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Characterizing maternal isolation-induced ultrasonic vocalizations in a gene–environment interaction rat model for autism

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Abstract

Deficits in social communication and language development belong to the earliest diagnostic criteria of autism spectrum disorders. Of the many risk factors for autism spectrum disorder, the contactin-associated protein-like 2 gene, *CNTNAP2*, is thought to be important for language development. The present study used a rat model to investigate the potential compounding effects of autism spectrum disorder risk gene mutation and environmental challenges, including breeding conditions or maternal immune activation during pregnancy, on early vocal communication in the offspring. Maternal isolation-induced ultrasonic vocalizations from *Cntnap2* wildtype and knockout rats at selected postnatal days were analyzed for their acoustic, temporal and syntax characteristics. *Cntnap2* knockout pups from heterozygous breeding showed normal numbers and largely similar temporal structures of ultrasonic vocalizations to wildtype controls, whereas both parameters were affected in homozygously bred knockouts. Homozygous breeding further exacerbated altered pitch and transitioning between call types found in *Cntnap2* knockout pups from heterozygous breeding. In contrast, the effect of maternal immune activation on the offspring's vocal communication was confined to call type syntax, but left ultrasonic vocalization acoustic and temporal organization intact. Our results support the "double-hit hypothesis" of autism spectrum disorder risk gene–environment interactions and emphasize that complex features of vocal communication are a useful tool for identifying early autistic-like features in rodent models.

KEYWORDS
autism spectrum disorders, *Cntnap2*, gene–environment interaction, maternal immune activation, poly I:C, rat model, ultrasonic vocalization

1 | INTRODUCTION

Autism spectrum disorder (ASD) comprises neurodevelopmental conditions characterized by deficits in social communication and restrictive, repetitive behaviors including sensory issues.[1] One of the earliest symptoms of ASD is a failure to meet language development milestones, ranging from delayed language acquisition, inability to use spoken language, preference for repeating words (echolalia) as well as

unusual tone or inflection.[2] Of the many risk factors for ASD, the *Contactin associated protein-like2* gene (*CNTNAP2*) is known to participate in speech and language development in humans.[3,4] The expression of the neurexin encoded by *CNTNAP2*, CASPR2, is enriched in language-related circuits of the brain[5] and its contribution to social vocal communication appears to be conserved across species, including ultrasonic vocalizations (USVs) in rodents.[6–9] *CNTNAP2* variants can result in a range of phenotypes, from a small lag in language acquisition to autism with speech-language delays and impairments.[4,10–13] However, the influence of common *CNTNAP2* variants on early language acquisition in the general population is not sufficient to explain the prevalence and etiology of language-related disorders such as ASD in children.

It has been hypothesized that the susceptibility for ASD and verbal deficits is substantially increased by the simultaneous co-occurrence of other genetic or environmental risk factors.[4,14] This "multiple-hit hypothesis" of autism proposes that genetic mutations or variants and environmental challenges affect common neurodevelopmental pathways, in concert leading to the core symptoms of ASD including vocal social communication deficits.[14–16] Although nowadays this theory is commonly accepted, studies investigating the effects of gene–environment interaction on the severity of ASD-related vocal communication deficits are scarce. In the present study, we used the *Cntnap2* knockout (KO) rat model to characterize if combined effects of ASD susceptibility gene mutation and one of two other risk factors—parental environment and maternal immune activation (MIA) during pregnancy—can exacerbate vocal communication deficits in the offspring.

The family environment is assumed to be one of the strongest predictors for alterations in language development including maternal resources available to the infant and hereditary predispositions, in particular a familial history of late language emergence.[17,18] Rodent USVs depend on the genetic background of the strain[19] and they are malleable by the pups' rearing environment, in particular through the dam's maternal behavior that acts as the main mediator of environmental cues.[20,21] Selective breeding has been used to examine gene–environment interactions in the USV communicatory potential related with emotionality traits, affective behaviors and other endophenotypes of autism.[22–24] Thus, we tested whether homozygous *Cntnap2* KO maternal genotype and rearing condition may be able to intensify USV abnormalities compared with offspring from heterozygous *Cntnap2* breeders.

MIA is one of the most well-known environmental etiological risk factors for ASD.[25,26] Gestational viral infections, especially early during pregnancy, trigger a maternal immune response that can perturb fetal brain development and can manifest in autism.[27,28] It has been suggested that individuals with genetic predisposition for autism may have less tolerance to prenatal stressors such as maternal immune responses and that their interaction increases the autism risk.[29] Indeed, rodent models with experimentally induced MIA yield offspring that demonstrates hallmarks of autism, including altered USV communicative behavior,[30] and have shown interactive effects with *Cntnap2* mutation affecting certain ASD-like social behaviors.[16] To investigate whether MIA exacerbates USV abnormalities in *Cntnap2* KO offspring we injected pregnant heterozygous *Cntnap2* rats with the viral mimic polyinosinic:polycytidylic acid (poly I:C) and compared their offspring with offspring from saline injected dams.

