluate cued fear conditioning.

During the training phase, mice were placed in a shock chamber with a stainless-steel rod floor inside a sound-attenuating box. After a 2-minute acclimation period, the mice underwent four trials, each consisting of a 30-second, 75 dB tone followed by a 1-second, 0.75 mA foot shock, with a 60-second inter-trial interval. Following the final shock, the mice remained in the chamber for an additional 60 seconds before being returned to their home cages. Twenty-four hours post-training, contextual fear conditioning was assessed by placing the mice back into the same chamber for 3 minutes, during which their freezing behavior was recorded. Cued fear conditioning was evaluated by placing the mice in a novel, modified context and presenting the tone; freezing behavior in

response to the tone was recorded.

Freezing behavior was scored using automated motion detection software (FreezeFrame and FreezeView). On Day 1, baseline freezing was calculated from the first 120 seconds after introducing the mice to the chamber. Freezing behavior during trials 1-4 was measured as the percentage of time spent freezing during each 30-second tone presentation. On Day 2, contextual fear conditioning was analyzed by recording freezing behavior over the entire 3-minute session.

Open field test

The open field test (OFT) was performed in a square arena ($60 \times 60 \times 60$ cm). Each mouse was gently placed in the center of the arena and allowed to explore freely for 10 minutes. An overhead camera tracked the animal's movement and position. The total distance traveled and the time spent in the central zone were automatically quantified using tracking software, serving as indices of general locomotor activity and anxiety-like behavior, respectively. The arena was thoroughly cleaned with 75% ethanol between trials to eliminate residual olfactory cues.

Elevated plus maze test

The elevated plus maze (EPM) test was conducted using a plus-shaped apparatus elevated 50 cm above the floor. The maze consisted of two open arms and two enclosed arms of equal dimensions, extending from a central square platform. Each mouse was placed on the central platform facing an open arm and allowed to explore freely for 5 minutes. Behavior was recorded via an overhead camera, and the number of entries into the open and closed arms and the time spent in each arm were analyzed using tracking software. The frequency of open-arm entries and the duration spent in the open arms were quantified as indicators of anxiety-like behavior.

Treadmill test

Exercise capacity was assessed using a treadmill test (TT). Prior to the formal test, mice completed a two-day acclimation protocol. On day 1, they ran at 10 m/min for 5 minutes on a flat surface (0° incline) without shocks. On day 2, they ran at 10 m/min for 5 minutes at a 5° incline with shocks. Following acclimatization, the mice ran on a treadmill starting at 10 m/min with a 5° incline. The speed was gradually increased by 1 m/min until reaching 22 m/min. Exhaustion was defined as the point at which a mouse received more than five shocks (0.4 mA) within a 5-second period. The total running time and distance covered until exhaustion were recorded as primary measures of exercise capacity.

Three-chamber social interaction test

The testing apparatus consisted of three equal-sized rectangular chambers ($20 \times 40 \times 22$ cm). C57BL/6 mice were allowed access to each chamber via doors ($12 \times 6 \times 0.5$ cm) located between adjacent chambers. During the habituation phase, the test mouse was placed in the central chamber and allowed to freely explore the entire test arena, which contained an empty wire cage in each of the side chambers, for 10 minutes. To control for innate side preferences, the time spent in the left and right chambers was recorded for each mouse. Mice showing a significant preference for one side were excluded from subsequent testing.

Following habituation, the test mouse was temporarily restrained in the center chamber. An unfamiliar, age- and sex-matched C57BL/6 mouse (Stranger 1) was placed inside one of the wire cages. The test mouse was then given 10 minutes to freely explore all three chambers. After the social approach test, the test mouse was again confined to the central chamber for a brief interval. A second unfamiliar mouse (Stranger 2) was introduced into the previously empty wire cage. The test mouse was then allowed to explore the entire arena for another 10 minutes. The assignment of Stranger 1 and Stranger 2 to the side chambers was randomized.

Sniffing time directed toward each cage was manually quantified by trained observers blinded to the experimental conditions. A social approach preference index was calculated as the percentage of: $(\text{Time sniffing Stranger 1} - \text{Time sniffing empty cage}) / (\text{Time sniffing Stranger 1} + \text{Time sniffing empty cage})$. A social novelty preference index was calculated as the percentage of: $(\text{Time sniffing Stranger 2} - \text{Time sniffing Stranger 1}) / (\text{Time sniffing Stranger 2} + \text{Time sniffing Stranger 1})$.

Forced swim test

The forced swim test (FST), a predictive model of depressive-like behavior, was conducted as previously described⁴². Each mouse was individually placed in a vertical Plexiglas cylinder (25 cm height $\times$ 18 cm diameter) filled with water to a depth of 15 cm ($25 \pm 1^\circ\text{C}$). The mice were videotaped for 6 minutes, and the total immobility time during the last 5 minutes was quantified via video analysis using the TopView Animal Behavior Analyzing System (CleverSys Inc., Reston, VA). The water was replaced after each trial to prevent olfactory cues. All behavioral assessments were performed by investigators blinded to the experimental group assignments.

Tail suspension test

The tail suspension test (TST) was performed in a chamber (60 $\times$ 60 $\times$ 60 cm). Mice were first acclimated to the testing room for 10–15 minutes. Each mouse was then suspended by the tail tip (approximately 1–2 cm from the end) using a hook for a 6-minute session. During the suspension, mouse behaviors were closely monitored and recorded, focusing on patterns of struggling, swinging, and immobility. The total immobility time during the last 5 minutes was quantified as a measure of depression-like behavior. All mice were handled carefully before and after testing to prevent injury.

Novelty-suppressed feeding test

The novelty-suppressed feeding test (NSFT) was conducted in an open-field arena (60 $\times$ 60 $\times$ 60 cm). Approximately 18–24 hours before testing, mice were individually housed and food-deprived, with water available ad libitum. At the start of the experiment, a high-fat, high-sugar food pellet unfamiliar to the mice was placed in the center of the testing arena. Each mouse was introduced into a corner of the arena, and the latency to initiate feeding (defined as the time from placement to the first bite of the food pellet) was recorded during a 6-minute session. The feeding latency during the final 5 minutes was compared between groups, with shorter latencies indicating reduced depression-like behavior.

Chronic variable stress protocol

To assess the susceptibility of mice to stress and depression-like phenotypes, a chronic variable stress (CVS) protocol was conducted over 2 weeks, as previously described¹⁰³. The protocol

consisted of seven different mild stressors applied in a randomized order daily, with the sequence varied each week to prevent habituation. The selected stressors were designed to be non-painful and to not interfere with food or water intake. These stressors included constant light exposure, fox odor exposure (1:5000, 2,4,5-trimethylthiazole; Sigma-Aldrich, St. Louis, MO), novel object exposure, restraint in a 50-mL conical tube, frequent cage changes, exposure to 100 dB white noise (Sleep Machine; Brookstone, Merrimack, NH), and overnight exposure to saturated bedding. Following the CVS regimen, mice from the control and experimental groups were evaluated for behavioral performance.

Maternal behavior observation

Maternal behavior was systematically observed in postpartum dams following an established protocol¹⁰⁴. Each dam was observed 4 times daily for 75 minutes per session between days 3 and 15 postpartum, including one observation during the dark phase (8:00 AM) and three during the light phase (9:15 AM, 1:30 PM, and 4:30 PM). The first two sessions occurred before pup separation (starting at 10:30 AM), while the latter two took place after separation, reflecting the natural pattern of increased nursing activity during the light phase. Behavior was sampled every 3 minutes using instantaneous recording (approximately 3 seconds per observation), yielding 25 records per session and 100 records per day. The following behaviors were recorded: (1) licking and grooming any pups, (2) nursing pups in passive and blanket postures, (3) nursing pups in arched-back nursing posture, (4) total nursing (i.e. nursing in active, passive, and arched-back posture), (5) nest building, (6) self-grooming, (7) staying in the nest in contact with the pups (in nest), (8) resting with no contact with the pups (resting), and (9) eating or drinking. These behavior categories were non-mutually exclusive. Data are expressed as the mean frequency of occurrence per behavior across the four daily sessions.

sEV isolation and characterization

The protocol for sEV isolation was optimized from established methods⁶⁸⁻⁷⁰. Briefly, raw breastmilk was sequentially centrifuged: first at $500 \times g$ for 10 min to remove cells and large debris, then twice at $3,000 \times g$ for 10 min each to pellet residual debris and the upper cream layer. The resulting supernatant was further centrifuged at $5,000 \times g$ for 30 min and $10,000 \times g$ for 30 min, and the final supernatant was stored at -80°C until further processing. For sEV isolation, the thawed supernatant was centrifuged at $17,000 \times g$ for 10 min to remove protein aggregates, followed by ultracentrifugation at $120,000 \times g$ for 90 min to pellet crude sEVs. The pellet was resuspended in sterile PBS and further purified by size-exclusion chromatography (SEC). A 1 mL sample was loaded onto a pre-equilibrated 20 mL Econo-Pac column (BioRad, Cat. #7321010) packed with CL-6B resin (Bestchrom, Cat. #AG0044). A total of 26 fractions were collected by eluting with 1 mL sterile PBS per fraction. Protein concentration and particle number in each fraction were quantified using the BCA assay and nanoparticle tracking analysis (NTA), respectively.

The BCA assay was performed according to the manufacturer's instructions (Thermo Fisher Scientific, Cat# 23225). Protein concentrations were determined based on a standard curve. For NTA, measurements were conducted on a Nanosight NS3000 system (Malvern Panalytical, Shanghai, China). Each fraction was diluted at least 20-fold in sterile PBS to achieve a final particle concentration between 2×10^8 and 8×10^8 particles/mL. Three random fields of view per sample

were recorded for 60 seconds each. Videos were analyzed using Nanosight NTA 3.4 software to determine the average particle concentration and size distribution. Due to the limited detection sensitivity of the Nanosight instrument for particles below 60 nm¹⁰⁵, only particles larger than 60 nm were counted.

To assess the purity of the sEV fractions, immunoblotting was performed for canonical sEV markers and the breastmilk whey protein lactoferrin^{68,69}. SEC fractions showing abundant sEV markers but low levels of lactoferrin were considered indicative of relatively pure breastmilk sEVs.

The morphological features of the sEV fractions (SEC fractions 6–8) were examined by transmission electron microscopy (TEM). An aliquot of the sample was applied to a copper grid and allowed to adsorb for one minute. Excess liquid was removed using filter paper, and the grid was negatively stained with uranyl acetate for one minute, after which the excess stain was blotted off. After air-drying at room temperature, the grid was imaged under a transmission electron microscope operating at an accelerating voltage of 80 kV.

As a cross-validation method, nano-flow cytometry (NanoFCM) was employed. Given that EVs possess a phospholipid bilayer membrane that can be disrupted by detergents such as Triton X-100, a decrease in particle count after Triton X-100 treatment reflects the EV purity of the sample⁷². Samples were mixed with Triton X-100 to a final concentration of 1% (v/v) and incubated on ice for 1 hour, while controls were treated with an equal volume of sterile PBS. NanoFCM analysis was performed under the following instrument settings: laser, 10 mW, 488 nm; SS Decay, 10%; sampling pressure, 0.8 kPa; sample period, 100 μ s; and recording time, 1 min.

Administration of sEVs *in vitro* and *in vivo*

For *in vitro* studies, sEVs were administered to cultured cells at a concentration of 2 μ g (by total protein) per 1×10^5 cells.

