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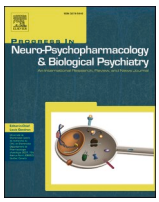

Tph2 deficiency leads to alterations in social adjustment and socio-affective communication in neonatal rats: No rescue effect of communal nesting

Tianhua Wang [a,b], Aaron L. Gnade [a], Judith R. Homberg [c], Marta C.F. Samina [d], Rogério C.R. Castro [c], Sharon M. Kolk [d], Natalia Alenina [e,f], Michael Bader [e,f,g,h], Jinye Dai [i], Markus Wöhr [a,b,j,k,*]

[a] *Philipps-Universität Marburg, Faculty of Psychology, Experimental and Biological Psychology, Social and Affective Neuroscience, D-35032 Marburg, Germany*
[b] *Philipps-Universität Marburg, Center for Mind, Brain, and Behavior (CMBB), D-35032 Marburg, Germany*
[c] *Radboud University Medical Center, Donders Institute for Brain, Cognition, and Behaviour, Department of Medical Neuroscience, NL-6525 EN Nijmegen, the Netherlands*
[d] *Radboud University, Donders Institute for Brain, Cognition, and Behaviour, DCN Section Neurobiology, NL-6525 AJ Nijmegen, the Netherlands*
[e] *Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Molecular Biology of Peptide Hormones, D-13125 Berlin, Germany*
[f] *German Center for Cardiovascular Research (DZHK), partner site Berlin, D-10785 Berlin, Germany*
[g] *Charité University Medicine Berlin, D-10117 Berlin, Germany*
[h] *University of Lübeck, Institute for Biology, D-23562 Lübeck, Germany*
[i] *Mount Sinai, Icahn School of Medicine, Department of Pharmacological Sciences and Department of Neuroscience, Friedman Brain Institute, NY-10029 New York, USA*
[j] *KU Leuven, Faculty of Psychology and Educational Sciences, Research Unit Brain and Cognition, Laboratory of Biological Psychology, Social and Affective Neuroscience Research Group, B-3000 Leuven, Belgium*
[k] *KU Leuven, Leuven Brain Institute, B-3000 Leuven, Belgium*

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ABSTRACT

Deficiency of tryptophan hydroxylase 2 (TPH2), the rate-limiting enzyme for serotonin (5-hydroxytryptamine, 5-HT) synthesis in the brain, was repeatedly reported to cause impairments in socio-affective communication and maternal affiliation across species, including mice, rats, and monkeys. We recently applied a rescue protocol in the *Tph2* knockout rat model and demonstrated that communal nesting ameliorates maternal affiliation impairments. Interestingly, however, this rescue strategy did not lead to improvements in socio-affective communication and was associated with an aggravated growth retardation phenotype in *Tph2*-deficient offspring. In the present study, we aimed to gain deeper insight into the interplay between socio-affective communication, nesting condition, and test context. To this aim, we studied *Tph2*$^{-/-}$ knockout, *Tph2*$^{+/-}$ heterozygous, and *Tph2*$^{+/+}$ wildtype rat pups of both sexes, randomly assigned to standard versus communal nesting. We performed detailed spectrographic analyses and compared the emission of isolation-induced ultrasonic vocalizations under social test conditions, i.e., the maternal preference test and the homing test, to non-social test conditions, i.e., the isolation box test. Our results show that *Tph2* deficiency causes prominent alterations in isolation-induced ultrasonic calling linked to reduced maternal responsiveness, including changes in acoustic features, e.g., increased call duration but reduced frequency modulation. Remarkably, irrespective of communal nesting, *Tph2*$^{-/-}$ pups typically displayed either no evidence for social adjustment or even changes opposite to *Tph2*$^{+/+}$ littermates, suggesting a reduction and/or delay in the capability and/or motivation to appropriately adjust to changes in the social environment. Such alterations in social adjustment likely contribute to growth retardation through reduced quality of mother-pup interactions.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter in the central nervous system of mammals. As an ancient neuromodulator, it modulates a broad set of physiological functions and psychological processes, ranging from regulating various

* Corresponding author at: KU Leuven, Faculty of Psychology and Educational Sciences, Research Unit Brain and Cognition, Laboratory of Biological Psychology, Social and Affective Neuroscience Research Group, Tiensestraat 102 - bus 3714, Leuven B-3000, Belgium.
E-mail address: markus.wohr@kuleuven.be (M. Wöhr).

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events in neurodevelopment and biological rhythmicity to controlling appetite, mood, cognitive functions, and social behavior (Gaspar et al., 2003; Okaty et al., 2019; Wöhr et al., 2015). A key regulator of the central 5-HT system is tryptophan hydroxylase 2 (TPH2), which is the initial and rate-limiting enzyme for 5-HT synthesis in the brain (Walther et al., 2003).

Tph2 deficiency was shown to result in growth retardation associated with low survival rates during early development in both mice (Alenina et al., 2009) and rats (Kaplan et al., 2016), followed by dysfunctions in emotion regulation and social coping (Alonso et al., 2023; Golebiowska et al., 2025; Peeters et al., 2019; Meng et al., 2022), with mostly corroborating evidence obtained in human studies (Kulikov and Popova, 2015; Lesch et al., 2012; Waider et al., 2011). Specifically, *Tph2* deficiency was repeatedly reported to cause impairments in socio-affective communication and maternal affiliation during early postnatal life across species, including mice (Mosienko et al., 2015), rats (Liu et al., 2023), and monkeys (Liu et al., 2023). For example, Liu et al. (2023) found that *Tph2* deficiency led to deficits in maternal affiliation in mice and rats, reflected by the lack of preference for the mother's odor. Similar features were also observed in *TPH2*-deficient infant monkeys who lacked preference for the mother's face. These studies highlight the importance of 5-HT in shaping socio-affective communication and behavior during early developmental stages in rodents and primates.

Socio-affective communication is a key element of the social behavior repertoire of rodents. Mice and rats communicate with each other through olfactory cues, e.g., scent marks, and auditory signals, most notably ultrasonic vocalizations (USV; Brudzynski, 2013; Premoli et al., 2023; Wöhr and Schwarting, 2013). *Tph2*-deficient mouse and rat pups were found to display prominent reductions in the emission of isolation-induced USV (Liu et al., 2023; Mosienko et al., 2015). Such isolation-induced USV are emitted by rodent pups after being separated from the mother and littermates and serve as a primary means of communication with their mother to promote maternal caregiving behavior (Smotherman et al., 1974; Wöhr and Schwarting, 2008).

In a recent study, we applied a rescue protocol in the *Tph2* knockout rat model and demonstrated that communal nesting ameliorates maternal affiliation impairments (Wang et al., 2024). This is in line with the prominent role of the social environment in shaping social behavior (Kiser et al., 2012). Communal nesting of two or more dams with their litters is an early form of social enrichment widely applied in laboratory rodents (Branchi et al., 2011). It has been shown to promote adult sociality (Branchi et al., 2013a) and to enhance resilience to stress (Branchi et al., 2013b), as compared to standard nesting where a single dam is rearing its litter alone.

Interestingly, however, this rescue strategy did not lead to improvements in socio-affective communication and was associated with an aggravated growth retardation phenotype in the *Tph2* knockout rat model (Wang et al., 2024). This work comprehensively illustrated socio-affective communication in rat pups tested under non-social conditions, and suggested that *Tph2* deficiency, causing deficits in socio-affective communication, hindered efficient mother-pup communication. This possibly contributed to the severe growth retardation in *Tph2*-deficient rat pups. However, the assessment of socio-affective communication was based on the analysis of isolation-induced USV in non-social test conditions, whereas mother-pup communication naturally occurs in environments with a variety of social components, i.e., the home cage for laboratory rats.

In the present study, we therefore aimed to gain deeper insight into the interplay between socio-affective communication, nesting condition, and test context. To this aim, we studied *Tph2*$^{-/-}$ knockout (KO), *Tph2*$^{+/-}$ heterozygous (HET), and *Tph2*$^{+/+}$ wildtype (WT) rat pups of both sexes, randomly assigned to standard nesting (SN) versus communal nesting (CN). We performed detailed spectrographic analyses and compared the emission of isolation-induced USV under social test conditions, i.e., the maternal preference test and the homing test, to non-social test conditions, i.e., the isolation box test.

To probe the impact of *Tph2* deficiency on socio-affective communication in social contexts, we conducted detailed spectrographic analyses for the isolation-induced USV emitted during the homing test and the maternal preference test, where either the mother's odor or the mother herself was present. Moreover, to investigate the effects of nesting conditions during the process, the litters were randomly assigned to SN or CN from the day of birth. Importantly, by associating the isolation-induced USV of the pups with the mother's preference in the maternal preference test, we directly assessed the impact of *Tph2* deficiency on mother-pup communication.

2. Materials and methods

2.1. Animals and housing

We assessed the effects of nesting conditions on social adjustment and socio-affective communication in *Tph2*$^{-/-}$ knockout (KO) and *Tph2*$^{+/-}$ heterozygous (HET) rat pups, as compared to *Tph2*$^{+/+}$ wildtype (WT) littermate controls, with balanced representation of sexes. To this aim, we used *Tph2*$^{+/-}$ rats carrying a 10-base pair deletion in exon 7, originally generated by means of zinc finger technology on a Dark Agouti background (Kaplan et al., 2016). *Tph2*$^{+/-}$ founders were provided by the Max Delbrück Center for Molecular Medicine, Germany (Sbrini et al., 2020). After arrival at the Philipps-Universität Marburg, Germany, we backcrossed *Tph2*$^{+/-}$ founders by pairing them with wildtype Dark Agouti rats (DA/OlaHsd, Janvier, France; DA/HanRj according to Janvier nomenclature). Genotyping was performed as previously described (for details, please see Wang et al., 2024).

Rats were housed in Makrolon Type IV cages under standard laboratory conditions in temperature- and humidity-controlled animal holding rooms (22 ± 2 °C, 40–70 %, 12:12 h light/dark cycle), with standard rodent chow (Altromin, Lage, Germany) and water available ad libitum. To mitigate the low survival rates reported for *Tph2*$^{-/-}$ rats (Kaplan et al., 2016), we applied intensive health monitoring, provided agar food, and reduced litter sizes after genotyping by randomly culling over-represented genotypes for large litters (for details on animal housing and health care, please see Wang et al., 2024).

To obtain offspring for experiments, we applied a heterozygous breeding strategy that resulted in litters with roughly the expected Mendelian ratios, typically consisting of ~20–30 % *Tph2*$^{-/-}$, ~40–50 % *Tph2*$^{+/-}$, and ~ 20–30 % *Tph2*$^{+/+}$ rat pups, as reported previously (Wang et al., 2024). To avoid litter effects, we included only litters with *Tph2*$^{+/-}$ and/or *Tph2*$^{-/-}$ rat pups and *Tph2*$^{+/+}$ littermate controls, with both sexes present, in the experiments. In total, $N = 15$ litters were included in the study, with $N = 94$ rat pups. For identification of pups, paw tattoos were applied (using non-toxic Ketchum permanent tattoo inks green paste, Ketchum Manufacturing Inc., Brockville, Canada; for details, please see Wang et al., 2024).

2.2. Experimental design

To study the effects of nesting conditions on social adjustment and socio-affective communication in a genotype- and sex-dependent manner, we applied an experimental design with four independent variables, namely sex, genotype, nesting condition, and social context. From the day of birth, i.e., postnatal day (P) 0, litters were randomly assigned to either standard nesting (SN, one mother with her litter; N = 9 litters) or communal nesting (CN, two mothers with their two litters; $N = 6$ litters; for details on nesting conditions, please see Wang et al., 2024).

As reported previously (Wang et al., 2024), we recorded isolation-induced ultrasonic vocalizations (USV) on P2, P4, P6, P8, P10, P12, and P14 during the isolation box test, i.e., under non-social test conditions with no social cues being present. This was paralleled by the assessment of developmental milestones, somatosensory reflexes, and thermoregulatory capabilities (for details, please see Wang et al., 2024).

On P7, we conducted the maternal preference test to study the emission of isolation-induced USV under social test conditions in the presence of other rats and to assess whether the mothers' preference for *Tph2*$^{-/-}$ rat pups versus *Tph2*$^{+/+}$ littermate controls is driven by differences in the emission of isolation-induced USV in such a competitive situation. The maternal preference test was conducted on P7 because rat pups reach substantial emission rates of isolation-induced USV at the end of the first week of life (for details, please see Wang et al., 2024). On P11, we performed the homing test as a proxy for maternal affiliation to study the emission of isolation-induced USV under social test conditions, i.e., with soiled bedding from the home cage with maternal odor being present, but in the absence of other rats. The homing test is typically conducted during the second week of life, i.e., at a developmental stage when the rat pups had acquired the necessary motor capabilities. In line with the experimental approach applied by Liu et al. (2023), we conducted the homing test at P11.

The test durations for the isolation box test, the maternal preference test, and the homing test were 10 min each. The same rat pups were used for the isolation box test and the homing test. For the maternal preference test, only pairs of *Tph2*$^{-/-}$ rat pups and *Tph2*$^{+/+}$ littermate controls were used. Of note, one *Tph2*$^{+/+}$ littermate control exposed to CN was injured while the two females present in the cage were competing for it at P10. It was excluded from all data analyses, except the maternal preference test conducted at P7. All behavioral tests were performed during the light phase. Experimenters were blind to genotypes during data acquisition and analysis.