Overall, we found that homozygous breeding and rearing condition impacted USVs from *Cntnap2* KO pups more than MIA. Homozygously bred *Cntnap2* KO pups demonstrated alterations in several categorical, acoustic and temporal USV characteristics, whereas the same parameters were largely intact in USVs from poly I:C offspring. Both environmentally challenged *Cntnap2* KO groups showed intensified abnormalities in call subtype distribution and transition probability between call types, reflective of call syntax structure (i.e., KOs from homozygous breeding and from poly I:C offspring compared with KOs from heterozygous breeding and saline offspring, respectively). Furthermore, we found call type-specific independent and synergistic effects of *Cntnap2* KO and MIA. Our results reinforce the usefulness of pup USVs—and in particular call type repertoire and transitions—as a preclinical approach to study ASD risk gene–environment interactions linked with vocal communication deficits.

2 | MATERIALS AND METHODS

2.1 | Animals

Male (M) and female (F) Sprague–Dawley wildtype (*Cntnap2* WT) and homozygous knockout (*Cntnap2* KO) rats were used in this study. Heterozygous breeders were obtained from Horizon Discovery (Boyertown, PA, USA, originally created at SAGE Laboratories, Inc. in conjunction with Autism Speaks; the line is now maintained by Envigo, RRID:RGD_11568646) and bred to obtain *Cntnap2* heterozygous and homozygous breeders. The model contains a five base pair deletion in exon six of the *Cntnap2* gene, created using the zinc finger nuclease target site CAGCATTTCCGCACC|aatgga|GAGTTTGACTACCTG. Rats were housed in a temperature-controlled room on a 12 h light/dark cycle with ad libitum food and water. Behavioral testing was performed during the light phase of the cycle (lights on at 07:00 h). All procedures were approved by the University of Western Ontario Animal Care Committee and were in accordance with the guidelines established by the Canadian Council on Animal Care.

2.2 | Cohort 1 (Interaction of *Cntnap2* mutation and breeding condition)

Adult *Cntnap2* heterozygous or homozygous female and male knockout rats were mated (1 female +1 male breeder per cage) to obtain either *Cntnap2* WT and KO^{het} pups, or *Cntnap2* KO^{hom} pups, respectively. The male breeders were separated from the females about 1 week before the expected day of birth. The day of parturition was designated as postnatal day 0 (PND0). All pups remained with their biological mother until weaning on PND21. Occurrence of physical developmental milestones including fur, lower incisor eruption,

TABLE 1 Number of animals for ultrasonic vocalization recordings in cohort 1

	Number of animals					
PND	WT F	WT M	KO^{het} F	KO^{het} M	KO^{hom} F	KO^{hom} M
3	2	3	4	5	3	2
5	4	4	4	6	3	2
8	4	6	6	6	3	2
12	4	6	6	6	9	6
15	4	6	6	6	9	6
21	4	6	6	6	9	6
Number of litters	4				2	
Average litter size	13				10	

TABLE 2 Number of animals for ultrasonic vocalization recordings in cohort 2

	Number of animals							
Offspring	Saline				Poly I:C			
	WT M				WT M			
	KO F				KO F			
	KO M				KO M			
	WT F	WT M	KO F	KO M	WT F	WT M	KO F	KO M
PND8	12	9	7	12	7	5	11	14
Number of litters	7				6			
Average litter size	11				11			

auditory startle, eye opening and ear twitch reflex was assessed in a subgroup of pups to control for potential global developmental delay[31] in *Cntnap2* KO rats (WT: PND3-12 $n = 5$, PND15, 28 $n = 8$, PND21 $n = 10$, KO^{het}: PND3-12, 28 $n = 9$, PND15 $n = 10$, PND $n = 12$, KO^{hom}: PND3-8 $n = 5$, PND12-21 $n = 20$, PND28 $n = 15$). USV recordings took place on PND3, 5, 8, 12, 15 and 21 (numbers of animals see Table 1).