For *in vivo* administration, normal pups received daily intragastric injections of PND-sEVs or HC-sEVs throughout the lactation period to assess phenotypic changes. The sEV dosage was calibrated to the pups' developmental stage, based on the progressively increasing daily breastmilk intake: Day 1: ~0.1 mL; Days 2–3: ~0.2 mL; Days 4–5: ~0.4 mL; Days 6–7: ~0.6 mL; Days 8–10: ~0.8 mL; and Days 11–21: ~1 mL. Specifically, the daily sEV dose was isolated from a volume of breastmilk equivalent to this age-specific intake, ensuring that the administered PND-sEVs mirrored physiological exposure to breastmilk-derived sEVs. For example, P0 pups received sEVs extracted from 0.1 mL of HC or PND breastmilk. During the first postnatal week, sEVs were delivered via gastric injection using an insulin syringe due to underdeveloped esophageal function; from the second week onward, sEVs were administered by gavage. Following each daily treatment, pups were returned to their mothers. Upon reaching adulthood, the offspring underwent behavioral tests to assess learning and memory, alongside molecular analyses to evaluate the morphology and function of newborn neurons in the DG. Behavioral tests were conducted on both male and female offspring, whereas all other experiments were performed exclusively on males.

To inhibit sEV secretion in breastmilk, lactating dams received intramammary injections of

AAV2/9-MMTV-EGFP-IRES-Cre at a dose of 1×10^{11} vg per mammary gland.

Culture of primary hippocampal neurons

Hippocampal tissues were dissected from mouse embryos at embryonic day 14.5 (E14.5) and digested with 0.25% trypsin at 37 °C for 10 minutes. The digestion was terminated by adding DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Cat. #10099141C). After washing with Dulbecco's PBS, the hippocampal tissues were dissociated into single cells using glass Pasteur pipettes. The resulting cells were filtered through a 40 μ m strainer and plated onto poly-D-lysine-coated coverslips in DMEM/F12 medium containing 10% FBS. After 2 hours of attachment, the medium was completely replaced with Neurobasal medium supplemented with 2% B27 and 1% GlutaMax. Thereafter, the medium was changed to half the volume every 3 days.

For AAV transduction, cultured cells were infected on day 4 with AAV particles expressing miRNAs under the hSyn promoter: AAV-hSyn-miR-15a-5p (1.02×10^{13} vg/mL, TaiTool Bioscience, Shanghai, China), AAV-hSyn-miR-15b-5p (1.65×10^{13} vg/mL), or AAV-hSyn-miR-205-5p (1.71×10^{13} vg/mL). Polybrene was added to the culture medium at a final concentration of 6 μ g/mL to enhance transduction efficiency. On day 9, cells were harvested for RNA extraction and Western blot analysis, or fixed for immunostaining.

The production of AAV begins with the cloning of the desired sequence into a plasmid containing inverted terminal repeats (ITRs). This recombinant plasmid is co-transfected into HEK293T cells together with the packaging plasmid (pAAV-RC) and the helper plasmid (pHelper). Following a 2-3 days incubation period to allow for viral assembly, the cells are harvested and lysed to release the crude viral particles. The lysate undergoes concentration and purification through ultracentrifugation or chromatography to obtain a high-titer, high-purity viral preparation suitable for *in vivo* applications. The genomic titer of the final AAV product is accurately determined using quantitative PCR.

Stereotaxic viral injection in adult and neonatal mice

scAAVs and retroviruses were delivered into the DG and SuM of adult mice using stereotaxic surgery. Briefly, mice were anesthetized with isoflurane and secured in a stereotaxic frame. Ophthalmic ointment was applied to prevent corneal drying, and the surgical site was shaved and disinfected with alcohol before skin incision. Viral suspensions were bilaterally infused into the target brain regions at a constant rate of 0.1 μ L/min using a microsyringe pump. The following coordinates relative to bregma were used: for the DG, anteroposterior (AP) -2.00 mm, mediolateral (ML) ± 1.40 mm, dorsoventral (DV) -2.00 mm; for the SuM, AP -2.4 mm, ML ± 0.6 mm, DV -4.85 mm. The injection pipette remained in place for 5 minutes post-infusion to allow for proper diffusion before being slowly withdrawn. Following surgery, mice recovered in a warm, clean environment before returning to their home cages.

For the injection of scAAVs and retroviruses into the target brain regions of pups on P0-P5, the skull is soft, but the sagittal and lambdoid sutures remain present and intersect at the lambda point. As described before, the pups were anesthetized and the head was secured by ear bars with rubber tips. Following the alignment of the bregma and lambda point, a small craniotomy was performed above

the target region using a 26G needle. The virus was then delivered into the brain at a rate of 30 nL/min at the following stereotaxic coordinates: DG (AP: -1.2 mm from bregma; ML: ± 0.8 mm; DV: -1.5 mm); SuM (AP: 1.8 mm anterior to lambda point; ML: ± 0.3 mm; DV: -4.2 mm).

Retrovirus, scAAV, and AAV vectors were produced by Taitool Bioscience (Shanghai, China), except for rAAV-hSyn-mCherry, which was supplied by BrainVTA (Wuhan, China). Retroviruses were generated by co-transfecting HEK293T cells with RV-EGFP or RV-tdTomato plasmid, pGag-pol packaging plasmid, and pVSV-G envelope plasmid using Lipofectamine 2000. Virus-containing medium was collected after 48 hours, filtered through a 0.45- μ m membrane, and concentrated by ultracentrifugation. scAAV vectors were produced by triple transfection of HEK293T cells with scAAV vector plasmid, adenoviral helper plasmid, and serotype helper plasmid. Cells were harvested after 72 hours, lysed, and vectors were purified via iodixanol gradient ultracentrifugation.

The control retrovirus vector expressed a shRNA sequence targeting firefly luciferase. The control scAAV vector was an empty vector encoding a scrambled RNA sequence and fluorescent marker. The control AAV vector was an empty vector encoding a scrambled RNA sequence and fluorescent marker.

Intraductal injection for mammary gland transduction

AAV vectors, including AAV-CMV-has-miR-302f, AAV-CMV-NC, AAV-MMTV-miRNA, AAV-MMTV-NC, AAV-MMTV-SP(miRNA), and AAV-MMTV-SP(NC), were delivered into the mammary glands of mice via intraductal injection at a dose of approximately 1×10^{11} vg per mammary gland. The procedure was performed according to established protocols^{64,65}. Briefly, mice were placed in a supine position after anesthesia, and abdominal hair surrounding the nipple was removed to fully expose the teats. The intraductal injection was conducted under a stereomicroscope for precise visualization. The target nipple was carefully identified, and any overlying keratinized skin was gently cleared using fine forceps to prevent duct blockage. Prior to injection, a microsyringe fitted with a 33-G metal needle was loaded with 10–20 μ L of the viral suspension. To compensate for potential minor fluid loss during the procedure, the actual loaded volume was increased to 11 μ L or 21 μ L depending on the gland position (21 μ L for the 4th pair and 11 μ L for the others). During injection, the duct was gently lifted and stabilized with fine forceps. The viral solution was infused slowly at a rate of approximately 40 μ L/min to avoid structural damage to the duct lumen caused by rapid fluid flow. All AAV vectors were produced by Taitool Bioscience (Shanghai, China), using the production methods described in the previous section.

Western blotting

To evaluate mGluR8 protein levels in hippocampal tissues, mice were anesthetized with isoflurane, and their brains were rapidly extracted and sectioned into 300 μ m acute slices in cold PBS. The hippocampal tissue was meticulously dissected and homogenized in RIPA lysis buffer (Yeasten, Cat. #20101ES60) supplemented with EDTA-free protease inhibitor cocktail (Roche, Cat. #4693132001) and phosphatase inhibitors (Roche, Cat. #4906837001). Homogenates were incubated on ice for 30 min to facilitate complete lysis, followed by centrifugation at $12,000 \times g$ for 15 min at 4 °C. The resulting supernatant was collected as the protein extract. For cultured cells, monolayers were washed with cold PBS and lysed on ice using the same RIPA buffer system for 10–20 min. Lysates

were homogenized and centrifuged at $12,000 \times g$ for 15 min at 4 °C to remove insoluble materials. Protein concentrations in all supernatants were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Cat. #23227).

Samples were mixed with 5 × SDS loading buffer and boiled for 10 minutes. Extracted proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes (Millipore, Cat. #IPVH00010). The membranes were blocked at room temperature for 1 hour in TBS buffer containing 0.1% Tween-20 (TBST) and 5% nonfat milk. Overnight incubation at 4 °C was performed with the following primary antibodies: mGluR8 (1:1000, Abcam, ab53094), PSD95 (1:1000, Abcam, ab2723), and β -tubulin (1:3000, Yeasen, 30302ES20). After washing the membranes three times in TBST, they were incubated at room temperature for 1 hour with HRP-conjugated secondary antibodies. The membranes were then treated with ECL substrate (Thermo Fisher Scientific, Cat. #34580) according to the manufacturer's instructions, and protein levels were visualized using the Tanon 5200 multi-detection system (Tanon Science & Technology, Shanghai, China). Data were quantified using ImageJ software, and relative protein levels were normalized to β -tubulin. All experiments were performed in triplicate. The key resource is listed in the Table S1.

BrdU labeling and immunohistochemistry

BrdU (Sigma-Aldrich, Cat. #B5002) was dissolved in saline and administered to adult mice via intraperitoneal injection at a dosage of 100 mg/kg. To assess neural stem cell (NSC) proliferation, mice received two BrdU injections at 12-hour intervals on P20 and P21, and were perfused intracardially 24 hours after the final injection. To examine NSC survival and neuronal differentiation, mice were injected with BrdU three times at 24-hour intervals on P19, P20, and P21, followed by intracardiac perfusion on P56 after the last injection.

Briefly, after deep anesthesia, mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were extracted, fixed overnight in PFA, and then transferred to a 30% (w/v) sucrose solution for storage at 4 °C. Subsequently, 40 μ m coronal sections were cut every 240 μ m from the brain tissue using a cryostat (Leica CM, model 1950), covering the hippocampal region (from -1.06 mm to -3.08 mm relative to bregma). For BrdU immunohistochemistry, DNA denaturation was performed before applying the primary antibody. Floating sections were rinsed three times (10 minutes each) in PBS and then exposed to citrate buffer (10 mM sodium citrate, pH 6.0) at 80 °C for 30 minutes. After cooling to room temperature, the sections were incubated with 2 M HCl for 15 minutes at 37 °C, followed by neutralization in 0.1 M boric acid (pH 8.5) for 15 minutes. The sections were rinsed again three times in PBS and incubated with blocking solution (PBS containing 5% BSA and 1% Triton X-100) at room temperature for 1 hour. For cultured hippocampal neurons, cells were fixed with ice-cold 4% PFA solution. Sections or cells were permeabilized with 0.2% Triton X-100 and blocked with 5% normal donkey serum. Following blocking, sections or cells were incubated overnight at 4 °C with the following primary antibodies: Nestin (1:200, Millipore, MAB353), BrdU (1:1000, Millipore, MAB4072; 1:1000, Abcam, ab6326), PCNA (1:500, Cell Signaling Technology, 13110), MCM2 (1:500, BD Biosciences, 610700), NeuN (1:500, Cell Signaling Technology, 24307), and DCX (1:500, Cell Signaling Technology, 91954). The next day, sections were rinsed and incubated with Alexa 488-, 546-, or 647-conjugated secondary antibodies (1:1000, Invitrogen) for 1 hour. After secondary antibody incubation, sections

were stained with DAPI (1 μ L/mL, Sigma) for 10 minutes and rinsed three times in PBS. After staining, sections were mounted, coverslipped, and stored at 4 °C in the dark until analysis. Images were acquired using a Leica TCS SP8-MaiTai MP confocal microscope and analyzed using ImageJ and Imaris software (Bitplane). For dendritic branching analysis, the dendritic processes of each RV-EGFP-labeled newborn neuron were reconstructed in 3D from Z-series confocal images acquired at 2 μ m intervals. The key resource is listed in the Table S1.

c-Fos immunohistochemistry

Two hours after the final lactation session, offspring mice were deeply anesthetized with 1% sodium pentobarbital (100 mg/kg body weight) and transcardially perfused with 50 mL of 0.1 M phosphate-buffered saline (PBS), followed by 50 mL of 4% paraformaldehyde (PFA) in PBS. The brains were harvested, post-fixed overnight in the same fixative at 4°C, and cryoprotected in 30% sucrose until saturated. Coronal sections (40 μ m thickness) were prepared using a Leica CM1950 cryostat and organized into three interleaved sets.