2.3. Isolation box test

For eliciting isolation-induced USV under non-social test conditions, we conducted the isolation box test on P2, P4, P6, P8, P10, P12, and P14. As previously described (Wang et al., 2024), the rat pups were separated from their mother and littermates and individually isolated in an isolation box for 10 min under room temperature. Importantly, no social cues were present during the isolation box test, and the box was cleaned with a 0.1 % acetic acid solution and dried with paper towels between pups. For broadband, high-resolution ultrasound recording of isolation-induced USV, an UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics, Berlin, Germany) was placed in the roof of the isolation box, 12 cm above the floor.

2.4. Maternal preference test

For eliciting isolation-induced USV under social test conditions in the presence of other rats, we applied the maternal preference test on P7. Importantly, social cues were present in the maternal preference test, i.e., the mother and another pup. Given the competitive nature of this situation, this allowed us not only to study the emission of isolation-induced USV under social test conditions but also to assess whether the mothers' preference for *Tph2*$^{-/-}$ rat pups versus *Tph2*$^{+/+}$ littermate controls is driven by differences in the emission of isolation-induced USV. As previously described (Wang et al., 2024), an open field (60x60x60 cm) with two wire cylinders (diameter: 10.5 cm; height: 12 cm; with clean bedding) positioned at opposite corners under dim red-light conditions (room temperature: 20–21 °C) was used for the maternal preference test. Each mother was individually placed in the center of the open field for 10 min and simultaneously exposed to one *Tph2*$^{-/-}$ rat pup and one *Tph2*$^{+/+}$ littermate control from her own litter, placed in separate wire cylinders. Of note, mothers were habituated to the open field the previous day, and each mother was exposed to all pairs of *Tph2*$^{-/-}$ rat pups and *Tph2*$^{+/+}$ littermate controls present in her litter, i.e., if possible, a mother was repeatedly tested, but no pup was used more than once. The open field and the wire cylinders were cleaned with 0.1 % acetic acid solution and dried with paper towels between rat pups. Maternal behavior was video recorded (EQ150, EverFocus, Taipei, Taiwan) and analyzed using The Observer XT 12 (Noldus, Wageningen, The Netherlands). For assessing maternal preference, a 2 cm radius around the wire cylinder was defined, and the time the mother spent with her nose within this radius around the cylinders was quantified and defined as a maternal investigation, reflecting attention directed toward the pup inside. After quantifying maternal investigation for each pup, the maternal preference was evaluated and a maternal preference index (MPI) was calculated as follows: MPI % = (duration of maternal investigation for one pup / duration of maternal investigation for both pups) x 100. Of note, data on maternal behavior were previously reported (Wang et al., 2024) and are included here for assessing whether the mothers' preference for *Tph2*$^{-/-}$ rat pups versus *Tph2*$^{+/+}$ littermate controls is driven by differences in the emission of isolation-induced USV. To this aim, the emission of isolation-induced USV was recorded separately for each rat pup and synchronized to the behavior displayed by the mother. For broadband, high-resolution ultrasound recording of isolation-induced USV, an UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics) was placed in the top of each of the two wire cylinders, 9.5 cm above the floor, i.e., two microphones were used, one for each of the two wire cylinders, to identify the sender emitting the isolation-induced USV.

2.5. Homing test

For eliciting isolation-induced USV under social test conditions in the absence of other rats, we performed the homing test on P11. As previously described (Wang et al., 2024), the pups were separated from their mother and littermates and individually isolated in a standard Makrolon Type III cage for 10 min under room temperature. Importantly, social cues were present in the homing test, i.e., soiled bedding from the home cage with maternal odor. The soiled bedding was evenly spread over 1/3 of the cage floor on one side, while the rest was covered with clean bedding. The cage was cleaned with 0.1 % acetic acid solution and dried with paper towels between rat pups. For behavior scoring, video recording was performed (IP camera RLC-410-5MP; Reolink, Hong Kong, China). For broadband, high-resolution ultrasound recording of isolation-induced USV, an UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics) was positioned 24 cm above the cage floor.

2.6. Ultrasound recording and analysis

The microphones used for ultrasound recording during the isolation box test, the maternal preference test, and the homing test were connected via an UltraSoundGate 416 USGH audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250,000 Hz in 16-bit format by Avisoft RECORDER (version 2.97; Avisoft Bioacoustics). For analyzing isolation-induced USV, we first used DeepSqueak (DS; version 3.1.0; Coffey et al., 2019) with a custom-trained faster regional convolutional neural network (FASTER-RCNN; for details on the accuracy of call detection by DS and manual corrections, please see Wang et al., 2024).

For isolation-induced USV emitted during the isolation box test and the maternal preference test, we next determined call emission rate (n/min) and calling time ratio (%), with the calling time ratio representing the proportion of time spent calling relative to the total test duration, e.g., the 10 min in the isolation box. Then, we assessed four acoustic call features for every single isolation-induced USV emitted, namely call duration (ms), peak frequency (kHz), peak amplitude (au), and frequency modulation (kHz; for definitions of acoustic call features, please see Wang et al., 2024). For the maternal preference test, the two ultrasound recordings, i.e., one for each wire cylinder, were analyzed in parallel and the source of every single isolation-induced USV was determined by an experienced observer comparing peak amplitude and potential overtones. If isolation-induced USV were detected in both ultrasound recordings, the isolation-induced USV were assigned to the rat pup in the wire cylinder from which the louder signal was obtained. Of note, data on isolation-induced USV emitted during the exposure to the

isolation box were previously reported (Wang et al., 2024) and are included here for the sake of comparison, i.e., for studying the effects of social context in modulating socio-affective communication in *Tph2*-deficient rat pups and wildtype littermate controls.

For the homing test, we applied a slightly different approach and extracted the isolation-induced USV emitted by rat pups while being in the soiled bedding zone or the clean bedding zone, respectively, with a self-written MATLAB (version 2022a) script. To this aim, we synchronized video and ultrasound recordings by beeping a timer under the camera and microphone. For behavior scoring, the cage floor was virtually divided into 3 zones, namely a soiled bedding zone (1/3), a central zone (1/3) with clean bedding, and a clean bedding zone (1/3). The time periods the pup spent in the soiled bedding zone and the clean bedding zone, respectively, were determined using The Observer XT 12 (Noldus, Wageningen, The Netherlands), and isolation-induced USV were sorted accordingly. Thus, call emission rate (n/min), calling time ratio (%), call duration (ms), peak frequency (kHz), peak amplitude (au), and frequency modulation (kHz; for definitions of acoustic call features, please see Wang et al., 2024) were assessed not only for the whole test arena, but also separately for the clean bedding zone and the soiled bedding zone. Of note, call emission rate (n/min) and calling time ratio (%) for the whole test arena were previously reported (Wang et al., 2024).

Finally, we determined call subtypes for isolation-induced USV emitted by rat pups during the maternal preference test and the homing test by means of density plots using our previously established approach (Wöhr, 2014). For the maternal preference test, two types of density plots were generated, i.e., one depicting peak frequency versus call duration and another one depicting peak frequency versus frequency modulation (>65,000 calls). For the homing test, the same two density plots were generated, but also separately for isolation-induced USV emitted by rat pups while being in the soiled bedding zone (>120,000 calls) or the clean bedding zone (only for *Tph2*$^{-/-}$ rat pups, >12,000 calls).

2.7. Statistical analysis

To gain first insight into the effects of social context in modulating socio-affective communication, we compared isolation-induced USV emitted during the exposure to the isolation box test with those recorded during the maternal preference test and the homing test. Specifically, we used ANOVAs for repeated measurements with the between-subject factors sex and genotype, and the within-subject factor social context, for comparing isolation-induced USV emitted during the maternal preference test with those recorded during the isolation box test conducted before and after the maternal preference test (i.e., before, during, and after social context change; five test days). A similar strategy was applied for the homing test and isolation-induced USV emitted during the homing test were compared to those recorded during the isolation box test conducted before and after the homing test (i.e., before, during, and after social context change; five test days). Emission rates and relevant acoustic call features, including call duration, peak frequency, and frequency modulation, were compared. Peak amplitude was not compared because recording distances of microphones and ultrasound gain settings differed across the three recording test conditions, i.e., maternal preference test, homing test, and isolation box test. ANOVAs with the between-subject factors sex, genotype, and nesting condition were conducted for comparing the isolation-induced USV emitted during the maternal preference test and the homing test (separately for the entire test arena, including both the clean and the soiled bedding zone, and the soiled bedding zone only). To compare the isolation-induced USV emitted by *Tph2*$^{-/-}$ rat pups depending on the location of the pup during call emission in the homing test, i.e., clean bedding zone versus soiled bedding zone, we used ANOVAs for repeated measurements with the between-subject factors sex and nesting condition, and the within-subject factor zone. For assessing whether the preference displayed by the mother in the maternal preference test is linked to the emission of isolation-induced USV, Pearson correlation coefficients were calculated. Pearson correlation coefficients were also calculated to assess whether maternal investigation was linked to the emission of isolation-induced USV. For ANOVAs including the between-subject factor genotype, *Tph2*$^{-/-}$ rat pups and *Tph2*$^{+/+}$ littermate controls were included for the sake of clarity (for the results obtained using ANOVAs including *Tph2*$^{+/-}$ rat pups as well, please see the supplementary Figures). In case of lack of sphericity, Greenhouse-Geisser correction was applied. ANOVAs were followed by *post-hoc* tests when appropriate. Of note, for ANOVAs for repeated measurements, all five test days (i.e., before, during, and after social context change) were included, yet post-hoc tests were applied exclusively for comparing P7 (maternal preference test) and P11 (homing test) to the days before and after, i.e., P7 vs. P6/ P8 and P11 vs. P10/ P12, respectively, for the sake of clarity. A p-value of <0.050 was considered statistically significant. Statistical analyses were performed using SPSS (version 29, IBM; Armonk, NY, USA). Figures were created using Prism (GraphPad Software, Boston, MA, USA) and BioRender (BioRender, Toronto, Canada).

3. Results

3.1. Developmental trajectories affected by the social context

To gain first insight into the effects of social context in modulating socio-affective communication in *Tph2*-deficient rat pups and wildtype littermate controls, we compared the emission of isolation-induced USV under social test conditions, i.e., during the maternal preference test and the homing test, with the emission of isolation-induced USV under non-social test conditions, i.e., during the isolation box test, previously reported (Wang et al., 2024). While social cues were present in the maternal preference test, i.e., the mother and another pup, and the homing test, i.e., soiled bedding from the home cage with maternal odor, no social cues were present during the isolation box test. As depicted in the experimental overview (Fig. 1), we compared the USV emitted during the maternal preference test conducted on P7 with the USV emitted by the same rat pups in the isolation box test on the experimental days before and after, i.e., on P4/ P6 and P8/ P10. A similar strategy was applied for the USV emitted during the homing test on P11, and the USV were compared to the USV emitted by the same rat pups in the isolation box test on P8/ P10 and P12/ P14.

We first analyzed the developmental trajectories of isolation-induced USV and found that these trajectories are affected by the maternal preference test on P7. Specifically, the emission rate of isolation-induced USV reached particularly high levels during the maternal preference test (context: $F_{4,168} = 12.699$; $p < .001$; Fig. 2A). Importantly, most prominent genotype differences in the emission of isolation-induced USV were observed during this test (Fig. 2A). Highest emission rates were seen in *Tph2*$^{+/+}$ littermate controls, reaching ~190 calls per minute. Much lower emission rates of only ~90 calls per minute were seen in *Tph2*$^{-/-}$ rat pups. Genotype differences were driven by at least two factors. Firstly, *Tph2* deficiency was associated with strongly reduced emission rates irrespective of test condition (genotype: $F_{1,42} = 180.200$; $p < .001$). Secondly, *Tph2* deficiency was associated with a lack of adjustments to changes in the social environment (context x genotype: $F_{4,140} = 8.346$; $p < .001$). In fact, emission rates were increased in *Tph2*$^{+/+}$ littermates but not *Tph2*$^{-/-}$ pups under the social test conditions of the maternal preference test. The combination of those two factors resulted in genotype differences that were more prominent during the maternal preference test, as compared to the isolation box test, reported previously (Wang et al., 2024). Specifically, emission rates in *Tph2*$^{+/+}$ littermates increased from ~150 calls per minute during the isolation box test on P6 to ~190 calls per minute during the maternal preference test on P7, followed by a slight reduction to ~170 calls per minute during the isolation box test on P8. In contrast, the emission rates in *Tph2*$^{-/-}$ pups remained mostly constant at a relatively low level of ~90 calls per