2.3 | Cohort 2: Interaction of *Cntnap2* mutation and MIA

Breeding was performed as described in References 32,33. In brief, adult male and female heterozygous *Cntnap2* rats were crossed. After pairing, a vaginal smear was collected from each female at 8 a.m. every morning and inspected under a light microscope to track the estrus cycle and check for the presence of sperm. If sperm was detected in the smear, the female was considered pregnant and that day was considered as Gestation Day (GD) 0.5. MIA was induced using poly I:C (P9582-5MG Lot#118M4035V, Sigma Aldrich, Saint Louis, Missouri), which had been previously aliquoted and stored at −20°C. Poly I:C aliquots were diluted in 0.9% saline to obtain a concentration of 4 mg/mL. Pregnant females were randomly assigned to receive either poly I:C or saline injections. On GD9.5 at around 10 a.m., the pregnant females were anesthetized with isoflurane (5% induction, 2% maintenance) and injected intravenously into the tail vein with either poly I:C (4 mg/kg) or saline (0.9%, 1 mL/kg). The injection procedure took an average of 5 minutes and rats were returned to their cages afterwards. GD9.5 roughly correlates to the end of the first trimester of human pregnancy where MIA has the most severe neurodevelopmental impact.[34] The day of parturition was designated as PND0 and USV recordings took place on PND8 (numbers of animals see Table 2). We chose to record USVs on PND8 to roughly match the time point of recording with previous studies investigating the effects of MIA on rat pup USVs (between PND3 and 11[35,36–39]).

It has previously been shown in Long-Evans pups that the size of the litter or the relative proportion of males in the litter did not alter the overall pattern of age-related USV changes,[40] therefore we did not cull the litters and used all the pups. Since all USV parameters of heterozygous pups were not statistically different from those of WT pups in Cohort 2 (not shown), all analyses described below focused on WT and KO offspring in both cohorts.

2.4 | Apparatus and recording procedure

Rat pup USVs were recorded in a sound-attenuated chamber using the Avisoft UltraSoundGate 116 microphone and Avisoft-RECORDER USGH software (version 4.2; Avisoft Bioacoustics; Glienicke/Nordbahn, Germany, Avisoft-RECORDER, RRID:SCR_014436) located in a temperature controlled room (22.5 ± 1.5°C). The microphone was affixed to the upper lid of the recording chamber, at a vertical distance of 20.5 cm from its floor. The recording chamber was constructed with black PVC boards (20 × 24 × 12 cm) and was lined with sound-absorbing foam to insulate the recording environment from external noise. A heating pad (37°C) was placed on the floor of the chamber

before the recordings to prevent temperature fluctuations of the animals. Pups were taken out individually from the cage in random order and placed in a 600 mL beaker that was lined on its bottom with paper towels to minimize scratching noises that would interfere with their USV signals in the recording. The pup inside the beaker was placed into the chamber for a 3-min recording. The audio signals were sampled at 250 kHz and stored as WAV files. After the recording, the beaker was cleaned thoroughly with 70% ethanol in between every recording to minimize effects on their USV because of olfactory cues.

2.5 | USV detection, call type classifier training and analysis

Audio files were analyzed offline using the DeepSqueak software suite[41] (version 2.6.2, RRID:SCR_021524) for MATLAB (version R2019a, The MathWorks, Inc., Natick, Massachusetts, RRID:SCR_001622). DeepSqueak uses a convolutional neural network for USV detection and identification.[41] The USVs were detected using DeepSqueak's multidetect function by its default detection networks "Long Rat Call Network_v2" (overlap 0.2 s) and "Short Rat Call Network_v2" (overlap 0.05 s), high frequency cut off 100 kHz, low frequency cut off 20 kHz. The slider was set to "high recall" and the contour threshold to 0.3 for all audio files. For better merging of the detection boxes, two adjustments were made prior to call detections in functions merge_boxes.m (line 23 from "OverlapMergeThreshold = .15" to "OverlapMergeThreshold = .05") and squeakDetect.m (line 177 from "Calls = merge_boxes(AllBoxes, AllScores, AllClass, AllPowers, audio_info, 1, score_cuttoff, 0)" to "Calls = merge_boxes(AllBoxes, AllScores, AllClass, AllPowers, audio_info, 1, score_cuttoff, 1)"). The detection files were passed to DeepSqueak's Post Hoc Denoiser, a neural network capable of discriminating USVs from common types of background noise.[41] All files were manually checked and inaccurate detections including false negatives were corrected on DeepSqueak Screener, a version of the original program that has additional functions for manual detection editing.[42]

A total of 13 call subtypes were selected for USV call classification (Figure S1), adapted from Wright et al[43] and Riede[44] and their classification requirements defined as follows:

1. Flat: mean slope between −0.2 and 0.2 kHz/ms with no significant modulation in frequency.
2. Short: duration of less than 12 ms.
3. Upward ramp: monotonic increase in frequency with a mean slope of 0.2 kHz/ms or higher.
4. Downward ramp: monotonic decrease in frequency with a mean slope of −0.2 kHz/ms or lower.
5. Inverted U: monotonic increase followed by a monotonic decrease in frequency, each of at least 5 kHz.
6. Complex: contain two or more directional changes in frequency of at least 3 kHz each.
7. Multistep: two or more instantaneous frequency changes.
8. Split: middle component contains an instantaneous frequency change to a lower frequency and has a harmonic.
9. Step up: instantaneous frequency change to a higher frequency.
10. Step down: instantaneous frequency change to a lower frequency.
11. Composite: comprise more than one of the categories or have short breaks (less than 20 ms) in them.
12. Trill: rapid frequency oscillations with a period of approximately 15 ms.
13. Trill jump: a trill that contains one or more higher-frequency components.