Immunostaining was performed using a rabbit anti-c-Fos primary antibody (1:1000; Abcam) followed by a goat anti-rabbit IgG Alexa Fluor 488 secondary antibody (1:1000; Thermo Fisher Scientific). After staining, sections were counterstained with DAPI and imaged on a Leica TCS SP8-MaiTai MP confocal microscope. c-Fos⁺ cells were quantified by an investigator blinded to the experimental groups. For each brain region, the number of c-Fos⁺ cells was averaged across all slices within an interleaved set and normalized to the mean value of the control group for each animal.

Quantification of fluorescent intensity

Fluorescent images were converted to 8-bit grayscale and analyzed using ImageJ software¹⁰⁶. The total gray value within a defined region of interest was measured, and the fluorescent intensity was calculated as the average gray value per unit area.

Sholl analysis

Neuronal projections were semi-automatically traced using the Simple Neurite Tracer plugin in ImageJ. The total dendritic length of each RV-EGFP-infected newborn neuron in the granule cell layer was quantified. For morphological evaluation via Sholl analysis, a series of concentric circles were drawn at 20 μ m intervals from the soma, and the number of dendrites crossing each circle was counted. All quantifications were performed by researchers blinded to the experimental conditions.

miRNA target prediction

miRNA target prediction was performed using the multiMiR R package, which integrates multiple databases of both predicted and experimentally validated miRNA-target interactions¹⁰⁷. To enhance the prediction reliability, only target genes identified by at least two independent databases were selected for further analysis. The putative binding sites between the selected miRNAs and their target genes were then visualized to facilitate subsequent validation.

Luciferase reporter assay

A luciferase reporter assay was conducted to validate the direct targeting of mGluR8 by miR-15a-5p, miR-15b-5p, and miR-205-5p. The wild-type (WT) 3'-untranslated region (3'-UTR) of mGluR8,

which contains the predicted miRNA binding sites, was cloned into the pmirGLO reporter construct. To confirm binding specificity, site-directed mutagenesis was performed on miRNA binding regions, and the resulting mutant (Mut) 3'-UTRs were inserted into the identical luciferase reporter construct.

For transfection experiments, HEK293T cells were maintained in DMEM (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum and plated in 24-well plates. After 24 hours, the cells were co-transfected with 0.5 µg of either the WT or Mut reporter vector and 100 pmol of the corresponding miRNA mimic (GenePharm) or a negative control RNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours post-transfection, luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Yeasen, 11402ES60) on a TECAN Spark microplate reader. Firefly luciferase activity was normalized to the internal Renilla luciferase activity in each well to control for transfection efficiency.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of 23 immune factors in serum samples from mice treated with HC-sEVs or PND-sEVs were quantified using a commercial ELISA kit (4A Biotech Co., Ltd.), strictly in accordance with the manufacturer's instructions.

RNA isolation and quantitative RT-PCR assay

Total RNA was extracted using TRIzol reagent (Invitrogen, #15596018) following previously described methods¹⁰⁸. For mRNA expression analysis, cDNA was synthesized from 1 µg of total RNA using AMV reverse transcriptase (TaKaRa) and oligo dT primers (TaKaRa). The reverse transcription reaction was carried out at 16 °C for 5 minutes, 42 °C for 60 minutes, and 85 °C for 5 minutes. Quantitative RT-PCR was performed on an Applied Biosystems 7300 Sequence Detection System using the SYBR Green PCR kit. The PCR conditions included an initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. All reactions were conducted in triplicate. The primers used for quantitative RT-PCR were as follows: GAPDH: 5'-TCACCACCATGGAGAAGGC-3' (forward) and 5'-GCTAAGCAGTTGGTGGTGCA-3' (reverse). mGluR8: 5'-CAGCACTCACGGTCAGAGT-3' (forward) and 5'-TGAATTCAGCCCTGTAGCAGA-3' (reverse). mRNA expression levels were normalized to GAPDH and calculated using the $\Delta\Delta CT$ method.

For miRNA expression analysis, quantitative RT-PCR was performed using TaqMan miRNA probes according to the manufacturer's instructions. Real-time PCR was conducted with a TaqMan PCR kit on the Applied Biosystems 7300 Sequence Detection System, following a standardized protocol. All reactions were run in triplicate. To calculate the absolute levels of target miRNAs, a series of synthetic miRNA mimics at defined concentrations were reverse-transcribed and amplified alongside the samples. The absolute concentration of miRNAs was determined based on a standard curve. The ID numbers for the TaqMan miRNA probes used are provided in Table S1.

Electrophysiological recording

Four weeks post-retroviral injection, acute brain slices were prepared, and electrophysiological recordings were performed at 32–34 °C. Briefly, mice were deeply anesthetized with isoflurane and transcardially perfused with ice-cold, oxygenated NMDG cutting solution (93 mM NMDG, 93 mM

HCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 30 mM NaHCO₃, 25 mM D-glucose, 20 mM HEPES, 5 mM Na-ascorbate, 2 mM thiourea, 3 mM Na-pyruvate, 10 mM MgSO₄, 0.5 mM CaCl₂; pH 7.35 adjusted with NMDG or HCl). The brain was rapidly removed and immersed in ice-cold NMDG cutting solution. Horizontal hippocampal slices (300 μ m) were cut using a Leica VT1200s vibratome, recovered at 34 °C in NMDG cutting solution for 10-13 minutes, and then maintained at 25 °C in oxygenated artificial cerebrospinal fluid (ACSF: 126 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 10 mM glucose) for 1 hour prior to electrophysiological recordings. Slices were transferred to a recording chamber and superfused with oxygenated ACSF (~3 mL/min). Slices were visualized using infrared optics on an upright microscope equipped with a 40 $\times$ water-immersion lens (BX51WI, Olympus). EGFP-labeled neurons were identified using a fluorescent microscope, and whole-cell patch-clamp recordings were performed.

For miniature excitatory postsynaptic current (mEPSC) recordings, the internal pipette solution contained 135 mM K-gluconate, 5 mM KCl, 0.5 mM CaCl₂, 10 mM HEPES, 2 mM Mg-ATP, 0.1 mM GTP, 5 mM EGTA (300 mOsm, pH adjusted to 7.3 with KOH). To isolate glutamatergic currents, GABAA receptors were blocked with bicuculline (10 μ M), and Na⁺ channels were blocked with tetrodotoxin (1 μ M) during recordings. The holding potential was set at -70 mV for mEPSC recordings.

All signals were acquired using a MultiClamp 700B amplifier (Molecular Devices), filtered at 1 kHz, and sampled at 5 kHz via a Digidata 1440A interface using Clampex 10.2 software (Molecular Devices). Data were accepted if series resistance fluctuated within 15% of initial values (25-35 M Ω). Miniature events were analyzed using MiniAnalysis 6.07 software (Synaptosoft). A 5-minute recording period was used for frequency and amplitude analysis, with peak events detected automatically using a 10 pA amplitude threshold. The number of neurons varied across experiments and is indicated in the corresponding figure legends.

Miniscope calcium imaging

For the training phase on Day 1, neuronal calcium signals were collected and time-synchronized with four consecutive behavioral events: a 30-second pre-stimulation tone period, a 1-second foot shock stimulation, and a 60-second post-stimulation interval. Calcium traces from each identified region of interest (ROI) were synchronized with annotated behavior and Z-scored across all behavioral epochs of the same type. Signals were processed with a Gaussian filter to reduce high-frequency noise while preserving the slow dynamics of calcium transients. Statistically significant calcium transients were identified using a custom Python script as previously described⁹³⁻⁹⁵, which estimated the noise level for each cell. From the aligned signal sequences, the peak frequency and amplitude of calcium events were separately quantified before and after the four stimulation rounds, with a detection threshold set at $Z > 2$ ($p < 0.05$).

For the exploration phase on Day 2, calcium signals were similarly processed by Z-score normalization and Gaussian filtering. The frequency and amplitude of peaks exceeding the same significance threshold ($Z > 2$) were then statistically analyzed.

Cross-day cell registration

Neuronal regions of interest (ROIs) recorded across Day 1 and Day 2 were matched and aligned using the ImageJ software. Based on the previously quantified peak frequency and amplitude of calcium events, cross-day differential analysis was performed to compare the activity of the same set of cells between the two recording sessions.

Exponential decay model fitting and analysis

Based on the hypothesis that calcium overload leads to decreased neuronal firing thresholds and increased firing frequency, we performed the following analyses using previously recorded neuronal amplitude and firing rate data from different cells:

Intergroup statistical comparison: The average peak amplitude and number of peaks for individual cells in the experimental and control groups were calculated and compared.

Model fitting: An exponential decay model was applied to fit the average peak amplitude and peak number of individual cells. The model is defined as:

$$f(x) = A \cdot e^{(-B \cdot x)} + C$$

where x represents the independent variable; A denotes the initial amplitude; B is the decay coefficient ($B > 0$, with larger values indicating faster decay); and C is the asymptote (the value that the decay curve approaches indefinitely without falling below or exceeding).

Fitting objective: Model parameters were estimated by minimizing the sum of squared residuals (SSR), calculated as:

$$SSR = \sum_{i=1}^n [y_i - f(x_i, A, B, C)]^2$$

where A, B, C are the parameters to be estimated; $f(x_i, A, B, C)$ is the predicted value from the model; and y_i is the experimentally observed value.

RNAscope *in situ* hybridization

Brain slices (14 μ m thick) were prepared from 4% paraformaldehyde (PFA)-fixed tissues and subjected to an ethanol gradient for dehydration. Probes were designed by ACDbio, including mRNA probes: Mm-mGluR8-C2 (catalog #521491-C2), Mm-mGluR7 (catalog #487841), negative control (catalog #320871), and positive control (catalog #320881); and miRNA probes: SR-miR-15b-5p-S1 (catalog #1109211-S1), SR-miR-15a-5p-S1 (catalog #1218691-S1), and SR-miR-205-5p-S1 (catalog #728541-S1). *In situ* hybridization was conducted using either the RNAscope Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics, catalog #323100) or the miRNAscope HD Reagent Kit-RED (Advanced Cell Diagnostics, catalog #324500), in accordance with the manufacturer's protocol. Images were captured on a Leica TCS SP8-MaiTai MP confocal microscope and quantified using ImageJ software.

sRNA deep sequencing

sRNA deep sequencing was performed on sEVs isolated from 50 mL of breastmilk. The sEVs were purified using differential ultracentrifugation (dUC) followed by size exclusion chromatography

(SEC). Fractions corresponding to sEVs (SEC fractions 6–8) were pooled and concentrated to 100 μ L using a centrifugal ultrafiltration device (Millipore Amicon Ultra, UFC5003BK). Total RNA was extracted from the concentrated sEVs using TRIzol Reagent (Thermo Fisher Scientific). Meanwhile, total RNA was also directly extracted from 50 mL of breastmilk using the same method. RNA concentration was quantified using a NanoDrop spectrophotometer.