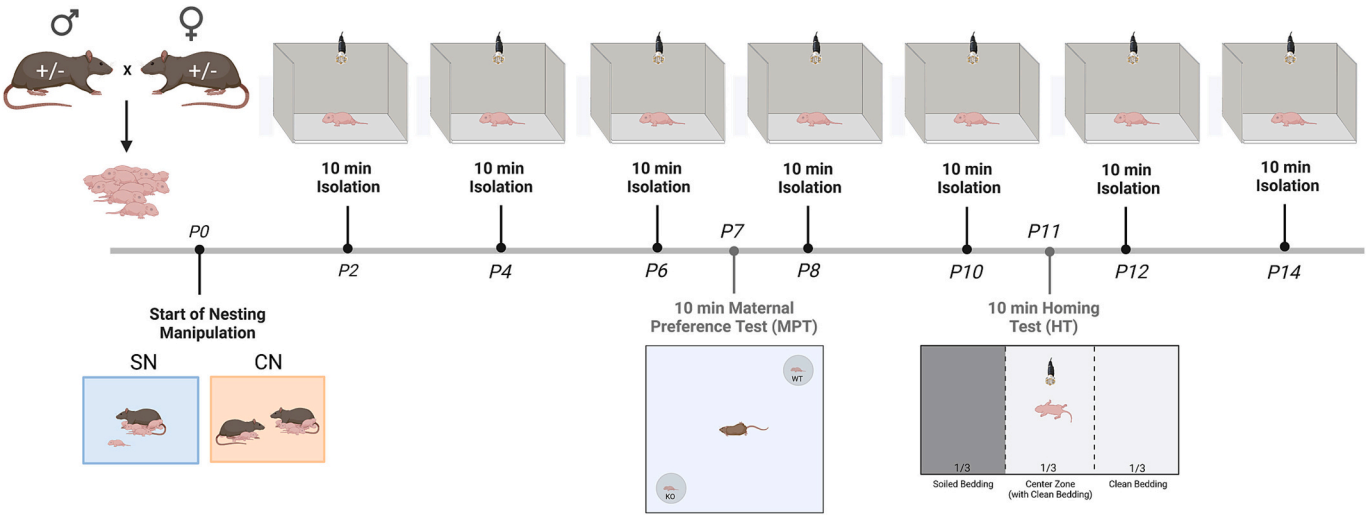

Fig. 1. Overview of the experimental design for assessing the effects of social context in modulating socio-affective communication in *Tph2*-deficient rat pups and wildtype littermate controls. Emission of isolation-induced ultrasonic vocalizations (USV) under social test conditions, i.e., during the maternal preference test (MPT) and the homing test (HT), was compared to their emission under non-social test conditions, i.e., during the isolation box test. While social cues were present in the maternal preference test, i.e., the mother, and the homing test, i.e., soiled bedding from the home cage with maternal odor, no social cues were present during the isolation box test. P = postnatal day, SN = standard nesting, CN = communal nesting.

minute, irrespective of test condition. Despite prominent sex differences, with females calling more than males, sex did not modulate the context and genotype effects on call emission rate (sex: $F_{1,42} = 7.653$; $p = .008$; all other *p*-values >.100; not shown in detail). Together, this suggests that *Tph2* deficiency is associated with a reduction and/or delay in the capability and/or motivation to appropriately adjust to changes in the social environment. *Tph2*$^{+/+}$ littermates but not *Tph2*$^{-/-}$ pups might adjust by increasing their emission rates to the more competitive test situation of the maternal preference test, where two rat pups compete for the attention of the mother.

A different pattern, however, was evident for the calling time ratio (Fig. 2B). While the social context led to prominent alterations in the developmental trajectory (context: $F_{3.245,136.284} = 6.335$; $p < .001$), and clear genotype differences were evident (genotype: $F_{1,42} = 112.588$; $p < .001$), the calling time ratio was reduced during the maternal preference test, as compared to the isolation box test. A slight reduction in the calling time ratio was seen in both *Tph2*$^{-/-}$ rat pups and *Tph2*$^{+/+}$ littermate controls, albeit the effects of social context tended again to be more prominent in *Tph2*$^{+/+}$ littermates (context x genotype: $F_{3.245,136.284} = 2.349$; $p = .070$). Calling time ratio was not affected by sex, and sex did not modulate the context and genotype effects (all p-values >.100; not shown in detail).

The acoustic features of the isolation-induced USV emitted during the maternal preference test were likewise modulated by social context. While *Tph2*$^{+/+}$ littermate controls typically displayed prominent adjustments, changes in the acoustic features of isolation-induced USV emitted by *Tph2*$^{-/-}$ rat pups were often absent or much weaker. Firstly, while the isolation-induced USV emitted by *Tph2*$^{-/-}$ pups maintained a slightly longer call duration of ~110 to ~120 ms from P4 to P10, including P7 during the maternal preference test, *Tph2*$^{+/+}$ littermates displayed a moderate decrease in call duration from ~120 to ~100 ms over the same period during the isolation box test, with an abrupt decline to ~85 ms on P7 during the maternal preference test (context: $F_{3.011,126.451} = 11.614$; $p < .001$; genotype: $F_{1,42} = 8.376$; $p = .006$; context x genotype: $F_{3.011,126.451} = 4.822$; $p = .003$; Fig. 2C). Secondly, the developmental patterns for peak frequency of isolation-induced USV emitted by *Tph2*$^{-/-}$ pups and *Tph2*$^{+/+}$ littermates differed over time. *Tph2*$^{-/-}$ pups showed a decrease of ~4 kHz from P4 to P7, followed by a moderate increase from P8 to P10, whereas a continuous decrease was observed in *Tph2*$^{+/+}$ littermates from P4 to P10, including P7 during the maternal preference test (context: $F_{2.332,97.951} = 9.323$; $p < .001$; genotype: $F_{1,42} = 0.010$; $p = .921$; context x genotype: $F_{2.332,97.951} = 4.476$; $p = .010$; Fig. 2D). Finally, while *Tph2*$^{-/-}$ pups displayed a relatively stable level of frequency modulation over time, *Tph2*$^{+/+}$ littermates displayed a moderate increasing trend, reaching ~7 kHz on P10. In fact, the peak level of close to ~8 kHz was also observed on P7 during the maternal preference test in *Tph2*$^{+/+}$ littermates (context: $F_{2.714,113.977} = 8.215$; $p < .001$; genotype: $F_{1,42} = 18.755$; $p < .001$; context x genotype: $F_{2.714,113.977} = 3.646$; $p = .018$; Fig. 2E). Of note, the acoustic features were not affected by sex, and sex did not modulate the context and genotype effects (all *p*-values >.100; not shown in detail).

In support of a modulatory role of social context, the developmental trajectories of isolation-induced USV were also affected by the homing test on P11. Both *Tph2*$^{-/-}$ rat pups and *Tph2*$^{+/+}$ littermate controls displayed prominent alterations, as compared to the isolation box test. However, the pattern of alterations differed depending on genotype. *Tph2*$^{-/-}$ pups maintained a relatively stable call emission rate of about 80 to 100 calls per minute across the four days of the isolation box test, with an abrupt peak of around 140 calls per minute on P11 during the homing test. In contrast, call emission rates observed in *Tph2*$^{+/+}$ littermates remained at about 180 to 190 calls per minute over all five days, including P11 (context: $F_{3.329,199.730} = 7.714$; $p < .001$; genotype: $F_{1,60} = 169.276$; $p < .001$; context x genotype: $F_{3.329,199.730} = 5.196$; $p = .001$; Fig. 2F). As for the calling time ratio, a relatively low level of about 15–18 % during the isolation box test and a mild increase to the level of ~22 % on P11 during the homing test were observed in *Tph2*$^{-/-}$ pups. Although *Tph2*$^{+/+}$ littermates displayed a relatively high calling time ratio of about 30–33 % in the isolation box test over time, a sharp reduction to a level similar to *Tph2*$^{-/-}$ pups (about 18–20 %) was evident on P11 during the homing test (context: $F_{4,240} = 5.861$; $p < .001$; genotype: $F_{1,60} = 122.056$; $p < .001$; context x genotype: $F_{4,240} = 16.631$; $p < .001$; Fig. 2G). Similar to the maternal preference test, sex did not modulate the context and genotype effects on call emission rate seen in response to the homing test, despite prominent sex differences, with females calling more than males (sex: $F_{1,60} = 8.546$; $p = .005$; all other p-values >.100; not shown in detail).

Moreover, the acoustic features of the isolation-induced USV emitted during the homing test were partially altered. The call duration of isolation-induced USV emitted by *Tph2*$^{-/-}$ rat pups ranged between ~100 and 120 ms during the isolation box test, and showed a slight reduction to ~90 ms during the homing test. The reduction of the call duration during the homing test was also observed in *Tph2*$^{+/+}$ littermate

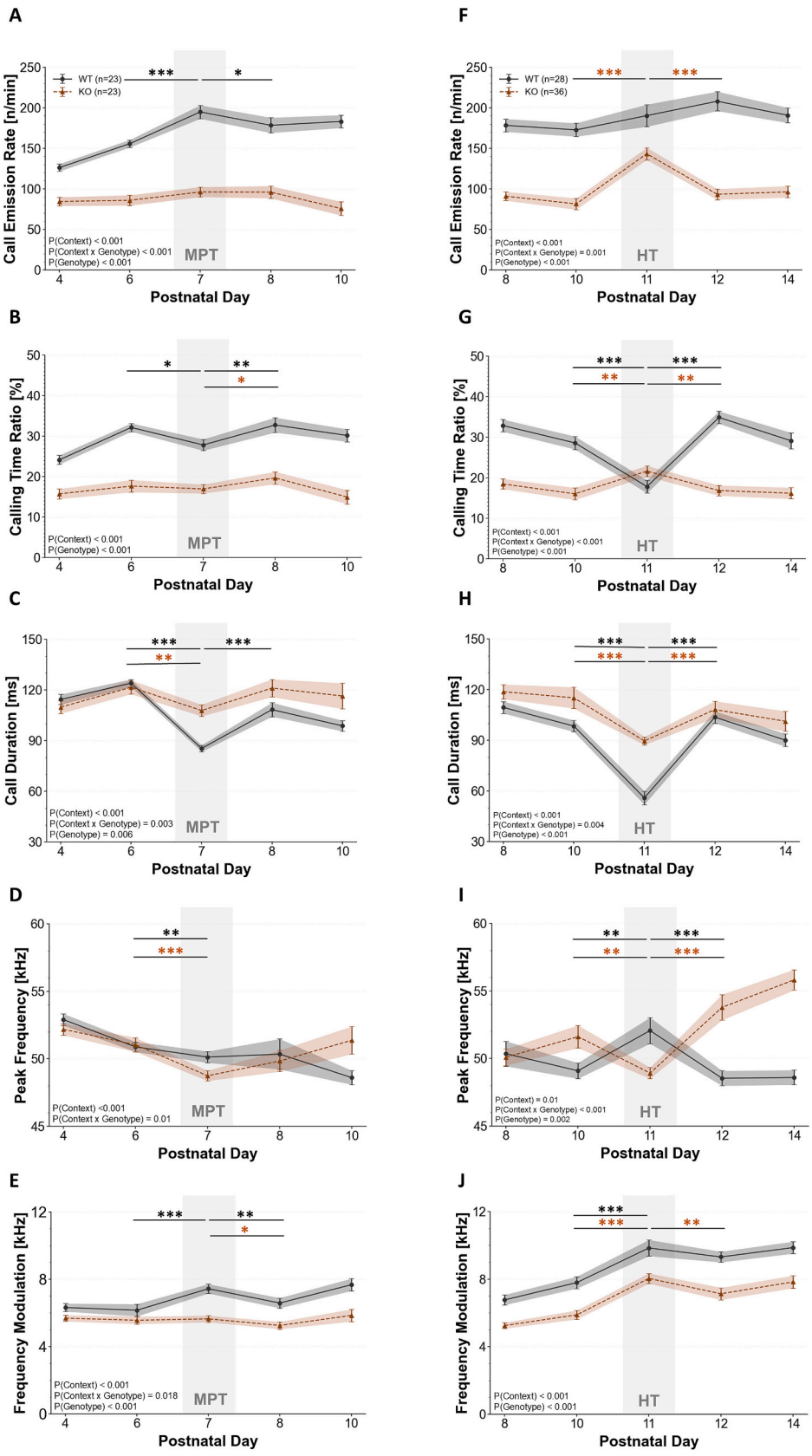

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Fig. 2. *Tph2* deficiency in neonatal rats leads to alterations in social adjustment and socio-affective communication, as reflected in the emission of isolation-induced ultrasonic vocalizations (USV) when exposed to social test conditions, i.e., maternal preference test and homing test, compared to non-social test conditions, i.e., isolation box test. Developmental trajectories of (A) call emission rate (n/min), (B) calling time ratio (%), (C) call duration (ms), (D) peak frequency (kHz), and (E) frequency modulation (kHz) of isolation-induced USV emitted by *Tph2*$^{-/-}$ knockout (KO; red triangle) rat pups and *Tph2*$^{+/+}$ wildtype (WT, black circle) littermate controls across P4 to P10 during the exposure to the isolation box and on P7 during the maternal preference test (MPT, light gray highlighting). Developmental trajectories of (F) call emission rate (n/min), (G) calling time ratio (%), (H) call duration (ms), (I) peak frequency (kHz), and (J) frequency modulation (kHz) of isolation-induced USV emitted by *Tph2*$^{-/-}$ knockout (KO; red triangle) rat pups and *Tph2*$^{+/+}$ wildtype (WT, black circle) littermate controls across P8 to P14 during the exposure to the isolation box and on P11 during the homing test (HT, light gray highlighting). P = postnatal day. Data are expressed as mean ± SEM. Within WT: $^{***(black)}$ $p < .001$; $^{**(black)}$ $p < .01$; $^{*(black)}$ $p < .05$. Within KO: $^{***(red)}$ $p < .001$; $^{**(red)}$ $p < .010$; $^{*(red)}$ $p < .050$. Of note, all five test days (i.e., before, during, and after social context change) were included in the ANOVAs for repeated measurements, yet *post-hoc* tests were applied exclusively for comparing P7 (maternal preference test) and P11 (homing test) to the days before and after, i.e., P7 vs. P6/ P8 and P11 vs. P10/ P12, respectively, for the sake of clarity. Number of rats in A-E: N(WT) = 23, N(KO) = 23; number of rats in F-J: N(WT) = 28, N(KO) = 36. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