TABLE 3 Numbers of calls used to train the classifier for automated, supervised call classification

Call type	Number
Flat	3092
Short	537
Upward Ramp	447
Downward Ramp	77
Inverted U	94
Complex	324
Trill	192
Trill Jump	144
Step Down	801
Step Up	225
Split	507
Multistep	407
Composite	508
TOTAL	8579

In total, 8579 calls including all 13 call subtypes (Table 3) were manually labeled during selection review and passed to "Tools → Network Training → Train Supervised Classifier" to train our classification network in DeepSqueak.[41] After the final training, the validation accuracy was 86.69% (validation frequency: 10 iterations). Automatic call classification was performed by this ClassifierNet for all audio files, and the call features including call type exported to Excel (2016, Microsoft, Redmond, Washington, RRID:SCR_016137) for further analysis in MATLAB and GraphPad Prism 9.3.1. (GraphPad Software, San Diego, California, RRID:SCR_002798).

2.6 | Simple categorical and spectral call features

USV characteristics including call number, principal frequency (kHz), frequency bandwidth (kHz) and call length (s) were exported for individual recordings using DeepSqueak.[41] The total numbers of accepted calls within a 3-min recording were averaged within experimental groups. The call principal frequency, and high and low frequency (kHz) were first log transformed for individual calls. The log transformed call principal frequency was averaged within recording and then experimental group to analyze group differences in call pitch. Frequency

bandwidth was calculated to quantify frequency modulations by subtracting log transformed low from high call frequency before building averages within recording and group. Frequency logarithms may better capture biological phenomena of fundamental frequency production and modulation than differences on a linear scale.[45] The call length was averaged for individual recordings and then experimental groups. All of the above-mentioned simple call features were calculated across all calls irrespective of different call types.

2.7 | Temporal organization

We defined three distinct inter-call interval (ICI) categories: short duration ICIs (≥20 ms) separating individual USVs,[44] medium duration ICIs (≥150 ms) separating sequences of USVs,[46] and long duration ICIs (≥2000 ms) separating bouts of USVs.[47,48] Our ICI criteria were based on previous findings (short ICIs: USV with a short pause can belong to the same motor activity associated with an acoustic utterance[44]; medium ICI: prototypical call sequences had an average ICI of 150–200 ms between calls[46]; long ICIs: 2-s pauses have been found as a natural threshold to define the end of a USV bout[47,48]). The USV temporal organization was quantified within each recording for all accepted calls irrespective of call type. The ICI between the next and preceding call was calculated by subtracting the end time (s) of the previous call from the begin time (s) of the following call. Call successions with ICIs shorter than 150 or 2000 ms were grouped into call sequences or bouts, respectively. Within each recording, we averaged the duration of sequences (begin time of first call in sequence subtracted from the end time of the last call in the sequence), the number of calls per sequence, the call rate per sequence (number of calls per sequence divided by sequence duration), and number of call sequences before averaging within groups. In the following step, each individual call sequence was assigned to the temporally corresponding bout that the sequence occurred in. The number of sequences per bout was extracted from each recording and averaged. Like for call sequences, we averaged the duration of bouts (begin time of first call in bout subtracted from the end time of the last call in the bout), the number of bouts and the sequence rate per bout (number of sequences per bout divided by bout duration) within recording before averaging within groups. For cohort 1, all ICIs were extracted for each animal on PND12. Within groups, the ICIs were sorted into 160 bins with bin width 0.05 s ranging from 0 to 8 s and histograms were displayed as relative frequency of numbers of ICIs (percentages) that lie within a bin.

2.8 | Call type distribution

For each recording, all calls of a respective subtype were added up. Then the number of USVs of each call type was normalized to the total amount of calls within a 3-min recording for individual pups and then averaged across animals within groups. The number of different call types out of the 13 possible types present in a recording were extracted and compared between groups.

2.9 | Syntax analysis

Syntax analysis was performed on supervised classified USVs. As for temporal organization analysis, an inter-bout interval of 2 s was chosen (maximum bout separation 2 s, exclude classes with frequency below 0.01). The transition probabilities between call types within call bouts was calculated by DeepSqueak and the conditional probabilities displayed in transition probability tables and syntax flow paths to reveal more complex patterns of calling that exceed simple categorical or spectral analysis.[41] Sums across columns of transition probability tables were calculated to quantify the summed probability for the next call to be of a certain type.