Library preparation and sequencing were carried out by BGI (Shenzhen, China). UMI-based small RNA libraries were constructed and sequenced on the DNBSEQ platform with single-end 50 bp reads (SE50). After quality control and adapter trimming, clean reads were obtained and mapped to reference genomes using Bowtie (v1.2.3). Sequential alignment was performed against the following databases: miRNAs to miRBase¹⁰⁹, rsRNAs to rRNAdb¹¹⁰, tsRNAs to GtRNAdb¹¹¹, and piRNAs to piRBase¹¹². All alignments were based on the hg19 reference genome. To minimize ambiguous mapping, reads were first aligned to miRNA references, followed by rsRNA, tsRNA, and piRNA databases. miRNA matches were defined as sequences with no more than one mismatch and no more than two nucleotide shifts. The fragment length distribution of sRNAs was analyzed to characterize RNA species composition. Differential expression analysis was conducted using the DESeq2 package¹¹¹, with a significance threshold of P-value < 0.05. Heatmaps were generated using the pheatmap R package to visualize expression patterns.

Transcriptome deep sequencing

Transcriptomic profiling of hippocampal tissues was conducted by BGI (Shenzhen, China) using RNA deep sequencing on an Illumina HiSeq 4000 platform. Raw sequencing reads were preprocessed with trim_galore (v0.3.3) to remove adapter sequences and low-quality bases. The resulting high-quality reads were aligned to the mouse reference genome (mm10) using STAR (v2.7.3a). Gene and transcript abundance was quantified with RSEM (v1.3). Differential expression analysis was performed using a T-test on normalized data, with statistical significance defined as P < 0.001. Visualization of expression patterns was achieved using the pheatmap R package. Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was conducted with the clusterProfiler R package¹¹³. Additionally, Fisher's exact test was applied to assess the enrichment of key miRNA target genes among the DEGs relative to the background gene set.

Single-cell RNA sequencing

Single-cell complementary DNA (cDNA) libraries were prepared using the Chromium Next GEM Single Cell 3' Reagent Kit v3.1 according to the manufacturer's instructions by OE Biotech Co., Ltd. (Shanghai, China). The 10 $\times$ Chromium scRNA-seq data were processed with CellRanger (v5.0.1; 10 $\times$ Genomics) for alignment, barcode assignment, and unique molecular identifier (UMI) counting, using the mouse reference genome (mm10). All libraries were sequenced on the NovaSeq 6000 Sequencing System (Illumina). Filtered count matrices were converted to sparse matrices using the Seurat R package (v4.2.0). Cells expressing fewer than 200 genes, more than 5,000 genes, or having over 5% mitochondrial reads were excluded from downstream analyses. To address batch effects across samples and experiments, data integration was performed using Seurat's canonical correlation analysis (CCA) integration method. Cell clusters were annotated based on marker genes identified using Seurat's "FindAllMarkers" function, with references from established literature. The distribution of marker genes was further validated using FeaturePlot, ensuring precise cell type

annotation for each cluster¹¹⁴. Dimensionality reduction was conducted using the “RunUMAP” function, with visualization through Uniform Manifold Approximation and Projection (UMAP). For subcluster analysis, cells of each type were extracted and re-clustered based on the first 30 principal components (PCs) at visually optimized resolutions.

Identification of signature genes

To identify marker genes specific to each cell cluster or subset, we calculated the log2 fold change (log2FC) between a given cell cluster/subset and other cells using the “FindMarkers” function in Seurat, which employs the Wilcoxon rank-sum test with default parameters. Genes were considered statistically significant if the adjusted P-value was less than 0.05.

Pseudotime analysis by Monocle

Developmental trajectories of excitatory neurons were inferred using Monocle2 (v2.18.0)⁸¹, following the recommended default parameters. Neural stem cells were included as the starting point. Integrated gene expression matrices for specific cell types were exported from Seurat into Monocle to construct a CellDataSet. The “setOrderingFilter” function was applied to sort cells based on the top 1,000 variable genes identified by the “differentialGeneTest” function. Dimensionality reduction was performed using the “reduceDimension” function with the “DDRTree” method. The “plot_cell_trajectory” function was used to visualize key genes along the pseudotime trajectory. Variations in expression levels of key genes across cell subsets were further visualized using the “plot_genes_branched_pseudotime” function.

Identification and mapping of E1 subpopulation

The E1 subpopulation was identified and mapped through cell type annotation using the anchor-based label transfer workflow in Seurat (v4.2.0). We employed a publicly available reference dataset (GSE146692)¹¹⁵ as a reference atlas. Anchors were identified with the “FindTransferAnchors” function (parameters: dims = 1:30, k.weight = 10), and cell labels were subsequently transferred using “TransferData”. Finally, cell-type labels were assigned to the query dataset based on the highest prediction scores.

Average distance calculation using RANN

The average inter-sample distance was calculated for the public datasets HIP (GSE223066)¹¹⁶, AMY (GSE246147; limited to HC samples)¹¹⁷, TH (GSE208707)¹¹⁸, and HY (GSE146692)¹¹⁵ using the Random Approximate Nearest Neighbors (RANN) algorithm via the RANN R package⁸⁷. This metric was computed for each dataset to evaluate the overall similarity between its constituent samples.

Statistical Analyses

Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were conducted using SPSS or GraphPad Prism 8.0 software. The specific statistical tests used, along with sample sizes, are described in the figure legends. A P value of ≤ 0.05 was considered statistically significant.

Data and code availability

The RNA sequencing data have been deposited in the National Genomics Data Center (China National Center for Bioinformation) under accession number: PRJCA035478 (mouse sample) and PRJCA036054 (human sample). The accession number is listed in the Table S1.

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Author Contributions Statement

X.C., Z.X.W., C.B.J, Z.P.C. and C.Y.Z. conceived and designed the study. Z.X.W., J.N.X., C.Y.T., L.J.L. and X.H.X. conducted the experiments. Z.X.W., C.Y.T., X.S.Z. and J.J.Y performed data analysis. R.Y.Y., D.J.C. and Z.X.W. carried out RNA sequencing analyses. A.X.Z. and J.H. provided essential materials. Z.P.C., Y.B.W, W.G., T.W., and J.H.G. contributed to the discussion and interpretation of results. X.C. and Z.X.W. wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

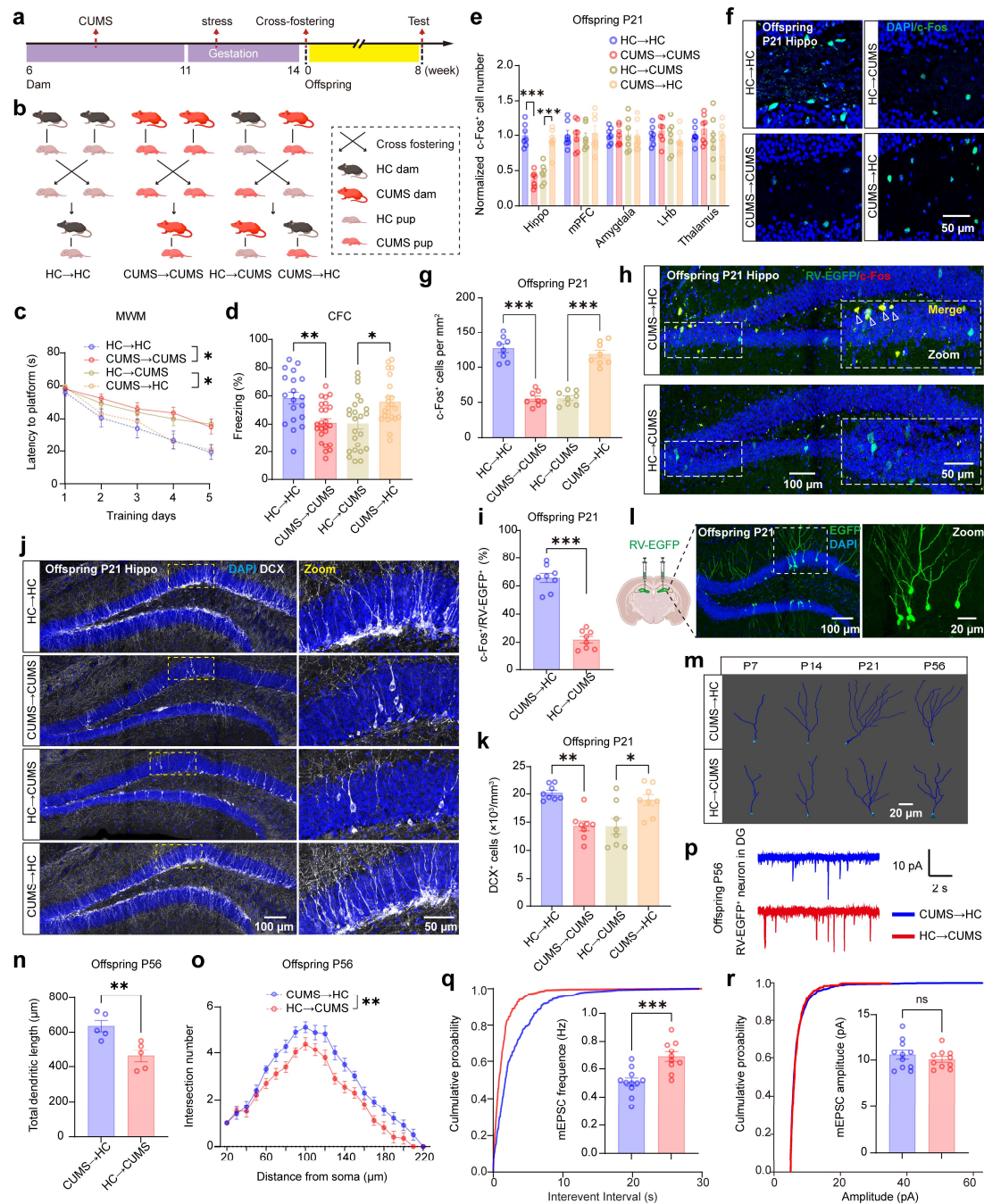


Figure 1. Effects of maternal PND on offspring behavior and hippocampal neurodevelopment in a cross-fostering model. (a) Schematic of the experimental design. Female C57BL/6J mice were exposed to CUMS paradigms to induce PND. Their offspring were cross-fostered at birth to control for genetic effects and underwent behavioral and neurobiological assessments at P56. (b) Cross-fostering scheme. Immediately after birth, offspring from CUMS or HC dams were cross-fostered by either CUMS or HC dams, generating four groups (HC→HC, CUMS→CUMS, HC→CUMS, and CUMS→HC) designated as birth mother→foster mother. (c) Latency to locate the hidden platform in the training trial of the MWM test (n = 19 for HC→HC; n = 24 for CUMS→CUMS and HC→CUMS; n = 22 for CUMS→HC). (d) Freezing percentage in the conditioning session of the MWM test. (e) Normalized c-Fos⁺ cell number in the hippocampus (Hippo), mPFC, Amygdala, LHb, and Thalamus for Offspring P21. (f) Immunofluorescence images of Offspring P21 Hippo showing DAPI (blue) and c-Fos (red) staining. (g) c-Fos⁺ cells per mm² in the hippocampus for Offspring P21. (h) Immunofluorescence images of Offspring P21 Hippo showing RV-EGFP (green) and c-Fos (red) staining. (i) c-Fos/RV-EGFP⁺ (%) in the hippocampus for Offspring P21. (j) Immunofluorescence images of Offspring P21 Hippo showing DAPI (blue) and DCX (green) staining. (k) DCX⁺ cells (×10³/mm²) in the hippocampus for Offspring P21. (l) Immunofluorescence images of Offspring P21 Hippo showing RV-EGFP (green) and DCX (red) staining. (m) Immunofluorescence images of Offspring P56 Hippo showing RV-EGFP (green) and DCX (red) staining. (n) Total dendritic length (μm) in the hippocampus for Offspring P56. (o) Intersection number in the hippocampus for Offspring P56. (p) Electrophysiological traces of Offspring P56 RV-EGFP⁺ neuron in DG showing mEPSCs. (q) Cumulative probability of mEPSC frequency (Hz) for Offspring P56. (r) Cumulative probability of mEPSC amplitude (pA) for Offspring P56.