controls, but to a greater extent, where the call duration of ~90 to 110 ms in the isolation box test reduced to ~55 to 60 ms during the homing test, representing an almost 50 % reduction (context: $F_{4,240} = 32.121$; $p < .001$; genotype: $F_{1,60} = 14.940$; $p < .001$; context x genotype: $F_{4,240} = 3.961$; $p = .004$; Fig. 2H). As for peak frequency, an increase from ~50 to ~56 kHz over time was observed in *Tph2*$^{-/-}$ pups during the isolation box test, while a slight reduction to ~49 kHz was evident on P11 during the homing test. In contrast, *Tph2*$^{+/+}$ littermates displayed a moderate decrease from ~50 to ~47 kHz in peak frequency across exposures to the isolation box test, but displayed an increase to ~52–49 kHz on P11 during the homing test (context: $F_{4,240} = 3.406$; $p = .010$; genotype: $F_{1,60} = 10.298$; $p = .002$; context x genotype: $F_{4,240} = 22.114$; $p < .001$; Fig. 2I). Finally, frequency modulation of the isolation-induced USV showed an increasing pattern across exposures to the isolation box test irrespective of genotype, while a similar slight increase was observed on P11 during the homing test (context: $F_{3.270,196.221} = 71.729$; $p < .001$; genotype: $F_{1,60} = 23.544$; $p < .001$; context x genotype: $F_{3.270,196.221} = 0.670$; $p = .584$; Fig. 2J; the result pattern obtained for *Tph2*$^{+/-}$ pups was similar to the one obtained for *Tph2*$^{+/+}$ littermates; for details, please see Supplementary Fig. S1; not shown in detail). Of note, sex affected the acoustic features. Firstly, isolation-induced USV emitted by females were shorter than the ones emitted by males (sex: $F_{1,60} = 5.152$; $p = .027$). Secondly, context effects on call duration were modulated by sex (context x sex: $F_{4.240} = 3.180$; $p = .014$; all other *p*-values >.100; not shown in detail).

In summary, during the maternal preference test and the homing test, the emission of isolation-induced USV and certain acoustic features (e.g., call duration and peak frequency) were altered in a genotype-dependent manner, as compared to the developmental trajectories typical for isolation-induced USV emitted in the isolation box test. When exposed to the mother during the maternal preference test on P7, *Tph2*$^{-/-}$ rat pups displayed relatively weak changes in USV emission and acoustic features. In contrast, a prominent increase in the emission rate was evident in *Tph2*$^{+/+}$ littermate controls, and their USV were characterized by shorter call duration and higher levels of frequency modulation. When being exposed to the mother's odor during the homing test on P11, *Tph2*$^{-/-}$ pups displayed a substantial increase in USV emission, along with a mild reduction in call duration, a mild increase in frequency modulation, and a decrease in peak frequency. In contrast, *Tph2*$^{+/+}$ littermates did not show the abrupt increase of the USV emission but displayed a substantial decrease in the calling time ratio due to the much shorter calls emitted during the homing test. Finally, the isolation-induced USV emitted by *Tph2*$^{+/+}$ littermates were characterized by higher levels of frequency modulation and higher peak frequency during the homing test.

3.2. Effects of nesting conditions: homing test

We next assessed the effects of nesting conditions on social adjustment and socio-affective communication during the homing test on P11 in the presence of maternal odor (Fig. 3A). The emission of isolation-induced USV was affected by genotype and nesting conditions, yet no robust evidence in support of an interaction was obtained (Fig. 3B). Specifically, *Tph2*$^{-/-}$ rat pups emitted fewer isolation-induced USV than *Tph2*$^{+/+}$ littermate controls (genotype: $F_{1,56} = 10.188$; $p = .002$; Fig. 3C). While nesting conditions affected the emission of isolation-induced USV, with emission rates being lower under CN than SN conditions (nesting: $F_{1,56} = 5.811$; $p = .019$), nesting conditions did not modulate the genotype effect (nesting x genotype: $F_{1,56} = 0.141$; $p = .709$). Calling time ratios were comparable in *Tph2*$^{-/-}$ pups and *Tph2*$^{+/+}$ littermates (genotype: $F_{1,56} = 3.777$; $p = .057$; Fig. 3D), and while CN exerted again an inhibitory effect, as compared to SN (nesting: $F_{1,56} = 5.780$; $p = .020$), no evidence for an interaction between nesting conditions and genotype effects was obtained (nesting x genotype: $F_{1,56} = 0.136$; $p = .713$). Moreover, *Tph2* deficiency affected all four acoustic features of isolation-induced USV assessed, i.e., call duration, peak frequency, peak amplitude, and frequency modulation. Specifically, *Tph2*$^{-/-}$ pups emitted much longer isolation-induced USV than *Tph2*$^{+/+}$ littermates, irrespective of nesting conditions (genotype: $F_{1,56} = 55.580$; $p < .001$; nesting: $F_{1,56} = 1.080$; $p = .303$; nesting x genotype: $F_{1,56} = 0.689$; $p = .410$; Fig. 3E). Isolation-induced USV emitted by *Tph2*$^{-/-}$ pups were further characterized by lower levels of peak frequency, in comparison to *Tph2*$^{+/+}$ littermates, again irrespective of nesting conditions (genotype: $F_{1,56} = 9.142$; $p = .004$; nesting: $F_{1,56} = 2.649$; $p = .109$; nesting x genotype: $F_{1,56} = 0.104$; $p = .749$; Fig. 3F). In contrast to peak frequency, the peak amplitude of isolation-induced USV emitted by *Tph2*$^{-/-}$ pups was substantially higher than in *Tph2*$^{+/+}$ littermates, under both SN and CN conditions (genotype: $F_{1,56} = 30.636$; $p < .001$; nesting: $F_{1,56} = 0.925$; $p = .340$; nesting x genotype: $F_{1,56} = 0.067$; $p = .797$; Fig. 3G). As for frequency modulation, *Tph2*$^{-/-}$ pups emitted isolation-induced USV with lower levels of frequency modulation, as compared to *Tph2*$^{+/+}$ littermates (genotype: $F_{1,56} = 10.431$; $p = .002$; Fig. 3H). Interestingly, however, frequency modulation was affected by nesting conditions and CN led to a reduction in the level of frequency modulation (nesting: $F_{1,56} = 18.986$; $p < .001$). Again, however, nesting conditions did not modulate the genotype effect (nesting x genotype: $F_{1,56} = 0.465$; $p = .498$; the result pattern obtained for *Tph2*$^{+/-}$ pups was similar to the one obtained for *Tph2*$^{+/+}$ littermates; for details, please see Supplementary Fig. S2; not shown in detail). Sex did not modulate the effects of communal nesting, with the exception of modulatory effects on peak frequency. Females emitted isolation-induced USV characterized by particularly high levels of peak frequency under CN conditions, whereas in males peak frequency was particularly high under SN conditions (nesting x sex: $F_{1,56} = 5.414$; $p = .024$; all other *p*-values >.100; not shown in detail).

In addition, a detailed spectrographic analysis of more than 120,000 individual isolation-induced USV emitted during the homing test was conducted. Density plots revealed four major clusters of call subtypes (Fig. 3I-P), overall similar to the ones reported previously for the isolation box test, but with characteristic alterations (Wang et al., 2024). Specifically, by plotting peak frequency against call duration two call clusters were revealed in *Tph2*$^{+/+}$ littermate controls (Fig. 3I). One prominent cluster was characterized by relatively low peak frequencies roughly between 45 and 55 kHz, a bit higher in frequency but similar to

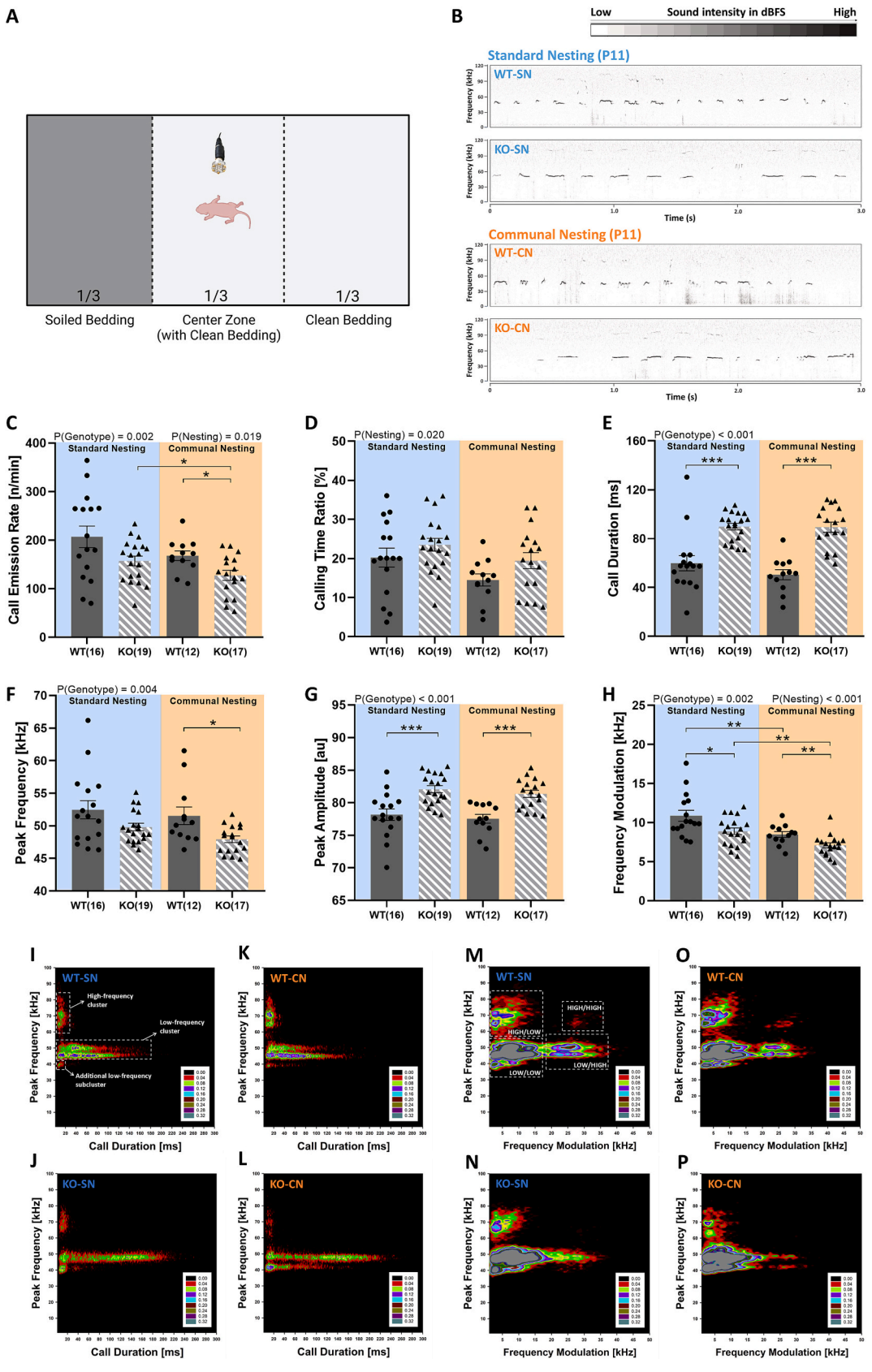

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Fig. 3. *Tph2* deficiency in neonatal rats causes robust changes in socio-affective communication under social test conditions, i.e., homing test, only mildly modulated by communal nesting, as reflected in the emission of isolation-induced ultrasonic vocalizations (USV) and the clustering of their subtypes during the homing test. (A) Overview of the homing test as a proxy for maternal affiliation in *Tph2*$^{-/-}$ knockout (KO) rat pups, compared to *Tph2*$^{+/+}$ wildtype (WT) littermate controls. (B) Exemplary spectrograms of isolation-induced USV emitted during the homing test, depending on genotype and nesting condition. (C) Call emission rate (n/min), (D) calling time ratio (%), (E) call duration (ms), (F) peak frequency (kHz), (G) peak amplitude (au), and (H) frequency modulation (kHz) of isolation-induced USV emitted during the homing test on postnatal day 11 by *Tph2*$^{-/-}$ knockout (KO; triangle) rat pups and *Tph2*$^{+/+}$ wildtype (WT, circle) littermate controls. (I-P) Density plots depicting the distribution of individual isolation-induced USV in *Tph2*$^{-/-}$ knockout (KO) rat pups, as compared to *Tph2*$^{+/+}$ wildtype (WT) littermate controls, depending on nesting condition, i.e., WT-SN (I, M; ~33,000 calls), WT-CN (K, O; ~20,000 calls), KO-SN (J, N; ~30,000 calls), and KO-CN (L, P; ~22,000 calls). Colour coding reflects frequencies as percentages. SN = standard nesting (blue), CN = communal nesting (orange). Data are expressed as mean ± SEM. *** $p < .001$; ** $p < .010$; * $p < .050$. Number of rats: N(WT-SN) = 16, N(KO-SN) = 19, N(WT-CN) = 12, N(KO-CN) = 17. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the one seen under the non-social test conditions of the isolation box test (Wang et al., 2024), yet with an additional subcluster roughly around 40 kHz. A second cluster was characterized by relatively high peak frequencies roughly between 60 and 80 kHz. This cluster was characterized by a much broader frequency range than a similar cluster observed under the non-social test conditions of the isolation box test (Wang et al., 2024). As previously reported (Wang et al., 2024), call durations in the low-frequency cluster were much longer than in the high-frequency cluster. The low-frequency cluster was the most prominent in all genotypes. Interestingly, however, the call durations represented in this cluster were affected by genotype. In *Tph2*$^{+/-}$ pups and *Tph2*$^{+/+}$ littermates, the majority of calls were shorter than 80 ms, whereas in *Tph2*$^{-/-}$ pups the calls were typically between 60 and 180 ms of duration, except for the additional subcluster. The high-frequency cluster was less prominent and characterized by call durations typically shorter than 20 ms in all genotypes. This was also the case for the additional subcluster roughly around 40 kHz. The relative prevalence, however, was affected by genotype. In *Tph2*$^{+/-}$ pups and *Tph2*$^{+/+}$ littermates, the high-frequency cluster was much more prominent than the additional subcluster, which was particularly weak in *Tph2*$^{+/+}$ littermates. In *Tph2*$^{-/-}$ pups, in contrast, the additional subcluster was much more prominent, while the high-frequency cluster was less prevalent. Of note, CN amplified such genotype differences, and in *Tph2*$^{-/-}$ pups exposed to CN, but not in those exposed to SN, the high-frequency cluster was barely visible (Fig. 3I-L; for *Tph2*$^{+/-}$ pups, please see Supplementary Fig. S2).