2.10 | Anxiety-related, exploratory and stereotypic behavior in adult rats

2.0- to 5.7-months-old female and male *Cntnap2* WT, KO^{het} and KO^{hom} rats were used to assess behavioral read-outs of anxiety, exploration and stereotypy (WT F: $n = 7$, M: $n = 7$, KO^{het} F: $n = 6$, M: $n = 5$, KO^{hom} F: $n = 6$, M: $n = 5$ rats; thereof 3 WT M, 1 KO^{het} M and all KO^{hom} were the same rats as pups used for USV recording earlier). Testing of spontaneous locomotion was performed as described in Scott, et al.[49] In brief, locomotor testing took place in a four-walled, plastic, open-top arena, in which rats were able to freely explore for 20 min. Rats' movements were tracked via ANY-Maze software (v4.29, Stoelting Co., Wood Dale, Illinois, RRID:SCR_014289). The proportion of time spent in the perimeter of the arena in contrast to the anxiety-inducing center[50] was used as an index of anxiety and calculated with the following equation:

$$\text{Perimeter preference} = \frac{\text{time}_{\text{perimeter}} - \text{time}_{\text{center}}}{\text{time}_{\text{perimeter}} + \text{time}_{\text{center}}}$$

The total distance traveled (m) was used as measure of exploratory behavior, and the number of full body rotations (circling) as measure of stereotypic behavior. All open-field locomotor data from *Cntnap2* WT and KO^{het} rats presented in the present study were re-analyzed from the first 10 min of the 20-min tests shown in Scott et al.[49] Data from *Cntnap2* KO^{hom} rats were acquired during the same time period as *Cntnap2* WT and KO^{het} rats.

2.11 | Statistical analysis

Unless otherwise stated, data are presented as group median and individual data points (animals). Statistical tests were performed in GraphPad Prism 9.3.1 and RStudio 2022.2.0.0 (PBC, Boston, MA, RRID:

SCR_000432), and figures were generated in GraphPad Prism 9.3.1 and in DeepSqueak 2.6.2[41] run with MATLAB (version R2019a). We used ARTool (Aligned Rank Transform, ART) to align-and-rank data for nonparametric factorial ANOVA[51] to examine main effects and interactions, and ART-C for post hoc pairwise comparisons (contrast tests[52]) to accommodate for non-normal distribution, unbalanced design and/or missing values. For cohort 1, the factor genotype comprises the three groups *Cntnap2* WT, KO^{het} and KO^{hom}. Statistical tests after the Aligned Rank Transform were based on the experimental design and included univariate analysis of variance (x-way ANOVA, repeated measures (RM) ANOVA, or Mixed-effects model), followed by multiple comparison tests with correction for type 1 error after Tukey's method for all Figures unless otherwise stated. Statistical tests on original data (i.e., without ART) were performed in Figure 4B,D (Friedman test followed by Dunn's multiple comparison tests), Figure 4 *F* (Mann Whitney test and Wilcoxon matched-pairs signed rank test), and in Figure S12A–C (Kruskal–Wallis test and Dunn's multiple comparisons tests) and Figure S14 (Simple linear regression). Presence or absence of developmental milestones was compared between *Cntnap2* WT (expected distribution) and KO^{het} or KO^{hom} (observed distributions) using the two-tailed binomial test (Figure S2). All statistical analyses are presented in the Figure legends or respective Tables. Statistical significance level was $\alpha = 0.05$, and resulting p values are reported in the legends using: $^{*}p = 0.05$; $^{**}p = 0.01$; $^{***}p = 0.001$; ns, not significant. Data in Figures 4A,C,E, 5 and 7 and Figures S3, S6, S7, S10, S11, S12D are descriptive.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3 | RESULTS

3.1 | Some basic acoustic call characteristics are exacerbated by the interplay of *Cntnap2* knock-out and maternal genotype

To characterize this ASD risk gene–environment interaction, *Cntnap2* heterozygous or homozygous females and males were mated. Both *Cntnap2* KO^{het} and KO^{hom} pups showed accelerated occurrence of fur, lower incisor eruption, auditory startle, eye opening and ear twitch reflex when compared with wildtype pups (Figure S2). Maternal isolation-induced USVs were recorded from WT and KO offspring on PND3, 5, 8, 12, 15 and 21. Call numbers in *Cntnap2* WT, KO^{het} and KO^{hom} pups increased with age and reached their peaks on PND12. The number of calls emitted by *Cntnap2* KO^{het} was similar to that of WT pups across PND3 to 21. In contrast, *Cntnap2* KO^{hom} showed a higher number of calls than WT and KO^{het} pups on PND8 and 12 (Figure 1A,B; see Tables 4–6 for statistical details).