CFC test ($n = 19$ for HC→HC; $n = 24$ for CUMS→CUMS and HC→CUMS; $n = 22$ for CUMS→HC). **(e)** Quantification of c-Fos⁺ cells across the indicated brain regions at P21 ($n = 7$ per group). **(f)** Representative images of c-Fos immunostaining in the DG. Scale bars: 50 μ m. **(g)** Quantification of c-Fos⁺ cells in the DG across the four offspring groups ($n = 8$ per group). **(h)** Representative images showing RV-EGFP and c-Fos co-localization in the DG. White arrows indicate double-positive cells. Scale bars: 100 μ m (overview), 50 μ m (zoom). **(i)** Overlapping ratios of c-Fos⁺ and RV-EGFP⁺ cells in the DG ($n = 8$ per group). **(j)** Left: Representative images of DCX staining in the DG across the four offspring groups. Scale bar: 100 μ m. Right: Magnified views of boxed areas, highlighting DCX⁺ cell morphology and density. Scale bars: 50 μ m. **(k)** Quantification of DCX⁺ cells in the DG across the four offspring groups ($n = 8$ per group). **(l)** Schematic and representative images of RV-EGFP labeling of newborn neurons. RV-EGFP was stereotactically injected into the DG of HC→CUMS and CUMS→HC offspring at P0 to label newborn neurons. Immunofluorescence images show EGFP⁺ newborn neurons in the DG at the indicated developmental time points (P7, P14, P21, and P56). Scale bars: 100 μ m (overview), 20 μ m (zoom). **(m)** Representative images showing the dendritic morphology of EGFP⁺ newborn neurons at the indicated developmental time points. Scale bars: 20 μ m. **(n)** Quantification of dendritic length in EGFP⁺ newborn neurons ($n = 5$ mice/group, 17-20 cells/mouse). **(o)** Sholl analysis of dendritic branching in EGFP⁺ newborn neurons ($n = 5$ mice/group, 17-20 cells/mouse). **(p)** Representative mEPSC traces from EGFP⁺ newborn neurons. Scale bars: 10 pA (vertical), 2 s (horizontal). **(q,r)** Cumulative probability plots of mEPSC inter-event intervals and amplitudes, with insets showing summary graphs of mEPSC frequency (q) and amplitude (r) ($n = 11$ cells from 4 mice for CUMS→HC; $n = 10$ cells from 4 mice for HC→CUMS). Data are presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparisons test (**d**, **e**, **g** and **k**), two-way ANOVA followed by Bonferroni's multiple comparisons test (**c** and **o**), or two-sided unpaired t-test (**i**, **n**, **q** and **r**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

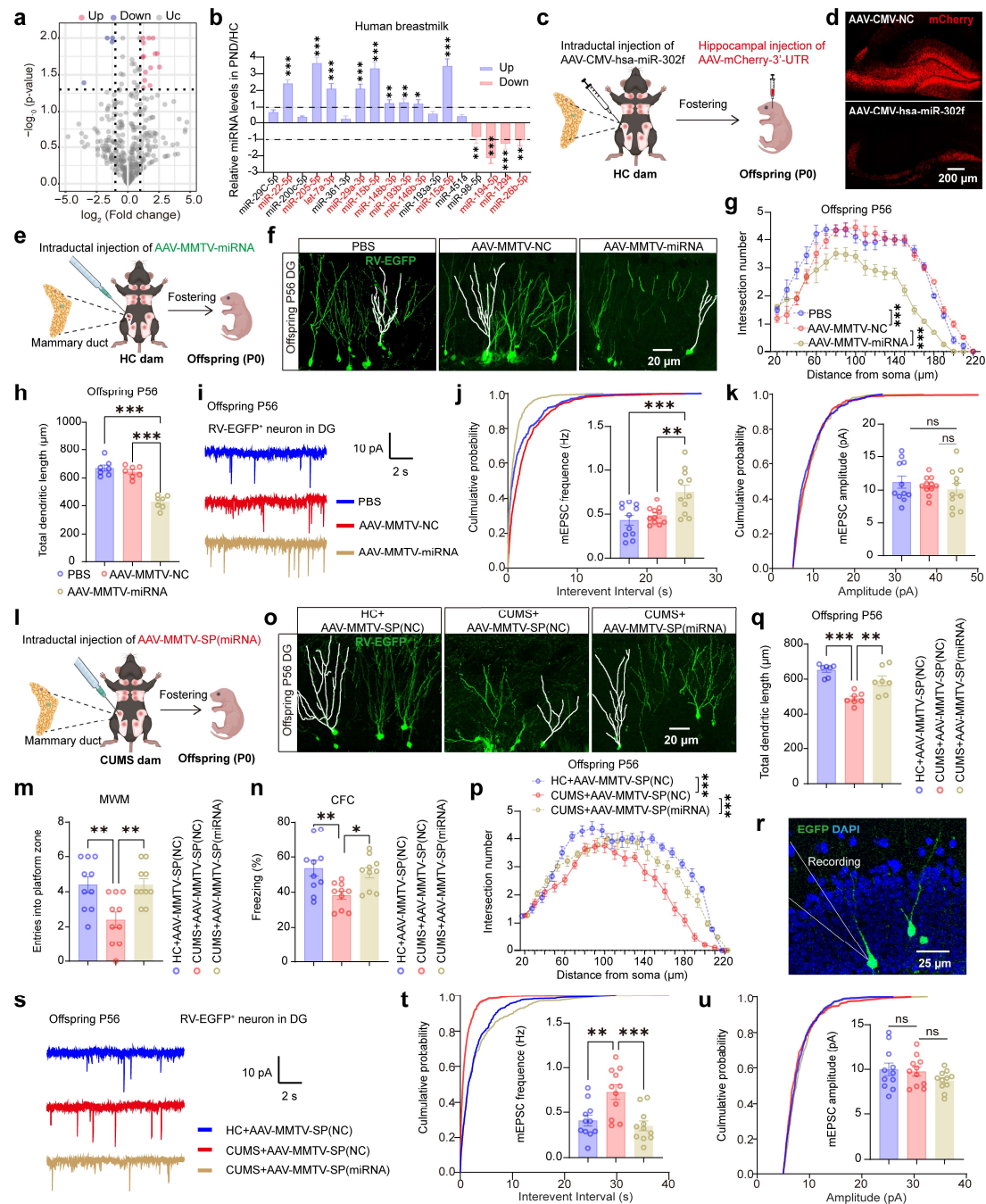


Figure 2. Stress-induced alterations in breastmilk miRNAs and their impact on offspring neurodevelopment. (a) Volcano plot from DESeq2 analysis of RNA sequencing data, comparing breastmilk from PND patients to HC mothers. Eighteen miRNAs were significantly differentially expressed (14 upregulated, 4 downregulated). (b) Quantitative RT-PCR analysis of miRNA levels in breastmilk from PND patients (n = 40) versus HC mothers (n = 51). Significantly changed miRNAs ($|\text{fold change}| > 2$, $P < 0.05$) are highlighted in red. The Y-axis represents $\log_2(\text{Fold Change})$, with the dashed line indicating the threshold set at $\log_2(\text{Fold Change}) = \pm 1$. (c) Schematic of the AAV-mCherry reporter assay. The reporter was stereotactically injected into the hippocampus of normal pups at P7, which were then breastfed by dams receiving intramammary injections of AAV-CMV-hsa_miR-302f or AAV-CMV-NC. mCherry expression in pup hippocampus was assessed after two weeks. (d) Representative confocal images of hippocampal mCherry expression

in pups fostered by AAV-CMV-hsa_miR-302f- versus AAV-CMV-NC-injected dams. Scale bars, 200 μ m. **(e)** Schematic of the experimental design. Three AAV-MMTV-miRNA-EGFP vectors to overexpress miR-15a-5p, miR-15b-5p, and miR-205-5p were pooled in equal proportions and administered via intraductal injection into the mammary glands of HC dams. Control groups received PBS or a negative control vector (AAV-MMTV-NC). Pups were fostered by these dams until adulthood. **(f)** Representative images showing the dendritic morphology of EGFP⁺ newborn neurons at P56. A representative neuron is demarcated by a white outline. Scale bars: 20 μ m. **(g)** Sholl analysis of dendritic branching in EGFP⁺ newborn neurons (n = 5 mice/group, 17-20 cells/mouse). **(h)** Quantification of dendritic length in EGFP⁺ newborn neurons (n = 5 mice/group, 17-20 cells/mouse). **(i)** Representative mEPSC traces from EGFP⁺ newborn neurons. Scale bars: 10 pA (vertical), 2 s (horizontal). **(j,k)** Cumulative probability plots of mEPSC inter-event intervals and amplitudes, with insets showing summary graphs of mEPSC frequency (j) and amplitude (k) (n = 11 cells from 4 mice per group). **(l)** Schematic of the experimental design. Three AAV-MMTV-SP(miRNA)-EGFP vectors, each containing tandemly arrayed, bulged binding sites for miR-15a-5p, miR-15b-5p, and miR-205-5p, were pooled in equal proportions and administered via intraductal injection into the mammary glands of CUMS-induced dams (CUMS + AAV-MMTV-SP(miRNA)). Control groups included HC or CUMS dams injected with a negative control vector (HC + AAV-MMTV-SP(NC) or CUMS + AAV-MMTV-SP(NC)). Pups were fostered by these dams until adulthood. **(m)** Number of entries into the platform zone in the probe trial of the MWM test (n = 10 mice per group). **(n)** Freezing percentage in the conditioning session of the CFC test (n = 10 mice per group). **(o)** Representative images showing the dendritic morphology of EGFP⁺ newborn neurons at P56. A representative neuron is demarcated by a white outline. Scale bars: 20 μ m. **(p)** Sholl analysis of dendritic branching in EGFP⁺ newborn neurons (n = 5 mice/group, 17-20 cells/mouse). **(q)** Quantification of dendritic length in EGFP⁺ newborn neurons (n = 7 mice/group, 17-20 cells/mouse). **(r)** Representative images of recorded newborn neurons, identified and confirmed by EGFP⁺ immunostaining signals (green) following RV-EGFP injection. Scale bars: 25 μ m. **(s)** Representative mEPSC traces from EGFP⁺ newborn neurons. Scale bars: 10 pA (vertical), 2 s (horizontal). **(t,u)** Cumulative probability plots of mEPSC inter-event intervals and amplitudes, with insets showing summary graphs of mEPSC frequency (t) and amplitude (u) (n = 11 cells from 4 mice per group). Data are presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparisons test (**b**, **h**, **j**, **k**, **m**, **n**, **q**, **t**, and **u**), or two-way ANOVA followed by Bonferroni's multiple comparisons test (**g** and **p**). *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