When plotting peak frequency against frequency modulation, four clusters of isolation-induced USV were evident in *Tph2*$^{+/+}$ littermate controls, i.e., two low-frequency clusters and two high-frequency clusters, differing in their level of frequency modulation (Fig. 3M). The level of frequency modulation present in the two low-frequency clusters was either below ~17 kHz or above ~20 kHz, extending up to ~35 kHz. For the two high-frequency clusters, the level of frequency modulation was either below ~15 kHz or above ~25 kHz, again extending up to ~30 kHz. In *Tph2*$^{+/+}$ littermates, the cluster characterized by low peak frequencies and low levels of frequency modulation was most prominent (LOW/LOW). The cluster with high peak frequencies but low levels of frequency modulation (HIGH/LOW) and the cluster with low peak frequencies but high levels of frequency modulation (LOW/HIGH) were similar in their prevalence. The weakest cluster was the cluster characterized by high peak frequencies and high levels of frequency modulation (HIGH/HIGH). Importantly, call clustering was affected by genotype. Call clustering was similar in *Tph2*$^{+/-}$ pups and *Tph2*$^{+/+}$ littermates, but clearly altered in *Tph2*$^{-/-}$ pups. While the LOW/LOW and HIGH/LOW clusters characterized by low levels of frequency modulation were likewise present in *Tph2*$^{-/-}$ pups, the LOW/HIGH and HIGH/HIGH clusters characterized by high levels of frequency modulation were weak or even completely absent, respectively. This change was associated with a tendency for a wider range of frequency modulation in the LOW/LOW cluster, extending up to ~20 kHz. Again, nesting conditions played a moderate modulatory role and tended to reduce the prevalence of the HIGH/LOW and LOW/HIGH clusters, and as a consequence amplified genotype differences (Fig. 3M-P; for *Tph2*$^{+/-}$ pups, please see Supplementary Fig. S2). Together, this shows that *Tph2* deficiency in rat pups affects call clustering in a social context and that genotype differences, although only moderately modulated, are amplified by nesting conditions.

Given that the experimental environment of the homing test contains both a social context (soiled bedding) and a non-social context (clean bedding), we next analyzed the USV emission within the soiled bedding zone specifically, in order to get a clearer picture of socio-affective communication through isolation-induced USV in a social context. The result pattern was very similar to the one obtained for all isolation-induced USV emitted independent of the zone (please see Supplementary Fig. S3; not shown in detail), probably because the vast majority of rat pups spent most of their time in the soiled bedding zone, except for a significant proportion of *Tph2*$^{-/-}$ pups (Wang et al., 2024; for density plots, please see Supplementary Fig. S4; not shown in detail).

Moreover, we compared the isolation-induced USV emitted by *Tph2*$^{-/-}$ rat pups between the two zones, i.e., the soiled bedding zone versus the clean bedding zone (Fig. 4A). Of note, the comparison was not conducted in *Tph2*$^{+/-}$ pups and *Tph2*$^{+/+}$ littermate controls due to an insufficient amount of valid data, as only three *Tph2*$^{+/+}$ littermates spent a substantial amount of time in the clean bedding zone. Firstly, call emission rate (zone: $F_{1,32} = 3.278$; $p = .080$; zone x nesting: $F_{1,32} = 0.144$; $p = .707$; Fig. 4B) and calling time ratio (zone: $F_{1,32} < 0.001$; $p = .983$; zone x nesting: $F_{1,32} = 2.561$; $p = .119$; Fig. 4C) were comparable between the two zones in both CN and SN conditions. Notably, the call emission rate was reduced by CN, as compared to SN (nesting: $F_{1,32} = 4.891$; $p = .034$), but not the calling time ratio (nesting: $F_{1,32} = 3.653$; $p = .065$). As for the acoustic features, *Tph2*$^{-/-}$ pups displayed shorter calls in the soiled bedding zone (zone: $F_{1,30} = 13.587$; $p = .001$; Fig. 4D). Although CN had no prominent modulatory effect (nesting: $F_{1,30} = 0.024$; $p = .877$; zone x nesting: $F_{1,30} = 1.733$; $p = .198$), the difference was more pronounced in SN ($t_{18} = 3.618$; $p = .002$) than in CN ($t_{14} = 1.531$; $p = .148$). Peak amplitude was similarly affected. *Tph2*$^{-/-}$ pups emitted USV characterized by lower peak amplitude in the soiled bedding zone (zone: $F_{1,30} = 7.007$; $p = .013$) without a prominent modulatory effect of CN (nesting: $F_{1,30} = 1.327$; $p = .259$; zone x nesting: $F_{1,30} = 1.708$; $p = .201$; Fig. 4F). However, the effect was more pronounced in SN ($t_{18} = 2.747$; $p = .013$) than CN ($t_{14} = 1.054$; $p = .310$). Peak frequency (zone: $F_{1,30} = 2.461$; $p = .127$; zone x nesting: $F_{1,30} = 1.639$; $p = .210$; Fig. 4E) and frequency modulation (zone: $F_{1,30} = 0.186$; $p = .669$; zone x nesting: $F_{1,30} = 0.330$; $p = .570$; Fig. 4G) were comparable between the soiled and the clean bedding zone. Interestingly, CN affected peak frequency (nesting: $F_{1,30} = 4.707$; $p = .038$) and frequency modulation (nesting: $F_{1,30} = 6.510$; $p = .016$), with lower levels being observed under CN than SN. No sex effects were observed, with the exception of a trend for peak frequency (nesting x sex: $F_{1,30} = 3.650$; $p = .066$; all other p-values >.100; not shown in detail). Of note, density plots for isolation-induced USV emitted by pups while in the soiled bedding zone of the homing test were similar to the density plots generated for all USV emitted during the homing test, and no prominent differences were evident when comparing the USV emitted by *Tph2*$^{-/-}$ pups depending on whether they were emitted while being in the clean versus the soiled bedding zone (for density plots, please see Supplementary Fig. S5; not shown in detail).

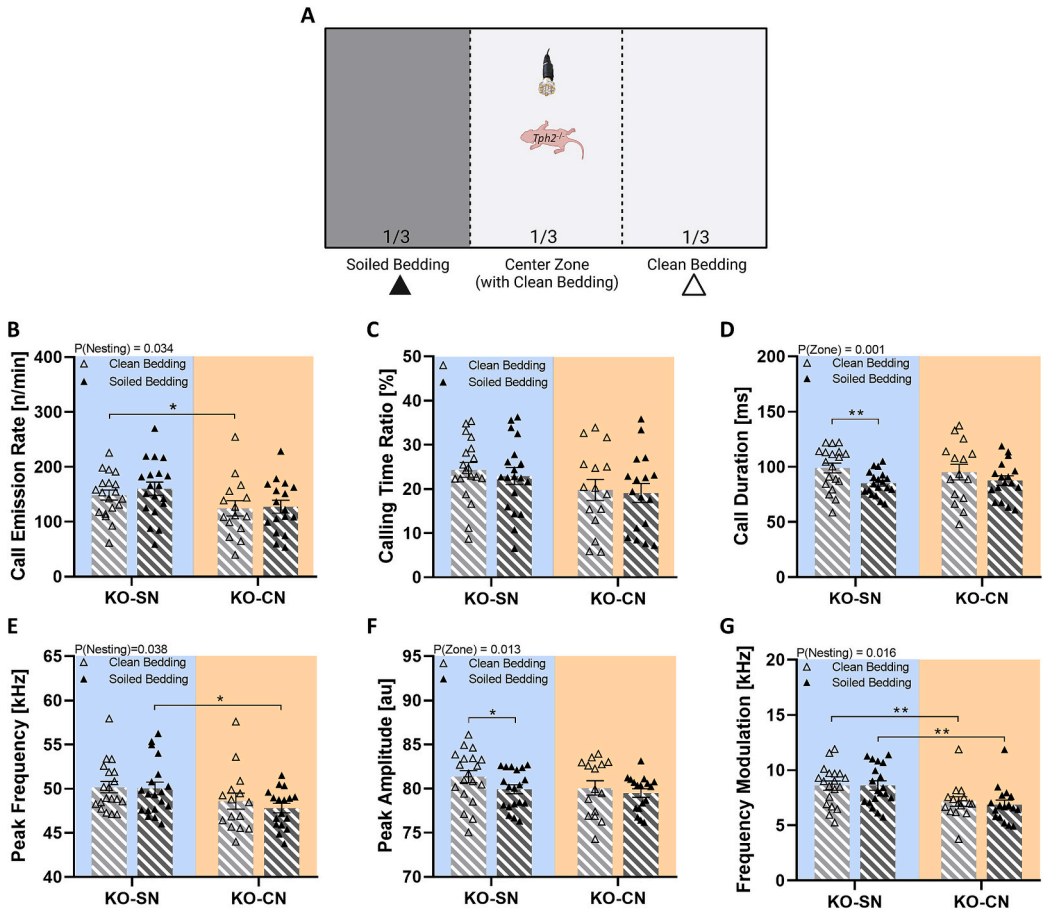

Fig. 4. *Tph2* deficiency in neonatal rats causes robust changes in socio-affective communication under social test conditions, i.e., homing test, only mildly modulated by pup location during call emission, as reflected in the emission of isolation-induced ultrasonic vocalizations (USV) during the homing test. (A) Overview of the homing test as a proxy for maternal affiliation in*Tph2*$^{-/-}$ knockout (KO) rat pups. (B) Call emission rate (n/min), (C) calling time ration (%), (D) call duration (ms), (E) peak frequency (kHz), peak amplitude (au), and (F) frequency modulation (kHz) of isolation-induced USV emitted during the homing test on postnatal day 11 by *Tph2*$^{-/-}$ knockout (KO; triangle) rat pups, depending on the location of the pup during call emission, i.e., clean bedding zone (white triangle) versus soiled bedding zone (black triangle). SN = standard nesting (blue), CN = communal nesting (orange). Data are expressed as mean ± SEM. ** p < .010; * p < .050. Number of rats: N (KO-SN) = 19, N(KO-CN) = 17. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. *Effects of nesting conditions: maternal preference test*

We also assessed the effects of nesting conditions on social adjustment and socio-affective communication during the maternal preference test on P7 in the presence of the mother. The alteration in socio-affective communication in the maternal preference test caused by *Tph2* deficiency was similar to the pattern observed in the homing test (Fig. 5A), with prominent changes in the emission of isolation-induced USV (Fig. 5B). While *Tph2*$^{+/+}$ littermate controls displayed a high call emission rate of ~200 calls per minute when exposed to the mother, *Tph2*$^{-/-}$ rat pups displayed a much lower call emission rate of only ~100 calls per minute (genotype: $F_{1,38} = 81.622$; $p < .001$; Fig. 5C). Importantly, this reduction of ~50 % occurred irrespective of nesting conditions (nesting: $F_{1,38} = 0.115$; $p = .736$; nesting x genotype: $F_{1,38} = 0.363$; $p = .551$). Calling time ratio was also reduced in *Tph2*$^{-/-}$ pups, as compared to *Tph2*$^{+/+}$ littermates (genotype: $F_{1,38} = 31.134$; $p < .001$; Fig. 5D). Again, this effect was seen irrespective of nesting conditions and seen in both SN and CN conditions (nesting: $F_{1,38} = 0.206$; $p = .652$; nesting x genotype: $F_{1,38} = 1.134$; $p = .294$). Regarding acoustic features, *Tph2*$^{-/-}$ pups emitted calls characterized by longer call durations, as compared to *Tph2*$^{+/+}$ littermates, regardless of nesting conditions (genotype: $F_{1,38} = 38.859$; $p < .001$; nesting: $F_{1,38} = 3.979$; $p = .053$; nesting x genotype: $F_{1,38} = 0.008$; $p = .930$; Fig. 5E). In contrast, peak frequency of the USV emitted by *Tph2*$^{-/-}$ pups was observed to be lower than in *Tph2*$^{+/+}$ littermates, in both SN and CN conditions (genotype: $F_{1,38} = 77.572$; $p = .009$; nesting: $F_{1,38} = 3.145$; $p = .084$; nesting x genotype: $F_{1,38} < 0.001$; $p = .993$; Fig. 5F). Likewise, frequency modulation was also lower in *Tph2*$^{-/-}$ pups than in *Tph2*$^{+/+}$ littermates, again regardless of nesting conditions (genotype: $F_{1,38} = 28.584$; $p < .001$; nesting: $F_{1,38} = 3.585$; $p = .066$; nesting x genotype: $F_{1,38} = 0.388$; $p = .537$; Fig. 5H). However, *Tph2*$^{-/-}$ pups emitted USV with a level of peak amplitude comparable to what was seen in *Tph2*$^{+/+}$ littermates, in both SN and CN conditions (genotype: $F_{1,38} = 0.605$; $p = .442$; nesting: $F_{1,38} = 0.005$; $p = .946$; nesting x genotype: $F_{1,38} = 1.313$; $p = .259$; Fig. 5G). No sex effects were observed, with the exception of a trend for call duration (sex: $F_{1,38} = 3.855$; $p = .057$; all other *p*-values >.100; not shown in detail).