On PND12—when the amount of USVs peaked in all three groups—the average call pitch was increased from 46 kHz in WT pups to 50 kHz in *Cntnap2* KO^{het} and to 53 kHz in KO^{hom} ($p < 0.0001$, $F(2, 31) = 15.18$, Figure 2A, see also Table 7 and Figure S14). Despite the higher call pitch in *Cntnap2* KO^{het} and KO^{hom}, the call bandwidth was similar between genotypes and breeding backgrounds (WT: 20 kHz, KO^{het}: 21 kHz, KO^{hom}: 20 kHz, Figure 2B, $p = 0.7888$, $F(2, 31) = 0.2391$). Moreover, mean call duration was 98 ms in *Cntnap2* KO^{het} and 82 ms in KO^{hom}, which was statistically not different from WT pups (79 ms, Figure 2C, $p = 0.0327$, $F(2, 31) = 3.829$, see also

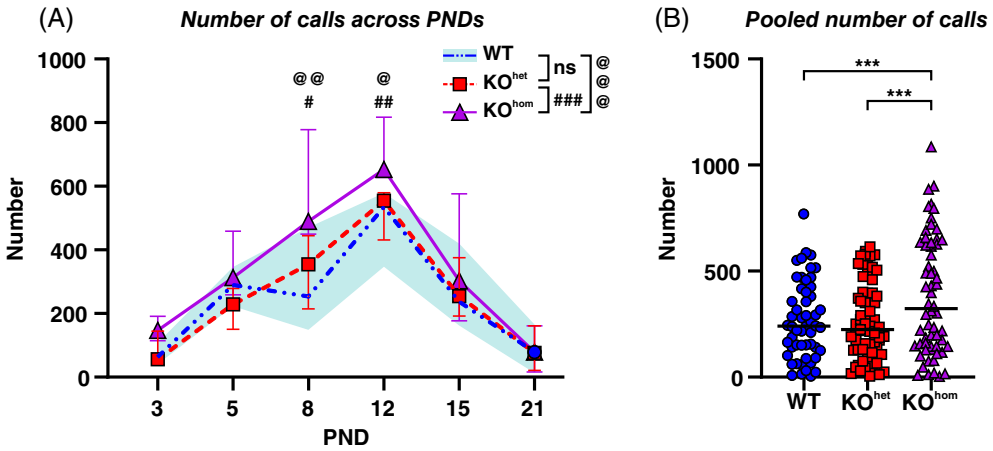

FIGURE 1 Ultrasonic vocalization profiles in *Cntnap2* WT, KO^{het} (both from heterozygous breeding), and KO^{hom} pups (from homozygous breeding) across PND3, 5, 8, 12, 15 and 21. (A) Developmental trajectories of ultrasonic vocalization (USV) call numbers (median ± interquartile range, IQR) from *Cntnap2* WT (blue line and area), KO^{het} (red squares and error bars), and KO^{hom} (purple triangles and error bars) peaked on PND12. *Cntnap2* KO^{hom} pups emitted more USVs than *Cntnap2* WT and KO^{het} pups, in particular on PND8 and 12 (ART ANOVA Mixed-effects model see Table 4; post hoc ART-C tests see Table 5 [contrast factor PND × genotype]. (B) The average number of calls pooled across PNDs was higher in *Cntnap2* KO^{hom} than *Cntnap2* WT or KO^{het} pups. Number of calls was similar between *Cntnap2* WT or KO^{het} pups (Table 6 [contrast factor genotype]). (A) WT: PND3 $n = 5$, PND5 $n = 8$, PND8, 12, 15, 21 $n = 10$, KO^{het}: PND3 $n = 9$, PND5 $n = 10$, PND8, 12, 15, 21 $n = 12$, KO^{hom}: PND3, 5, 8 $n = 5$, PND12, 15, 21 $n = 15$ female and male rat pups; (B) WT: $n = 53$, KO^{het}: $n = 67$, KO^{hom}: $n = 60$ female and male rat pups. Data expressed as (A) median ± IQR and (B) individual animals and PNDs (symbols) and median (horizontal black line). *p* values, @, # $p < 0.05$, @@, ##$p < 0.01$, @@@, ###$p < 0.001$, $^{***}p < 0.001$, ns not significant

TABLE 4 Statistical comparisons of ultrasonic vocalization call numbers for effects of sex, genotype, PND, sex × genotype, sex × PND, genotype × PND, genotype × PND × sex, in *Cntnap2* WT, KO^{het} and KO^{hom} pups (ART ANOVA)

Source of variation	*p* Value	*p* Value summary	*F* (DFn, DFd)	Effect size ηp2
Sex	0.9781	ns	$F(1, 32.49674) = 0.000759$	0.4369
Genotype	0.0001	***	$F(2, 31.96855) = 12.4$	<0.01
PND	<0.0001	***	$F(5, 123.90314) = 81.3$	0.7664
Sex × genotype	0.229	ns	$F(2, 32.18007) = 1.54$	0.0876
Sex × PND	0.135	ns	$F(5, 123.90453) = 1.72$	0.1536
Genotype × PND	0.0222	*	$F(10, 121.08504) = 2.2$	0.0649
Genotype × PND × sex	0.149	ns	$F(10, 120.74795) = 1.49$	0.1101

Note: Mixed-effects model, Analysis of Deviance Table, *p* values, *$p < 0.05$, ***$p < 0.001$, ns not significant.