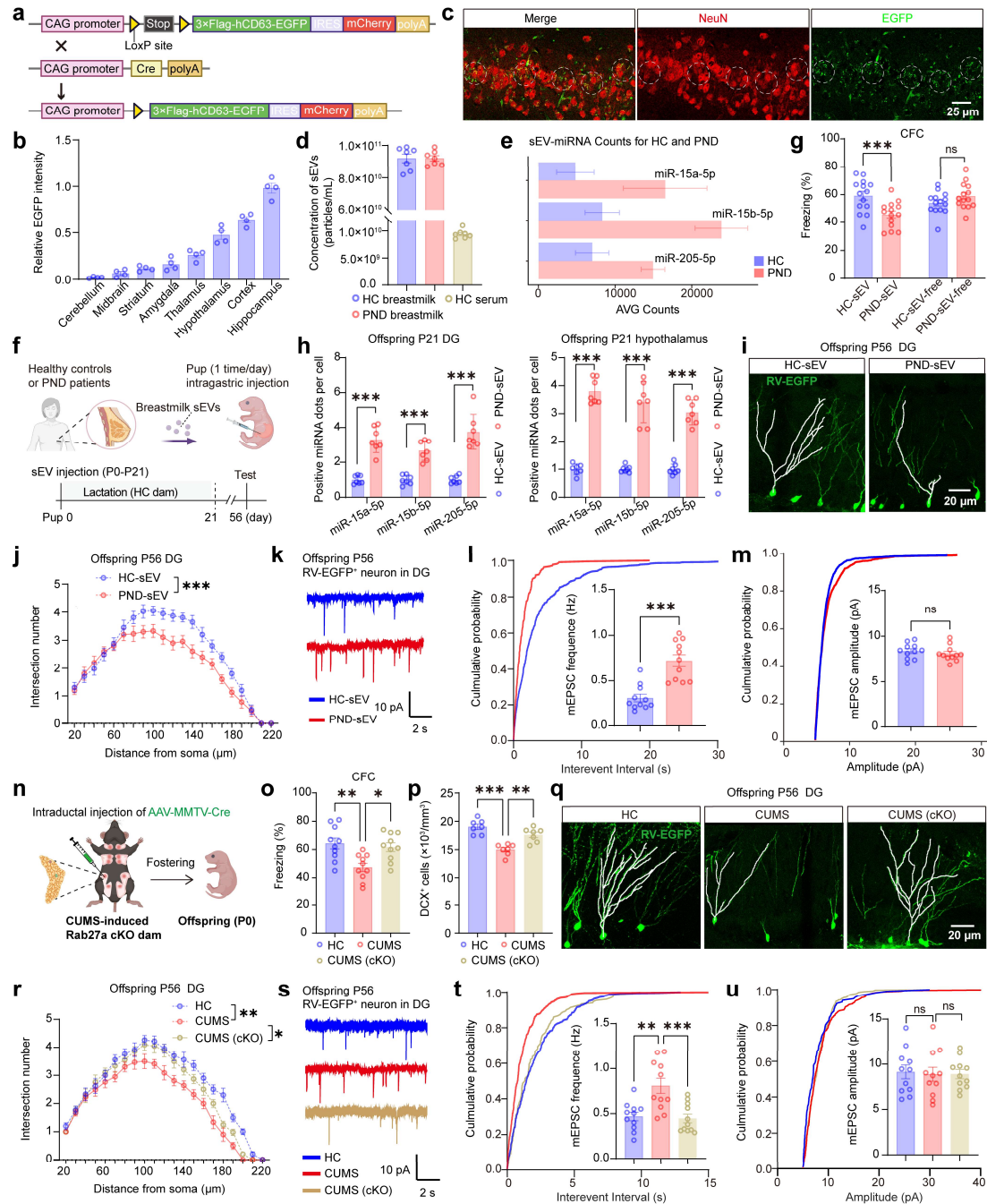


Figure 3. sEV-mediated transfer of breastmilk miRNAs and its impact on offspring neurodevelopment. (a) Schematic of the Cre-dependent EV tracking reporter system, in which Rosa26-CD63^{Flag}EGFP-mCherry knock-in mice were crossed with CAG-Cre mice to enable fluorescent labeling of breastmilk sEVs. (b) Quantification of EGFP intensity in pup brain regions after fostering by female reporter dams, indicating sEV distribution (n = 4 mice per group). (c) Representative confocal images of the pup hippocampus showing EGFP signal (from sEVs) and NeuN (neuronal marker). Scale bars: 25 μ m. (d) sEV concentration in breastmilk from PND patients and HC mothers, and in serum from HC mothers, as measured by NTA (n = 7 per group). (e) Abundance of miR-15a-5p, miR-15b-5p, and miR-205-5p in PND-sEVs relative to HC-sEVs, as determined by sRNA sequencing. (f) From P0 to P21, normal pups fostered by HC dams received daily intragastric injections of PBS, HC-sEVs, PND-sEVs (from SEC fractions 6-8), or sEV-free

fractions (from SEC fractions 1-5 and 9-26). The adult offspring then underwent behavioral and neurobiological assessments. **(g)** Freezing percentage in the conditioning session of the CFC test ($n = 14$ mice per group). **(h)** RNAscope *in situ* hybridization of miR-15a-5p, miR-15b-5p, and miR-205-5p, combined with NeuN immunostaining, to visualize the distribution of these miRNAs in the hippocampal DG of pups treated with HC-sEVs or PND-sEVs. Quantification of positive dots for miR-15a-5p, miR-15b-5p, and miR-205-5p per NeuN⁺ cells is shown ($n = 7$ mice per group, averaging 50 cells per mouse). **(i)** Representative images showing the dendritic morphology of EGFP⁺ newborn neurons at P56. A representative neuron is demarcated by a white outline. Scale bars: 20 μ m. **(j)** Sholl analysis of dendritic branching in EGFP⁺ newborn neurons ($n = 5$ mice/group, 17-20 cells/mouse). **(k)** Representative mEPSC traces from EGFP⁺ newborn neurons. Scale bars: 10 pA (vertical), 2 s (horizontal). **(l,m)** Cumulative probability plots of mEPSC inter-event intervals and amplitudes, with insets showing summary graphs of mEPSC frequency (l) and amplitude (m) ($n = 11$ cells from 4 mice per group). **(n)** Schematic of the strategy to block sEV secretion into breastmilk and evaluate its impact on offspring phenotypes. Mammary-specific suppression of sEV secretion was achieved by injecting AAV2/9-MMTV-EGFP-IRES-Cre into the mammary glands of Rab27a cKO females. These Rab27a cKO and wild-type females were then subjected to CUMS to induce PND (CUMS (cKO) versus CUMS), and their fostered normal pups were monitored for phenotypic changes, with pups fostered by HC dams serving as healthy controls. **(o)** Freezing percentage in the conditioning session of the CFC test ($n = 10$ mice per group). **(p)** Quantification of DCX⁺ cells in the DG ($n = 7$ per group). **(q)** Representative images showing the dendritic morphology of EGFP⁺ newborn neurons at P56. A representative neuron is demarcated by a white outline. Scale bars: 20 μ m. **(r)** Sholl analysis of dendritic branching in EGFP⁺ newborn neurons ($n = 5$ mice/group, 17-20 cells/mouse). **(s)** Representative mEPSC traces from EGFP⁺ newborn neurons. Scale bars: 10 pA (vertical), 2 s (horizontal). **(t,u)** Cumulative probability plots of mEPSC inter-event intervals and amplitudes, with insets showing summary graphs of mEPSC frequency (t) and amplitude (u) ($n = 11$ cells from 4 mice per group). Data are presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparisons test (**g, o, p, t** and **u**), two-way ANOVA followed by Bonferroni's multiple comparisons test (**j** and **r**), or two-sided unpaired t-test (**h, l** and **m**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

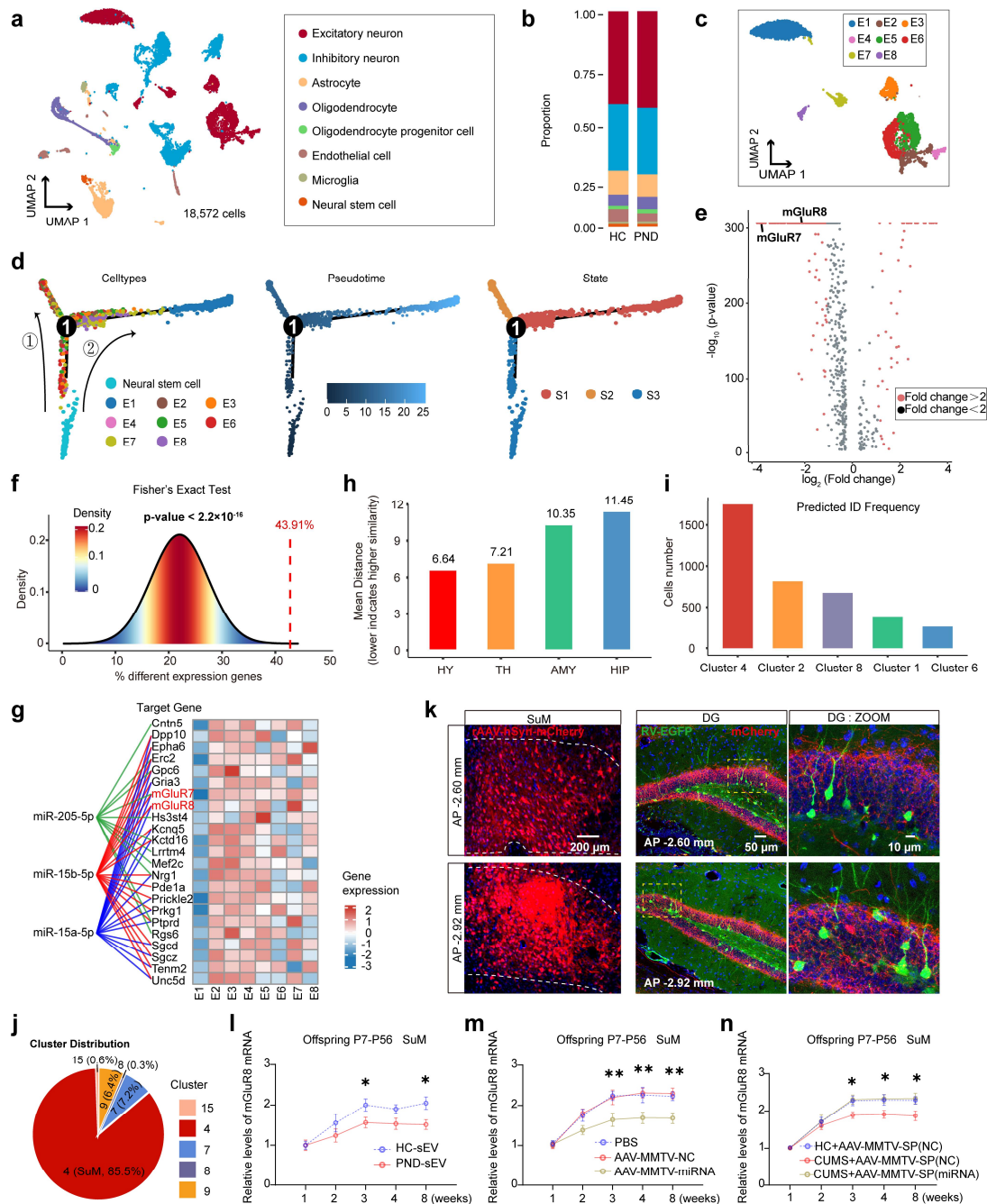


Figure 4. Single-cell RNA sequencing identifies the E1 subpopulation of excitatory neurons based on its characteristic transcriptional signature. (a) Unsupervised clustering of cell populations, consisting of 8 annotated cell types. (b) Bar plot showing the percentage of different cell types in pups treated with HC-sEVs or PND-sEVs. (c) UMAP showing the segmentation of excitatory neurons into 8 subpopulations, with colors representing different subpopulations. (d) Semisupervised pseudotime trajectory of excitatory neuron subpopulations by Monocle2. The arrowhead direction represents the trend of cell pseudo-temporal differentiation. The trajectory is colored by cell subtypes (left), pseudotime (middle), and cell states (right). (e) Volcano plot showing the differentially expressed genes in the E1 subpopulation compared to E2-E8 subpopulations in the integrated excitatory neuron cluster, based on P-value < 0.05. Genes with |fold change| > 2 are marked in red, while others are marked in gray. (f) Density plot showing the proportions of predicted

target genes of miR-15a-5p, miR-15b-5p, and miR-205-5p among 583 genes randomly sampled from the mouse genome (10,000 simulations). The target genes of these miRNAs were predicted using the multiMiR R package. The red dashed line indicates the actual proportion (43.91%) of these miRNA target genes among the 583-gene set characteristic of the E1 subpopulation (256 out of 583 genes). The random proportions (peaking around 21%) are significantly lower than the actual proportion. Statistical significance was assessed using Fisher's exact test ($p < 2.2 \times 10^{-16}$).