Moreover, through detailed spectrographic analyses of more than 65,000 individual isolation-induced USV emitted during the maternal preference test, call clustering was again found to be impacted by *Tph2* deficiency. When plotting peak frequency against call duration, two call clusters were evident in *Tph2*$^{+/+}$ littermate controls (Fig. 5I). One prominent cluster was characterized by relatively low peak frequencies roughly between 45 and 55 kHz, similar to the low-frequency cluster in the homing test, and again accompanied by an additional subcluster roughly around 40 kHz. A second cluster was characterized by relatively high peak frequencies roughly between 70 and 80 kHz. As compared to the homing test, this cluster was characterized by relatively low prevalence and a much smaller frequency range. Again, however, call durations in the low-frequency cluster were much longer than in the high-

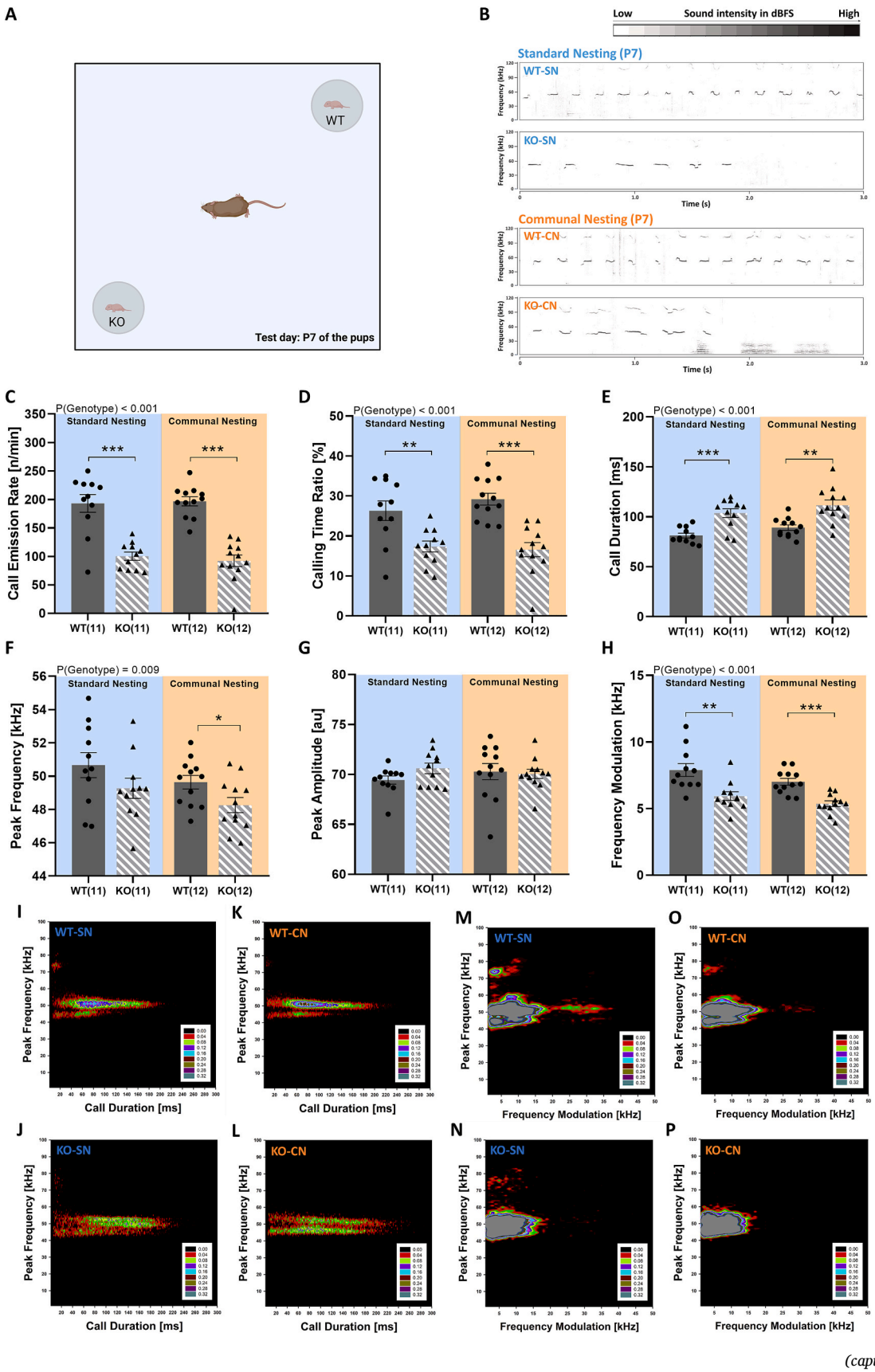

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Fig. 5. *Tph2* deficiency in neonatal rats causes robust changes in socio-affective communication under social test conditions, i.e., maternal preference test, only mildly modulated by communal nesting, as reflected in the emission of isolation-induced ultrasonic vocalizations (USV) and the clustering of their subtypes during the maternal preference test. (A) Overview of the maternal preference test to close the communicative loop between mother and pup and to compare maternal preferences between *Tph2*$^{-/-}$ knockout (KO) rat pups and *Tph2*$^{+/+}$ wildtype (WT) littermate controls. (B) Exemplary spectrograms of isolation-induced USV emitted during the maternal preference test, depending on genotype and nesting condition. (C) Call emission rate (n/min), (D) calling time ratio (%), (E) call duration (ms), (F) peak frequency (kHz), (G) peak amplitude (au), and (H) frequency modulation (kHz) of isolation-induced USV emitted during the maternal preference test on postnatal day 7 by *Tph2*$^{-/-}$ knockout (KO; triangle) rat pups and *Tph2*$^{+/+}$ wildtype (WT, circle) littermate controls. (I-P) Density plots depicting the distribution of individual isolation-induced USV in *Tph2*$^{-/-}$ knockout (KO) rat pups, as compared to *Tph2*$^{+/+}$ wildtype (WT) littermate controls, depending on nesting condition, i.e., WT-SN (I, M; ~21,000 calls), WT-CN (K, O; ~24,000 calls), KO-SN (J, N; ~11,000 calls), and KO-CN (L, P; ~11,000 calls). Colour coding reflects frequencies as percentages. SN = standard nesting (blue), CN = communal nesting (orange). Data are expressed as mean ± SEM. *** $p < .001$; ** $p < .010$; * $p < .050$. Number of rats: N(WT-SN) = 11, N(KO-SN) = 11, N(WT-CN) = 12, N(KO-CN) = 12. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

frequency cluster. The low-frequency cluster was much more prominent than the high-frequency cluster. Interestingly, the call durations represented in the low-frequency cluster were affected by genotype. This was also the case for the additional subcluster. In *Tph2*$^{+/+}$ littermates, the majority of calls had a duration between 50 and 120 ms, whereas in *Tph2*$^{-/-}$ pups the calls were typically between 80 and 180 ms of duration. In contrast, the high-frequency cluster was characterized by call durations typically shorter than 20 ms in all genotypes. Its relative prevalence, however, was affected by genotype. Albeit weak, the high-frequency cluster was still clearly detectable in *Tph2*$^{+/+}$ littermates. In *Tph2*$^{-/-}$ pups, in contrast, the high-frequency cluster was barely detectable. As for the homing test, CN amplified such genotype differences by a shift in prevalence away from the high-frequency cluster. In fact, CN was associated with an almost complete lack of the high-frequency cluster in both genotypes. In *Tph2*$^{-/-}$ pups, however, the exposure to CN further led to a prominent increase in prevalence of the additional subcluster, which, in fact, became more prominent than the low-frequency cluster (Fig. 5I-L).

When plotting peak frequency against frequency modulation, three out of the four previously described clusters of isolation-induced USV were evident in *Tph2*$^{+/+}$ littermate controls (Fig. 5M). While the two low-frequency clusters differing in their level of frequency modulation were evident, i.e., LOW/LOW and HIGH/LOW, only one of the two high-frequency clusters was detectable, namely the one characterized by a low level of frequency modulation, i.e., LOW/HIGH, but not the one characterized by a high level of frequency modulation, i.e., HIGH/HIGH. The level of frequency modulation present in the two low-frequency clusters was either below ~17 kHz or above ~20 kHz, extending up to ~35 kHz. For the one high-frequency cluster, the level of frequency modulation was clearly below ~15 kHz. In *Tph2*$^{+/+}$ littermates, the LOW/LOW cluster was clearly most prominent, and while this was also the case for *Tph2*$^{-/-}$ pups, call clustering was strongly affected by genotype. Most importantly, in contrast to the three clusters present in *Tph2*$^{+/+}$ littermates, only two clusters were present in *Tph2*$^{-/-}$ pups. Specifically, the two clusters with low levels of frequency modulation, i.e., LOW/LOW and HIGH/LOW, were present in *Tph2*$^{-/-}$ pups but not the one with high levels of frequency modulation, i.e., LOW/HIGH. As in the homing test, this change was associated with a tendency for a wider range of frequency modulation in the LOW/LOW cluster, extending up to ~20 kHz. Again, nesting conditions played a moderate modulatory role and tended to reduce the prevalence of weak clusters further. In *Tph2*$^{+/+}$ littermates exposed to CN, the HIGH/LOW and LOW/HIGH clusters were still detectable but weak. In *Tph2*$^{-/-}$ pups, exposure to CN amplified the genotype differences again, and out of the four call clusters previously described only one remained, i.e., the LOW/LOW cluster (Fig. 5M-P). Together, these data resemble the patterns obtained in the homing test, supporting the idea that *Tph2* deficiency in rat pups affects call clustering in a social context and that genotype differences, although only moderately modulated, are amplified by nesting conditions.

3.4. Closing the communicative loop between mother and pup

Importantly, the isolation-induced USV emitted by the pups during the maternal preference test (Fig. 6A) modulated the mother's interest and thus preference, with a stronger effect seen for USV emitted by *Tph2*$^{+/+}$ littermate controls (Fig. 6B). Firstly, our correlational analyses provided evidence in support of the notion that the mothers displayed a preference for pups emitting particularly high rates of isolation-induced USV (Fig. 6C). When correlating the MPI with the number of isolation-induced USV emitted by the pups, we obtained a strong positive correlation, particularly under CN conditions ($r = 0.590$, $p = .002$), while no such strong positive correlation was obtained under SN conditions ($r = 0.117$, $p = .605$). Typically, the mother displayed a preference for *Tph2*$^{+/+}$ littermate controls over *Tph2*$^{-/-}$ rat pups, with 6 out of 11 *Tph2*$^{+/+}$ littermate controls having a MPI of >50 %, while only 3 out of 11 *Tph2*$^{-/-}$ rat pups having a MPI of >50 %. Importantly, the greater the difference in the number of isolation-induced USV emitted by the pups tested simultaneously, the greater the difference in the MPI ($r = 0.457$, $p = .028$; Fig. 6D). Together, our findings suggest that mothers show a preference for the pup that is vocalizing more, typically a *Tph2*$^{+/+}$ littermate control, and that this preference increases with the difference in the number of isolation-induced USV emitted by the two pups tested simultaneously.

While the mothers demonstrated a preference for the pups emitting more isolation-induced USV during the maternal preference test, as reflected by a higher proportion of maternal investigation time allocated to them, the effect of pup USV emission on general maternal investigation during the maternal preference test suggested a more complex association. When correlating the number of USV emitted by each pup with the absolute maternal investigation duration for the individual pup or both pups, we found that as *Tph2*$^{+/+}$ pups emitted more USV, the mothers were investigating pups less in general ($r = -0.419$, $p = .047$; Fig. 6E). Remarkably, this reduction in investigation time was not accompanied by a shift in interest toward *Tph2*$^{-/-}$ pups, as indicated by a decrease in total maternal investigation time for both pups ($r = -0.427$, $p = .042$; Fig. 6E). In contrast, no significant correlation was observed between maternal investigation duration and USV emission for *Tph2*$^{-/-}$ pups, either individually ($r = 0.120$, $p = .585$; Fig. 6F) or collectively ($r = -0.148$, $p = .501$; Fig. 6F). These results suggest that the role of USV emission in modulating maternal behavior is more complex during the maternal preference test, particularly under stressful conditions where maternal search but no retrieval behavior is possible because the pups are not directly accessible for the mother in their wire cylinders. Nevertheless, even under stress, mothers still allocated more attention to pups emitting higher rates of USV.