TABLE 5 Post hoc pairwise comparisons of ultrasonic vocalization call numbers from *Cntnap2* WT, KO^{het} and KO^{hom} pups across PND3, 5, 8, 12, 15 and 21 (ART-C factor PND × genotype)

PND	Genotype comparison	Mean diff.	95.00% CI of diff.	Summary	Adjusted *p* value
3	WT vs. KO^{het}	−6.600	−31.11 to 17.91	ns	0.7565
	WT vs. KO^{hom}	−26.90	−56.43 to 2.627	ns	0.0706
	KO^{het} vs. KO^{hom}	−20.30	−51.99 to 11.39	ns	0.2334
5	WT vs. KO^{het}	22.18	−4.538 to 48.89	ns	0.1127
	WT vs. KO^{hom}	−13.63	−48.75 to 21.50	ns	0.5286
	KO^{het} vs. KO^{hom}	−35.80	−71.60 to 0.0006359	ns	0.0500
8	WT vs. KO^{het}	−15.59	−57.69 to 26.51	ns	0.6124
	WT vs. KO^{hom}	−60.20	−105.2 to −15.22	**	0.0096
	KO^{het} vs. KO^{hom}	−44.61	−80.55 to −8.671	*	0.0168
12	WT vs. KO^{het}	−6.983	−31.42 to 17.46	ns	0.7408
	WT vs. KO^{hom}	−28.35	−51.66 to −5.035	*	0.0183
	KO^{het} vs. KO^{hom}	−21.37	−34.95 to −7.783	**	0.0021
15	WT vs. KO^{het}	−1.350	−36.52 to 33.82	ns	0.9948
	WT vs. KO^{hom}	−14.20	−52.29 to 23.89	ns	0.6250
	KO^{het} vs. KO^{hom}	−12.85	−51.21 to 25.51	ns	0.6855
21	WT vs. KO^{het}	2.250	−30.82 to 35.32	ns	0.9834
	WT vs. KO^{hom}	−2.300	−36.75 to 32.15	ns	0.9844
	KO^{het} vs. KO^{hom}	−4.550	−33.11 to 24.01	ns	0.9172

Note: Tukey's multiple comparisons test, *p* values, *$p < 0.05$, **$p < 0.01$, ns not significant.

TABLE 6 Post hoc pairwise comparisons of ultrasonic vocalization call numbers from *Cntnap2* WT, KO^{het} and KO^{hom} pups (ART-C factor genotype)

Genotype comparison	Mean diff.	95.00% CI of diff.	Summary	Adjusted *p* value
WT vs. KO^{het}	1.636	−19.35 to 22.63	ns	0.9815
WT vs. KO^{hom}	−41.76	−63.29 to −20.24	***	<0.0001
KO^{het} vs. KO^{hom}	−43.40	−63.69 to −23.10	***	<0.0001

Note: Tukey's multiple comparisons test, *p* values, ***$p < 0.001$, ns not significant.

Table 7). Note that female pups showed shorter call durations than males, irrespective of group (sex $p = 0.0006$, $F(1, 31) = 14.76$, sex × genotype $p = 0.9407$, $F(2, 31) = 0.06121$).

In summary, while USVs did not display developmentally delayed peaking in numbers or altered call bandwidths in *Cntnap2* KO pups from both heterozygous or homozygous breeding backgrounds, we found that the homozygous parental genotype resulted in a higher number of USVs and further exacerbated altered call pitch also found in *Cntnap2* KO pups from heterozygous breeding. Interestingly, the homozygous breeding background seem to revert the slightly