(g) Heatmap of selected genes from the top 50 downregulated transcripts in the E1 subpopulation, showing their expression patterns across eight excitatory neuron subpopulations and their predicted targeting by miR-15a-5p, miR-15b-5p, and miR-205-5p. **(h)** Average inter-sample distances computed using the Random Approximate Nearest Neighbors (RANN) method for public datasets: hippocampus (HIP), amygdala (AMY), thalamus (TH), and hypothalamus (HY). Smaller values indicate higher transcriptional similarity between samples within each dataset. **(i)** Bar chart showing the distribution of E1 subpopulation cells across clusters in the GSE146692 dataset after Seurat-based mapping. Bar height represents the number of E1 cells assigned to each cluster. **(j)** Pie chart illustrating the regional composition of cells annotated as SuM in the GSE146692 dataset. Cluster 4 constitutes the largest proportion, indicating its dominance within SuM-derived cells. **(k)** Representative fluorescence images showing anterograde tracing of the SuM→DG circuit. Images display the DG granule cell layer across a series of anterior–posterior coordinates, illustrating close contacts between mCherry-labeled SuM axons and EGFP-labeled newborn neurons. In the SuM, mCherry (red) indicates neurons infected with rAAV-hSyn-mCherry. In the DG, EGFP (green) labels mature newborn neurons, and mCherry (red) represents axonal projections originating from the SuM. Scale bars: 200 μ m (SuM), 50 μ m (DG), 10 μ m (DG:ZOOM). **(l–n)** The developmental trajectory of mGluR8 mRNA levels in SuM neurons was determined by quantitative RT-PCR from P7 to P56. Data are presented for three experimental cohorts: HC-sEV and PND-sEV; PBS, AAV-MMTV-NC, and AAV-MMTV-miRNA; and PBS, AAV-MMTV-SP(NC), and AAV-MMTV-SP(miRNA) ($n = 3$ mice per group, with 65 cells analyzed per mouse at each time point). Data are presented as mean $\pm$ SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test (**l–n**). * $p < 0.05$; ** $p < 0.01$.

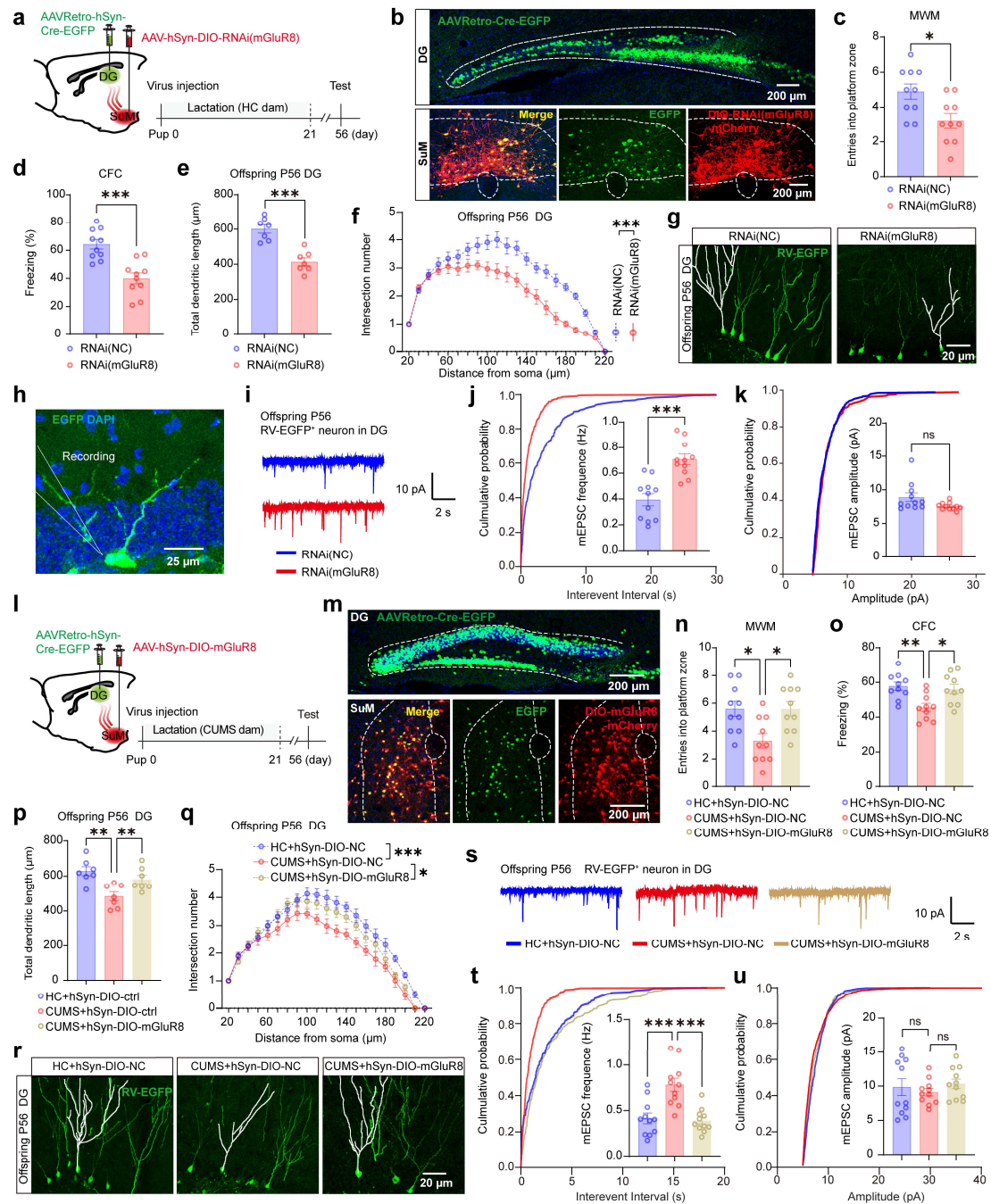


Figure 5. Presynaptic mGluR8 in the SuM→DG Circuit mediates PND-related deficits and governs neurodevelopment in offspring. (a) Schematic of the experimental strategy for presynaptic mGluR8 knockdown. AAV-hSyn-DIO-RNAi(mGluR8) was bilaterally injected into the SuM of P0 pups, and AAVRetro-hSyn-Cre was delivered into the DG to enable Cre-dependent knockdown of mGluR8 in SuM-DG projecting terminals. Control pups received AAV-hSyn-DIO-RNAi(NC) in the SuM. All pups were fostered by HC dams and assessed in adulthood. (b) Representative fluorescence images showing EGFP-labeled Cre expression in the DG and retrograde-traced labeling in the SuM, where it co-localized with DIO virus-derived mCherry in SuM neurons. Scale bars: 200 μ m. (c) Number of entries into the platform zone in the probe trial of the MWM test ($n = 10$ mice per group). (d) Freezing percentage in the conditioning session of the CFC test ($n = 10$ mice per group). (e) Quantification of dendritic length in EGFP⁺ newborn neurons

(n = 7 mice/group, 17-20 cells/mouse). **(f)** Sholl analysis of dendritic branching in EGFP⁺ newborn neurons (n = 5 mice/group, 17-20 cells/mouse). **(g)** Representative images showing the dendritic morphology of EGFP⁺ newborn neurons at P56. A representative neuron is demarcated by a white outline. Scale bars: 20 μ m. **(h)** Representative images of recorded newborn neurons, identified and confirmed by EGFP⁺ immunostaining signals (green) following RV-EGFP injection. Scale bars: 25 μ m. **(i)** Representative mEPSC traces from EGFP⁺ newborn neurons. Scale bars: 10 pA (vertical), 2 s (horizontal). **(j,k)** Cumulative probability plots of mEPSC inter-event intervals and amplitudes, with insets showing summary graphs of mEPSC frequency (j) and amplitude (k) (n = 11 cells from 4 mice per group). **(l)** Schematic of the presynaptic mGluR8 overexpression rescue strategy. AAV-hSyn-DIO-mGluR8 was bilaterally injected into the SuM of P0 pups, and AAVRetro-hSyn-Cre was delivered into the DG to enable Cre-dependent overexpression of mGluR8 in SuM-DG projecting terminals. Control pups received AAV-hSyn-DIO-NC in the SuM. All pups were fostered by CUMS dams and assessed in adulthood. **(m)** Representative fluorescence images showing EGFP-labeled Cre expression in the DG and retrograde-traced labeling in the SuM, where it co-localized with DIO virus-derived mCherry in SuM neurons. Scale bars: 200 μ m. **(n)** Number of entries into the platform zone in the probe trial of the MWM test (n = 10 mice per group). **(o)** Freezing percentage in the conditioning session of the CFC test (n = 10 mice per group). **(p)** Quantification of dendritic length in EGFP⁺ newborn neurons (n = 7 per group). **(q)** Sholl analysis of dendritic branching in EGFP⁺ newborn neurons (n = 5 mice/group, 17-20 cells/mouse). **(r)** Representative images showing the dendritic morphology of EGFP⁺ newborn neurons at P56. A representative neuron is demarcated by a white outline. Scale bars: 20 μ m. **(s)** Representative mEPSC traces from EGFP⁺ newborn neurons. Scale bars: 10 pA (vertical), 2 s (horizontal). **(t,u)** Cumulative probability plots of mEPSC inter-event intervals and amplitudes, with insets showing summary graphs of mEPSC frequency (t) and amplitude (u) (n = 11 cells from 4 mice per group). Data are presented as mean $\pm$ SEM. Statistical analysis was performed using two-sided paired t-test (**c-e** and **j-k**), one-way ANOVA followed by Bonferroni's multiple comparisons test (**n-p** and **t-u**) or two-way ANOVA followed by Bonferroni's multiple comparisons test (**f** and **q**). *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