4. Discussion

In our recently published study, we demonstrated that 5-HT deficiency leads to severe deficits in socio-affective communication in rat pups exposed to a non-social environment, i.e., the isolation box test, and that the deficits could not be rescued by communal nesting (CN), as compared to standard nesting (SN; Wang et al., 2024). In the present

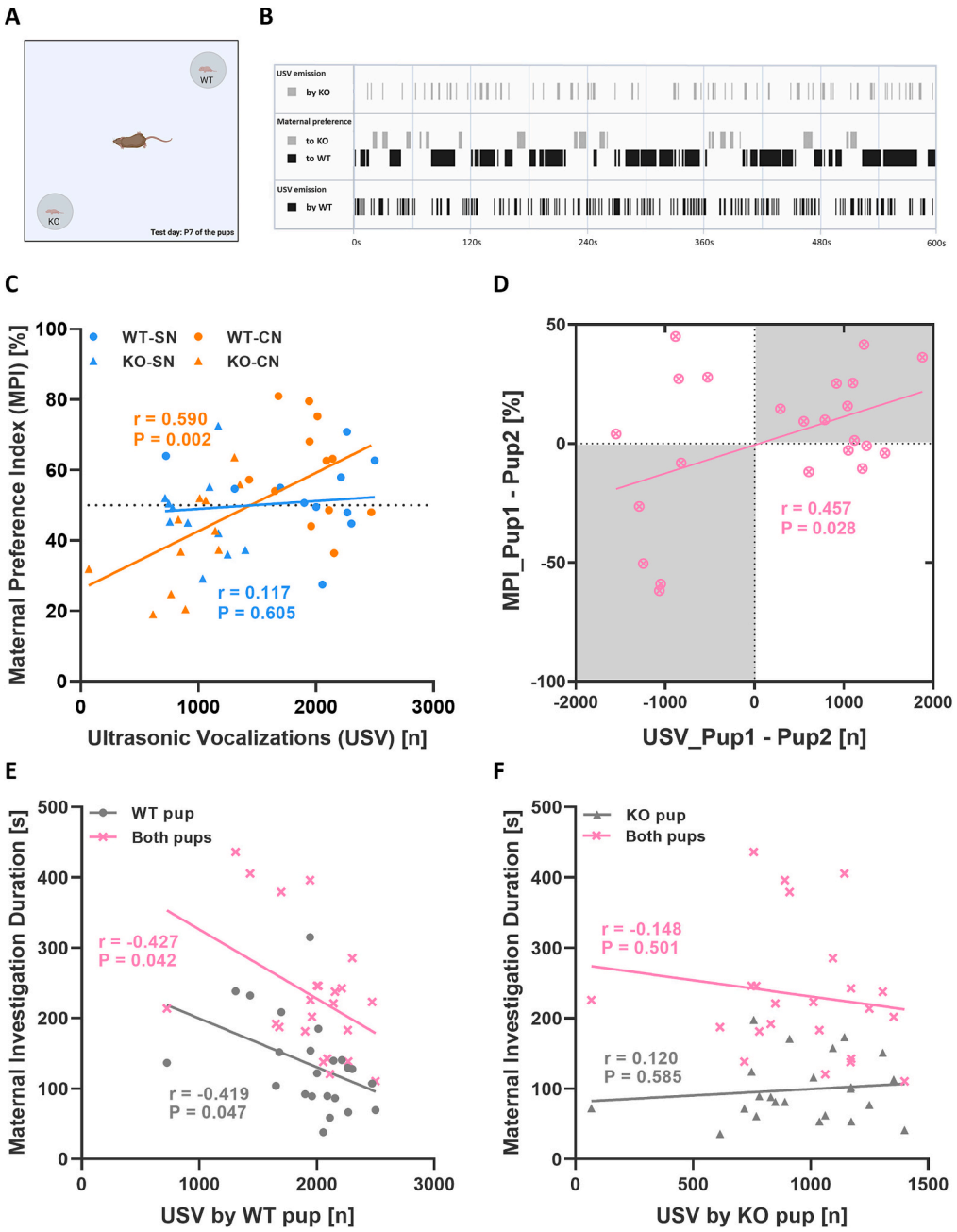

Fig. 6. *Tph2* deficiency in neonatal rats is associated with changes in maternal behavior, as reflected in preferences displayed by the mothers in response to the emission of isolation-induced ultrasonic vocalizations (USV) during the maternal preference test. (A) Overview of the maternal preference test to close the communicative loop between mother and pup and to compare maternal preferences between *Tph2*$^{-/-}$ knockout (KO) rat pups and *Tph2*$^{+/+}$ wildtype (WT) littermate controls. During the maternal preference test, the *Tph2*$^{+/-}$ heterozygous (HET) mother was simultaneously exposed to a *Tph2*$^{-/-}$ (KO; triangle) and a *Tph2*$^{+/+}$ (WT, circle) rat pup. (B) Exemplary ethogram depicting the emission of isolation-induced USV emitted by the KO (light gray) and the WT (dark gray) rat pup, together with the time-synced maternal preference for the KO (light gray) and the WT (dark gray) rat pup. (C) Scatter plot depicting the relationship between the maternal preference index (MPI) and the number of isolation-induced USV emitted by the rat pup. (D) Scatter plot depicting the relationship between the difference in the maternal preference index and the difference in the number of isolation-induced USV emitted between pups. Pup1 and Pup2 represent the pup inside cylinder1 and cylinder2, respectively. Light gray highlighted squares represent conditions, in which the mother preferred the pup that displayed higher emission rates of isolation-induced USV than the other pup present. (E) Scatter plot depicting the relationship between maternal investigation duration for either the *Tph2*$^{+/+}$ pup (circle) or both *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ pups (crosses) and the number of isolation-induced USV emitted by the *Tph2*$^{+/+}$ pup. (F) Scatter plot depicting the relationship between maternal investigation duration for either the *Tph2*$^{-/-}$ pup (triangle) or both *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ pups (crosses) and the number of isolation-induced USV emitted by the *Tph2*$^{-/-}$ pup. SN = standard nesting (blue), CN = communal nesting (orange). r = Pearson correlation coefficient. Number of rats: N(WT-SN) = 11, N(KO-SN) = 11, N(WT-CN) = 12, N(KO-CN) = 12. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

study, we therefore aimed to gain deeper insight into the interplay between socio-affective communication, nesting condition, and test context. To this aim, we studied *Tph2*$^{-/-}$ knockout (KO), *Tph2*$^{+/-}$ heterozygous (HET), and *Tph2*$^{+/+}$ wildtype (WT) rat pups of both sexes, randomly assigned to SN versus CN. We performed detailed spectrographic analyses and compared the emission of isolation-induced ultrasonic vocalizations (USV) under social test conditions, i.e., during the maternal preference test and the homing test, with the emission of

isolation-induced USV under non-social test conditions, i.e., during the isolation box test, previously reported (Wang et al., 2024). While social cues were present in the maternal preference test, i.e., the mother, and the homing test, i.e., soiled bedding from the home cage with maternal odor, no social cues were present during the isolation box test.

4.1. *Tph2* deficiency led to alterations in social adjustment

Firstly, we demonstrated that *Tph2* deficiency results in severe deficits in isolation-induced USV in neonatal rats under social test conditions, a phenomenon that has been recently reported under non-social test conditions, i.e., the isolation box test (Wang et al., 2024). Interestingly, socio-affective communication deficits were seen in *Tph2*$^{-/-}$ rat pups, while *Tph2*$^{+/-}$ pups were mostly indistinguishable from *Tph2*$^{+/+}$ littermate controls (for the results obtained in *Tph2*$^{+/-}$ pups, please see the supplementary Figures). This is consistent with previous reports in both mice (Mosienko et al., 2014) and rats (Kaplan et al., 2016), showing that partial *Tph2* deficiency results in only marginal or undetectable reductions in brain 5-HT levels and preserved behavioral phenotypes. These patterns likely reflect homeostatic adaptations that buffer against reduced TPH2 activity, thereby sustaining central 5-HT levels and maintaining functional 5-HT signaling in rodents (Mosienko et al., 2014). Despite prominent sex differences in the emission of isolation-induced USV, sex did not play a prominent role in modulating the effects of *Tph2* deficiency. Importantly, related socio-affective phenotypes caused by *Tph2* deficiency during early postnatal life have been observed in mice (Mosienko et al., 2015), rats (Liu et al., 2023), and monkeys (Liu et al., 2023). The high level of consistency of findings across sexes, social and non-social test conditions, and a broad variety of mammalian species underscores the substantial impact of *Tph2* deficiency on socio-affective communication during early development.

By comparing isolation-induced USV under social and non-social test conditions, we further demonstrated that *Tph2* deficiency was associated with alterations in social adjustment in neonatal rats. This was evidenced by a delayed adaptive change in call emission rates in *Tph2*$^{-/-}$ rat pups in response to social stimuli, e.g., the mother or maternal odor. Whereas *Tph2*$^{+/+}$ littermate controls demonstrated the ability to adjust to social stimuli by increasing call emission rates on postnatal day (P) 7 while competing for the mother's attention during the maternal preference test, *Tph2*$^{-/-}$ pups did not show any adjustment. The lack of change in the emission of isolation-induced USV displayed by *Tph2*$^{-/-}$ pups in this socially competitive environment indicates that *Tph2* deficiency is associated with a reduction and/or delay in the capability and/or motivation to appropriately adjust to changes in the social environment. In *Tph2*$^{-/-}$ pups, the adaptive increase in call emission rates in response to social stimuli was only observed at a later stage, on P11, during the homing test. Interestingly, *Tph2*$^{+/+}$ littermates did not exhibit an increase in call emission rates during the homing test, possibly due to the contact quieting effect, a phenomenon in which isolated rat pups emit fewer isolation-induced USV when exposed to maternal odor (Hofer and Shair, 1987; Oswalt and Meier, 1975). This aligns with the observations that *Tph2*$^{+/+}$ littermates displayed a strong preference for the mother's odor and spent the majority of the time in the soiled bedding zone, while *Tph2*$^{-/-}$ pups did not, as previously reported (Wang et al., 2024).

The deficits in social adjustment associated with *Tph2* deficiency were further evidenced by delayed and opposing adaptive changes in acoustic features of isolation-induced USV under social test conditions. During the maternal preference test on P7, *Tph2*$^{+/+}$ littermate controls emitted calls with shorter call duration and higher frequency modulation to attract the mother, whereas *Tph2*$^{-/-}$ pups did not display any adjustments in acoustic features. Similar adaptive changes in call duration and frequency modulation were observed in *Tph2*$^{-/-}$ pups only later, during the homing test on P11. Remarkably, *Tph2*$^{+/+}$ littermates maintained these adjustments in acoustic features during the homing test, but with a more pronounced reduction in call duration, contributing to a comparable calling time ratio to *Tph2*$^{-/-}$ pups. In addition, both *Tph2*$^{-/-}$ pups and *Tph2*$^{+/+}$ littermates exhibited adjustments in peak frequency in response to maternal odor on P11, but in opposite directions. While *Tph2*$^{+/+}$ littermates displayed an increase in peak frequency, *Tph2*$^{-/-}$ pups exhibited a reduction in peak frequency during the homing test. Notably, these bidirectionally opposing changes resulted in a convergence of peak frequency values across experimental conditions.

Liu et al. (2023) also assessed isolation-induced USV in response to different social odors in *Tph2*-deficient rats and found that these rats do not show deficits in social odor recognition. They observed a substantial reduction of isolation-induced USV emission in *Tph2*$^{+/+}$ pups exposed to maternal odor or unfamiliar male odor, as compared to clean bedding, while *Tph2*$^{-/-}$ pups maintained very low levels of USV emission in response to these social odors, similar to their response to clean bedding. Although the reductions in isolation-induced USV emission in response to maternal or male odor align with previous findings (Shair et al., 1997), it remains unclear whether the low levels of USV emission in *Tph2*$^{-/-}$ pups exposed to these social odors stem from their low baseline emission rates or a specific response to social odors. Notably, the pups tested by Liu et al. (2023) were not subjected to isolation prior to the exposure to different social odors, while in our study, rat pups underwent multiple brief isolation periods during the isolation box test before the maternal preference test and the homing test. This procedural difference may explain the contrasting responses to maternal odor observed in our study.

A number of studies have linked 5-HT to social adjustment. Animal studies demonstrated the essential role of 5-HT in both immediate coping behavior in response to social stress and the long-term development of social adaptability (Albertini et al., 2023; Wöhr et al., 2015; Wood et al., 2013). In depressed patients, an association between the HTR2A gene and social adjustment was identified (Antypa et al., 2013). Additionally, clinical studies provided evidence in support of the notion that serotonin reuptake inhibitors (SSRIs) can improve social adjustment in these patients (Briley and Moret, 2010). Moreover, neurodevelopmental disorders with early onset in humans, most notably autism spectrum disorder (ASD), are characterized by deficits in adjusting behavior to suit different social contexts (American Psychiatric Association, 2013). Interestingly, impaired social adjustment was found in genetic mouse models for ASD, e.g., BTBR mouse pups (Wöhr, 2015) and *Shank1*$^{-/-}$ mouse pups (Sungur et al., 2016).