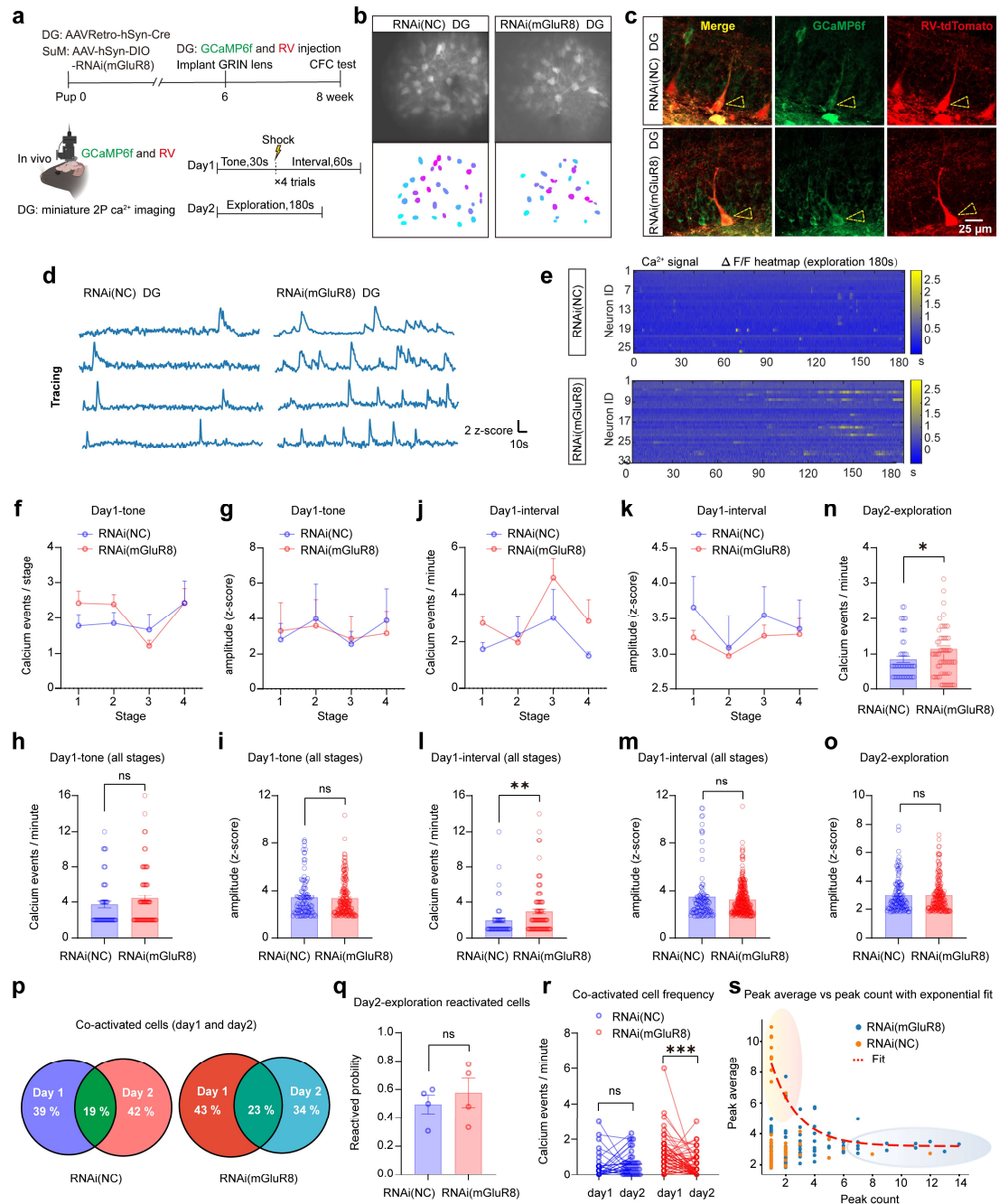


Figure 6. *In vivo* calcium imaging reveals a “high-frequency, low-amplitude” activation profile in DG neurons. (a) Schematic of the experimental workflow for investigating mGluR8 function in SuM→DG projections. Viral injection strategy: AAVRetro-hSyn-Cre was injected into the SuM to retrogradely label SuM→DG projecting neurons, and AAV-hSyn-DIO-RNAi(mGluR8) was injected into the DG for presynaptic mGluR8 knockdown. The control group received AAV-hSyn-DIO-RNAi(NC) in the DG. A genetically encoded calcium indicator (GCaMP6f) and RV-tdTomato were co-injected into the DG for calcium imaging. *In vivo* imaging and behavioral testing: Miniature two-photon calcium imaging was used to record DG neuronal activity during a CFC paradigm. On Day 1, mice underwent 4 trials of tone stimulation (30 s) paired with a foot shock, with 60 s inter-trial intervals. On Day 2, neuronal activity was recorded during a 180 s exploration period in the same context without stimulation. (b) Representative average time-series

calcium images of the DG. **(c)** Representative fluorescence images of the DG. GCaMP6f fluorescence signal (green) indicates labeled DG neurons; merged images show co-localization of viral labels. Scale bars: 25 μm . **(d)** Example calcium traces of DG neurons. **(e)** Calcium activity heatmaps of DG neurons during the 180 s exploration phase on Day 2. Each row represents the calcium dynamics of a single neuron (labeled by Neuron ID). **(f,g)** Calcium event frequency (f, stage = 30 s) and amplitude (g) in DG neurons during each stage of the tone stimulation phase on Day 1. **(h,i)** Calcium event frequency (h) and amplitude (i) in DG neurons across all stages of the tone stimulation phase on Day 1. **(j,k)** Calcium event frequency (j) and amplitude (k) in DG neurons during each stage of the interval phase on Day 1. **(l,m)** Calcium event frequency (l) and amplitude (m) in DG neurons across all stages of the interval phase on Day 1. **(n,o)** Calcium event frequency (n) and amplitude (o) in DG neurons during the exploration phase on Day 2. **(p)** Proportion of reactivated DG neurons (active on both Day 1 and Day 2). **(q)** Proportion of co-activated neurons relative to all activated neurons during the Day 2 exploration phase. **(r)** Calcium event frequency in context-related DG neurons (activated during the interval phase on Day 1). **(s)** Frequency-amplitude distribution of calcium events, fitted with an exponential model. Each point represents the amplitude of a calcium event at a given frequency. Dashed lines indicate the exponential fit. $n = 4$ mice per group for all figures. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using two-sided paired t-test (**r**), or two-sided unpaired t-test (**h, i, l, m, n, o** and **q**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

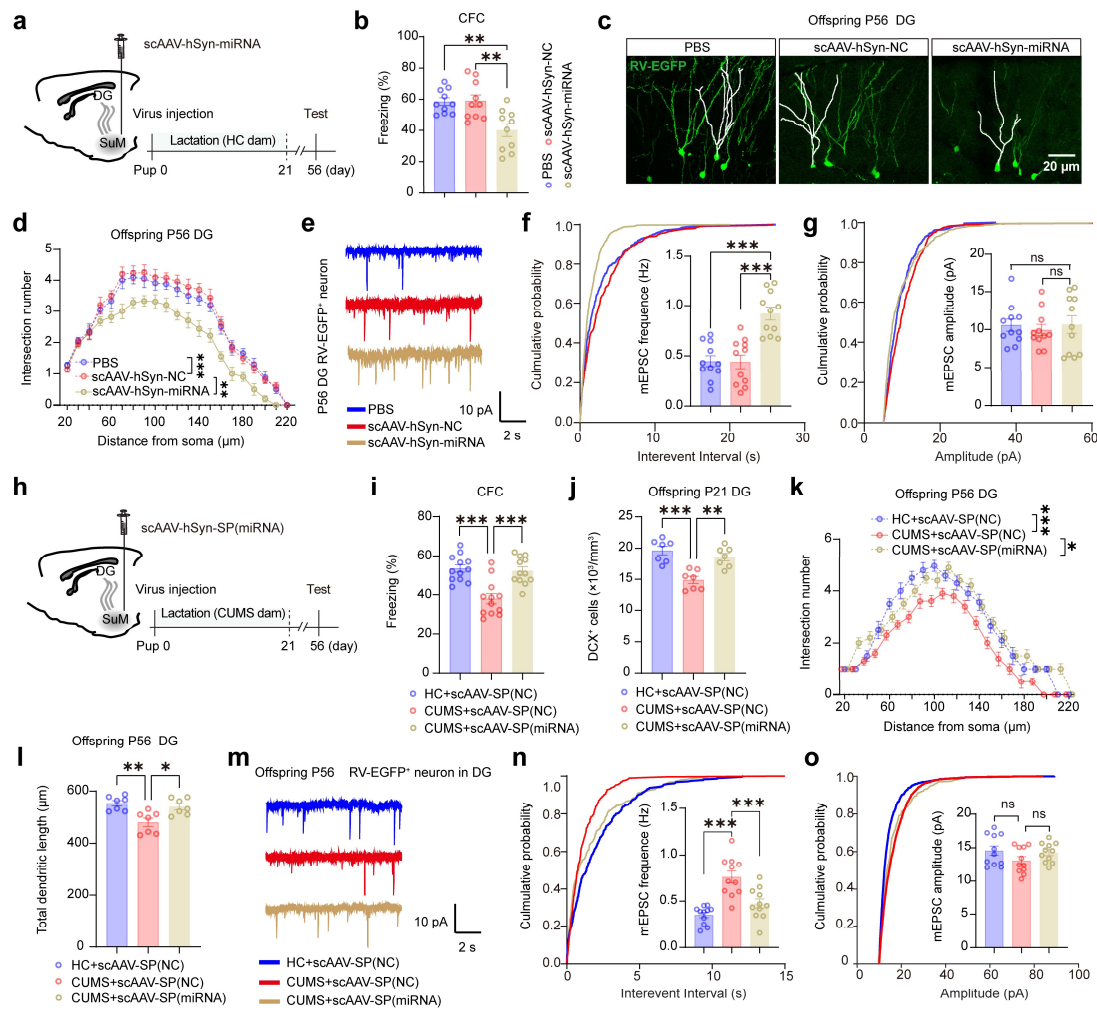


Figure 7. Bidirectional manipulation of miRNA activity influences offspring neurodevelopment via mGluR8 targeting. (a) Schematic of the strategy for overexpressing miRNAs. A pooled mixture of three scAAV-hSyn-miRNA vectors (encoding miR-15a-5p, miR-15b-5p, and miR-205-5p) was injected into the SuM of healthy pups at P0. Control pups received either PBS or scAAV-hSyn-NC. All pups were fostered by HC dams and assessed in adulthood. (b) Freezing percentage in the conditioning session of the CFC test (n = 10 mice per group). (c) Representative images showing the dendritic morphology of EGFP⁺ newborn neurons at P56. A representative neuron is demarcated by a white outline. Scale bars: 20 μ m. (d) Sholl analysis of dendritic branching in EGFP⁺ newborn neurons (n = 5 mice/group, 17-20 cells/mouse). (e) Representative mEPSC traces from EGFP⁺ newborn neurons. Scale bars: 10 pA (vertical), 2 s (horizontal). (f,g) Cumulative probability plots of mEPSC inter-event intervals and amplitudes, with insets showing summary graphs of mEPSC frequency (f) and amplitude (g) (n = 11 cells from 4 mice per group). (h) Schematic of the miRNA sequestration strategy. A pooled mixture of three scAAV-hSyn-SP(miRNA) vectors, each expressing a sponge for miR-15a-5p, miR-15b-5p, or miR-205-5p, was injected into the SuM of P0 pups. Control pups received scAAV-hSyn-SP(NC). Pups were fostered by either CUMS or HC dams and assessed in adulthood (CUMS + scAAV-hSyn-SP(miRNA) versus CUMS + scAAV-hSyn-SP(NC) versus HC + scAAV-hSyn-SP(NC)). (i) Freezing percentage in the conditioning session of the CFC test (n = 12 mice per group).

(j) Quantification of DCX⁺ cells in the DG (n = 7 per group). **(k)** Sholl analysis of dendritic branching in EGFP⁺ newborn neurons (n = 5 mice/group, 17-20 cells/mouse). **(l)** Quantification of dendritic length in EGFP⁺ newborn neurons in the HC + scAAV-hSyn-SP(NC), CUMS + scAAV-hSyn-SP(NC), and CUMS + scAAV-hSyn-SP(miRNA) groups at P56 (n = 7 mice/group, 17-20 cells/mouse). **(m)** Representative mEPSC traces from EGFP⁺ newborn neurons. Scale bars: 10 pA (vertical), 2 s (horizontal). **(n,o)** Cumulative probability plots of mEPSC inter-event intervals and amplitudes, with insets showing summary graphs of mEPSC frequency (n) and amplitude (o) (n = 11 cells from 4 mice per group). Data are presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparisons test (**b**, **f-g**, **i-j**, **l** and **n-o**), or two-way ANOVA followed by Bonferroni's multiple comparisons test (**d** and **k**). *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

Supplementary Files

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