4.2. *Tph2* deficiency led to alterations in socio-affective communication

The reduction and/or delay in the capability and/or motivation to appropriately adjust to changes in the social environment driven by *Tph2* deficiency was accompanied by deficits in socio-affective communication under social test conditions. The deficits were first evident in *Tph2*$^{-/-}$ pups as a reduction in isolation-induced USV during the maternal preference test and the homing test. During the maternal preference test on P7, *Tph2*$^{-/-}$ pups did not exhibit an adaptive increase in call emission rates that was seen in *Tph2*$^{+/+}$ littermate controls, resulting in a significant reduction in isolation-induced USV as seen in the isolation box (Wang et al., 2024). However, during the homing test on P11, *Tph2*$^{-/-}$ pups displayed a stronger increase in call emission rates in response to maternal odor than *Tph2*$^{+/+}$ littermates, resulting in less pronounced genotype differences, as compared to the isolation box (Wang et al., 2024).

The deficits in socio-affective communication under social test conditions were also reflected in changes in the acoustic features of USV emitted by *Tph2*$^{-/-}$ pups. During the maternal preference test and the homing test, *Tph2*$^{-/-}$ pups emitted isolation-induced USV that were longer and characterized by lower peak frequency and frequency modulation. Peak amplitude was higher in *Tph2*$^{-/-}$ pups during the homing test but comparable with *Tph2*$^{+/+}$ littermate controls during the maternal preference test. These alterations were somewhat different

from the patterns observed in the isolation box, where *Tph2*$^{-/-}$ pups emitted slightly longer calls characterized by higher peak frequency but lower peak amplitude and frequency modulation (Wang et al., 2024). The discrepancy between social and non-social test conditions can possibly be attributed to differences in the capabilities and/or motivation for social adjustment exhibited by *Tph2*$^{-/-}$ pups, as compared to *Tph2*$^{+/+}$ littermates across early developmental stages.

A comparison of isolation-induced USV emitted by *Tph2*$^{-/-}$ pups during the homing test revealed differences in call duration and peak amplitude between soiled and clean bedding zones. Specifically, *Tph2*$^{-/-}$ pups emitted shorter calls characterized by lower amplitude in the soiled bedding zone, resembling the phenotype seen in *Tph2*$^{+/+}$ littermate controls during the homing test. This suggests that *Tph2*$^{-/-}$ pups may retain the ability to recognize maternal odor.

Moreover, *Tph2* deficiency affected call clustering under social test conditions, following a pattern slightly different from what was seen under non-social test conditions (Wang et al., 2024). In the maternal preference test and the homing test, the low-frequency clusters ranging from 45 kHz to 55 kHz were the most prominent across all three genotypes, with *Tph2*$^{-/-}$ pups exhibiting a lower prevalence of call subtypes characterized by higher frequency modulation. This pattern was similar to that seen under non-social test conditions (Wang et al., 2024). However, the low-frequency clusters were predominantly composed of longer calls in *Tph2*$^{-/-}$ pups, as compared to *Tph2*$^{+/+}$ littermate controls, during the homing test, a feature not evident in the isolation box test (Wang et al., 2024). Additionally, in contrast to the higher prevalence of high-frequency clusters seen in *Tph2*$^{-/-}$ pups under non-social test conditions (Wang et al., 2024), their prevalence was reduced in *Tph2*$^{-/-}$ pups under social test conditions, as compared to *Tph2*$^{+/+}$ littermates. Alterations in call clustering associated with *Tph2* deficiency have also been reported in mice, yet only under non-social test conditions (Mosienko et al., 2015), and it is therefore unknown whether *Tph2* deficiency exerts similar effects on call clustering under social test conditions in mice. While changes in call clustering under non-social test conditions were also reported in several genetic mouse models displaying behavioral alterations relevant to neurodevelopmental disorders (Aerts et al., 2024; Fyke et al., 2021; Sungur et al., 2016; Wöhr, 2015; Wöhr et al., 2022), only one study revealed alterations in call clustering under social test conditions (Wöhr, 2015). To our knowledge, this is the first study demonstrating alterations in acoustic features and call clustering under social test conditions in a genetic rat model with relevance to ASD.

Importantly, nesting conditions did not exert prominent effects, and CN did not rescue the deficits in socio-affective communication driven by *Tph2* deficiency under social test conditions, in line with the results from non-social test conditions (Wang et al., 2024). In fact, CN appeared to slightly impair socio-affective communication under social test conditions. For example, the lowest call emission rates in the homing test were seen in *Tph2*$^{-/-}$ pups reared under CN conditions, which were not only lower than the call emission rates displayed by *Tph2*$^{+/+}$ littermate controls reared under CN conditions but also *Tph2*$^{-/-}$ pups reared under SN conditions. Moreover, the mild aggravation caused by CN was also reflected in the acoustic features of isolation-induced USV, where CN led to a further reduction in frequency modulation, associated with a reduced prevalence of the call cluster characterized by high levels of frequency modulation. The reductions in call emission rate and frequency modulation might be driven by changes in maternal caregiving behavior associated with communal nesting. For example, Wöhr and Schwarting (2008) reported that rat pups reared by mothers exhibiting particularly high levels of maternal caregiving behavior emitted fewer calls, albeit with higher frequency modulation. In contrast, Shahrier and Wada (2021) observed that rat pups reared by mothers presumed to exhibit deficits in maternal caregiving behavior due to ethanol exposure emitted a higher number of calls, often characterized by particularly high levels of frequency modulation.

Other nesting-related alterations evident during the homing test were subtle and appeared to be genotype-specific. For example, *Tph2*$^{-/-}$ pups reared under CN conditions exhibited an even stronger reduction in peak frequency than *Tph2*$^{-/-}$ pups reared under SN conditions, resulting in a peak frequency lower than in *Tph2*$^{+/+}$ littermates under CN but not SN conditions. Other alterations displayed by *Tph2*$^{-/-}$ pups were associated with the different zones of the homing test: *Tph2*$^{-/-}$ pups emitted shorter calls characterized by lower amplitude in the soiled bedding zone, particularly under SN conditions. Remarkably, the adjustments in isolation-induced USV in response to maternal odor were not associated with a preference for maternal odor under SN conditions in *Tph2*$^{-/-}$ pups, as previously reported (Wang et al., 2024). Therefore, we conclude that the lack of preference for the mother's odor observed in *Tph2*$^{-/-}$ pups was not due to an inability to detect social information (e.g., impaired olfaction), but rather a deficit in eliciting adjustments in behavior to social stimuli. To our knowledge, this is the first study revealing the effect of CN on socio-affective communication under social test conditions. One study assessing the effect of CN on isolation-induced USV in non-social test conditions demonstrated that CN inhibits pup ultrasonic calling in a rat line selectively bred for high rates of isolation-induced USV but not in a rat line selectively bred for low rates (Martinez et al., 2015). However, no unselected control line was included in this study.

4.3. *Tph2* deficiency in rat pups hindered mother-pup interactions

Pup ultrasonic calling functions in communicating with the mother to regulate mother-pup interactions and to stimulate maternal caregiving behavior in mice (Sewell, 1970; Smith, 1976) and rats (Smotherman et al., 1974; Wöhr and Schwarting, 2008), most notably maternal search and retrieval behavior. One can speculate that the alterations in socio-affective communication caused by *Tph2* deficiency would influence maternal caregiving behavior. By associating the USV emission of the pups and the maternal preference index (MPI) of the *Tph2*$^{+/-}$ mother in the maternal preference test, we observed that the mother preferred pups that emitted more isolation-induced USV, particularly under CN conditions. Two potential explanations may account for the stronger effect under CN conditions. Firstly, CN was associated with a moderate modulation of acoustic features, e.g., frequency modulation, during the maternal preference test, thereby increasing the relative importance of call emission rates in attracting maternal attention. Secondly, CN might have affected maternal behavior, enhancing the responsiveness of the mother to signals emitted by the pup, such as isolation-induced USV. Moreover, the difference in MPI for the pups was positively correlated with the difference in the number of isolation-induced USV they emitted. These results provide evidence in support of the notion that pup ultrasonic calling serves an important communication function in attracting the mother and in eliciting maternal caregiving behavior. However, *Tph2*$^{+/+}$ littermate controls, which exhibited higher call emission rates, did not always receive more attention from the mother. In 9 out of 26 trials, the mother preferred *Tph2*$^{-/-}$ pups, despite their lower call emission rates. This suggests that there might be other factors influencing maternal caregiving behavior, possibly associated with *Tph2* heterozygosity of the mother. In fact, it was previously shown that *Tph2*$^{-/-}$ female mice exhibit impaired maternal caregiving behavior (Alenina et al., 2009), yet there are no systematic studies suggesting impaired maternal caregiving behavior in *Tph2*$^{+/-}$ female mice or rats, to our knowledge.

Although *Tph2*$^{-/-}$ pups received less attention from the mother and therefore presumably less maternal care, they also appeared less affected by the mother's negative response to stress. Correlating absolute maternal investigation time with pup ultrasonic calling revealed that mothers spent less time investigating pups when *Tph2*$^{+/+}$ littermate controls emitted more calls, while calls emitted by *Tph2*$^{-/-}$ rat pups had no effect. This suggests that, in a stressful environment where maternal search but no retrieval behavior is possible, the mother might be more sensitive to isolation-induced USV emitted by *Tph2*$^{+/+}$ littermates,

followed by attempts to avoid the stress. The call emission rates, along with subtle changes in acoustic features and call clustering, may contribute to the mother's sensitivity to isolation-induced USV. Studies in mice showed that isolation-induced USV falling into a specific frequency range are more efficient than others in inducing maternal caregiving behavior (Ehret, 1987; Ehret, 1992; Ehret and Haack, 1982). Importantly, although mothers spent less time investigating pups when *Tph2*$^{+/+}$ littermates vocalized excessively, they still allocated more than half of the total investigation time on *Tph2*$^{+/+}$ littermates rather than shifting their care to *Tph2*$^{-/-}$ pups. Together, this is in line with the communicative function of isolation-induced USV in eliciting maternal search and retrieval behavior. Our findings suggest that mothers show a preference for the pup that is vocalizing more, typically a *Tph2*$^{+/+}$ littermate control, and that this preference increases with the difference in the number of isolation-induced USV emitted by the two pups tested simultaneously.

5. Limitations

Our study has a number of strengths, including the detailed spectrographic analysis and systematic comparison of isolation-induced USV under social versus non-social test conditions, a comprehensive assessment of the effects of nesting conditions in both male and female rat pups, and the incorporation of a maternal preference test to model the reciprocal nature of communication. However, it also has limitations. Firstly, the USV data under social test conditions was not collected at the exact same age as the USV data recorded under non-social test conditions. Nevertheless, the zigzag-shaped pattern clearly speaks for an effect of social context and against a nonspecific developmental effect. Secondly, it is difficult to compare the USV phenotype under social test conditions with previous findings (Oswalt and Meier, 1975; Hofer and Shair, 1987; Liu et al., 2023) due to differences in experimental design and setups. Most importantly, unlike one-time exposure to social odor, which elicits a strong contact quieting effect in rat pups exposed to maternal odor (Hofer and Shair, 1987; Liu et al., 2023; Oswalt and Meier, 1975), the rat pups in our study may have developed a new strategy to attract the mother's attention by keeping or potentiating the level of USV emission when exposed to the mother or maternal odor, likely as a result of experiencing multiple short isolation periods. Finally, it would be interesting to see whether the alterations in social adjustment and socio-affective communication observed in neonatal rats persist into juvenility and adulthood.

6. Conclusion

Our results show that *Tph2* deficiency causes prominent alterations in isolation-induced ultrasonic calling linked to reduced maternal responsiveness, including changes in acoustic features, e.g., increased call duration but reduced frequency modulation. Remarkably, irrespective of communal nesting, *Tph2*$^{-/-}$ pups typically displayed either no evidence for social adjustment or even changes opposite to *Tph2*$^{+/+}$ littermates, suggesting a reduction and/or delay in the capability and/or motivation to appropriately adjust to changes in the social environment. Such alterations in social adjustment likely contribute to growth retardation through reduced quality of mother-pup interactions.

Author contribution

MW acquired funding and designed the study. TW performed the experiments. TW and ALG analyzed the data. TW and MW compiled the figures. TW and MW wrote the manuscript with the help of JRH, ALG, MCFS, RCRC, SMK, NA, MB, and JD. TW, JRH, ALG, MCFS, RCRC, SMK, NA, MB, JD, and MW read, critically revised, and approved the manuscript.

CRediT authorship contribution statement

Tianhua Wang: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Aaron L. Gnade:** Writing – review & editing, Formal analysis. **Judith R. Homberg:** Writing – review & editing. **Marta C.F. Samina:** Writing – review & editing. **Rogério C.R. Castro:** Writing – review & editing. **Sharon M. Kolk:** Writing – review & editing. **Natalia Alenina:** Writing – review & editing. **Michael Bader:** Writing – review & editing. **Jinye Dai:** Writing – review & editing. **Markus Wöhr:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Ethics approval and consent to participate

All procedures were conducted in strict compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, the European regulations for animal experiments (2010/63/EU), and the legal requirements of Germany. Procedures were approved by the local authorities (G14/2022).

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Declaration of competing interest

The other authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2025.111469.

Data availability

Data will be made available on request.

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