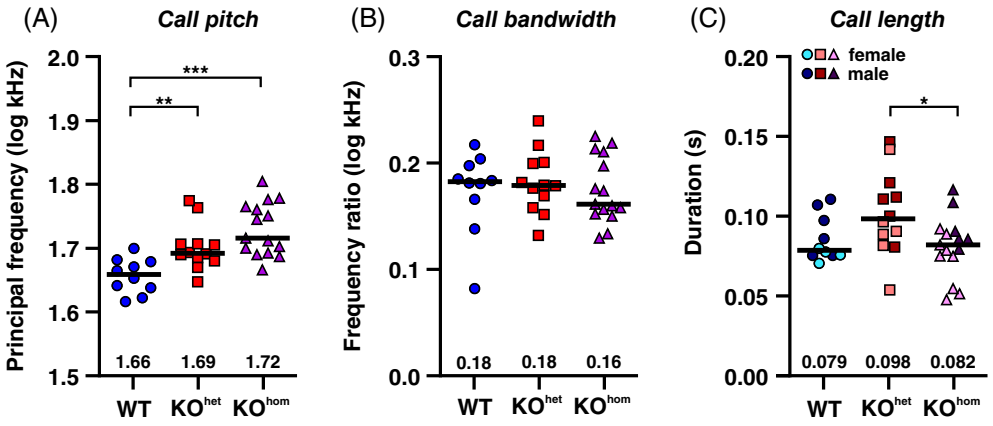

FIGURE 2 Acoustic parameters of maternal isolation-induced calls emitted by *Cntnap2* WT and KO^{het} and KO^{hom} pups on PND12. (A) Call pitch was significantly different between genotypes, with *Cntnap2* KO^{het} and KO^{hom} showing higher principal frequencies than WT pups. (B) Call bandwidth was similar between *Cntnap2* WT, KO^{het} and KO^{hom} pups. (C) Call length was significantly different between genotypes, with *Cntnap2* KO^{het} showing longer call duration than KO^{hom}. Note that on PND12, females (light symbols, median [IQR], 0.079 [0.071–0.089] s) emitted shorter calls than males (dark symbols, 0.099 [0.084–0.111] s), irrespective of genotype and rearing background. WT: $n = 10$, KO^{het}: $n = 12$, KO^{hom}: $n = 15$ female and male rat pups. Data expressed as individual animals (symbols) and median (horizontal black line and number insets). p values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

TABLE 7 Post hoc pairwise comparisons of ultrasonic vocalization call parameters duration, pitch and type from *Cntnap2* WT, KO^{het} and KO^{hom} pups on PND12 (ART-C factor genotype)

Call parameter	Genotype comparison	Predicted (LS) mean diff.	95.00% CI of diff.	Summary	Adjusted p value
Pitch	WT vs. KO^{het}	−12.08	−20.76 to −3.404	**	0.0048
	WT vs. KO^{hom}	−18.67	−27.02 to −10.31	***	<0.0001
	KO^{het} vs. KO^{hom}	−6.583	−14.42 to 1.251	ns	0.1132
Duration	WT vs. KO^{het}	−8.708	−19.80 to 2.387	ns	0.1468
	WT vs. KO^{hom}	2.181	−8.496 to 12.86	ns	0.8706
	KO^{het} vs. KO^{hom}	10.89	0.8734 to 20.90	*	0.0308
Number of different call types	WT vs. KO^{het}	−11.63	−19.88 to −3.373	**	0.0043
	WT vs. KO^{hom}	−18.40	−26.34 to −10.46	***	<0.0001
	KO^{het} vs. KO^{hom}	−6.778	−14.23 to 0.6713	ns	0.0802

Note: Tukey's multiple comparisons test, p values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns not significant.

increased call duration found in heterozygously bred *Cntnap2* KOs. Taken together, our results suggest compounding effects of *Cntnap2* gene mutation and maternal genotype on certain basic acoustic and categorical call characteristics.

3.2 | Compounding effects of *Cntnap2* gene mutation and maternal genotype on temporal structure of USV bouts

The temporal organization of pup vocalizations, such as the call rate and inter-call intervals (ICIs), is crucial for the elicitation of maternal care behavior.[46,48,53,54] Given the higher number of calls within the 3-min recordings from *Cntnap2* KO^{hom} pups (Figure 1) and the slightly increased call length in *Cntnap2* KO^{het} pups (Figure 2C), we next analyzed how *Cntnap2* mutation and/or the breeding background altered the temporal structure of vocalizations. To this end, we defined three distinct inter-call intervals: short duration ICIs separating individual USVs (≥20 ms[44]), medium duration ICIs separating sequences of USVs (≥150 ms[46]) and long duration ICIs separating bouts of USVs (≥2000 ms,[47,48] see Figure 3A) The defined medium duration ICIs coincided with the ICI frequency distribution, where the majority of ICIs fell into the 100 to 150 ms bin for all three groups (Figure S3). Based on these ICIs, we calculated features of call sequences and call bouts, including their numbers and durations, and compared them between *Cntnap2* WT, KO^{het} and KO^{hom} pups on PND12 (Figure 3B–I). There were no significant differences for the duration of call sequences, which were 0.90 s in WT, 0.83 s in KO^{het} and 0.88 s in KO^{hom} ($p = 0.5652$; Figure 3B). The number of calls per sequence was also not altered (WT: 5.24,

KO^{het}: 4.42, KO^{hom}: 5.00; $p = 0.1362$, Figure 3C), and the resulting call rate within a sequence was similar across genotypes, with 6.78 Hz in WT, 6.56 Hz in KO^{het}, and 6.90 Hz in KO^{hom} ($p = 0.5525$, Figure 3D). Interestingly, female pups emitted calls at a faster rate than males (7.15 Hz in females versus 6.46 Hz in males, $p = 0.0043$, $F(1, 31) = 9.503$), irrespective of genotype and rearing background, coinciding with the shorter duration of calls in females on PND12 (see Figure 2C). In summary, the temporal

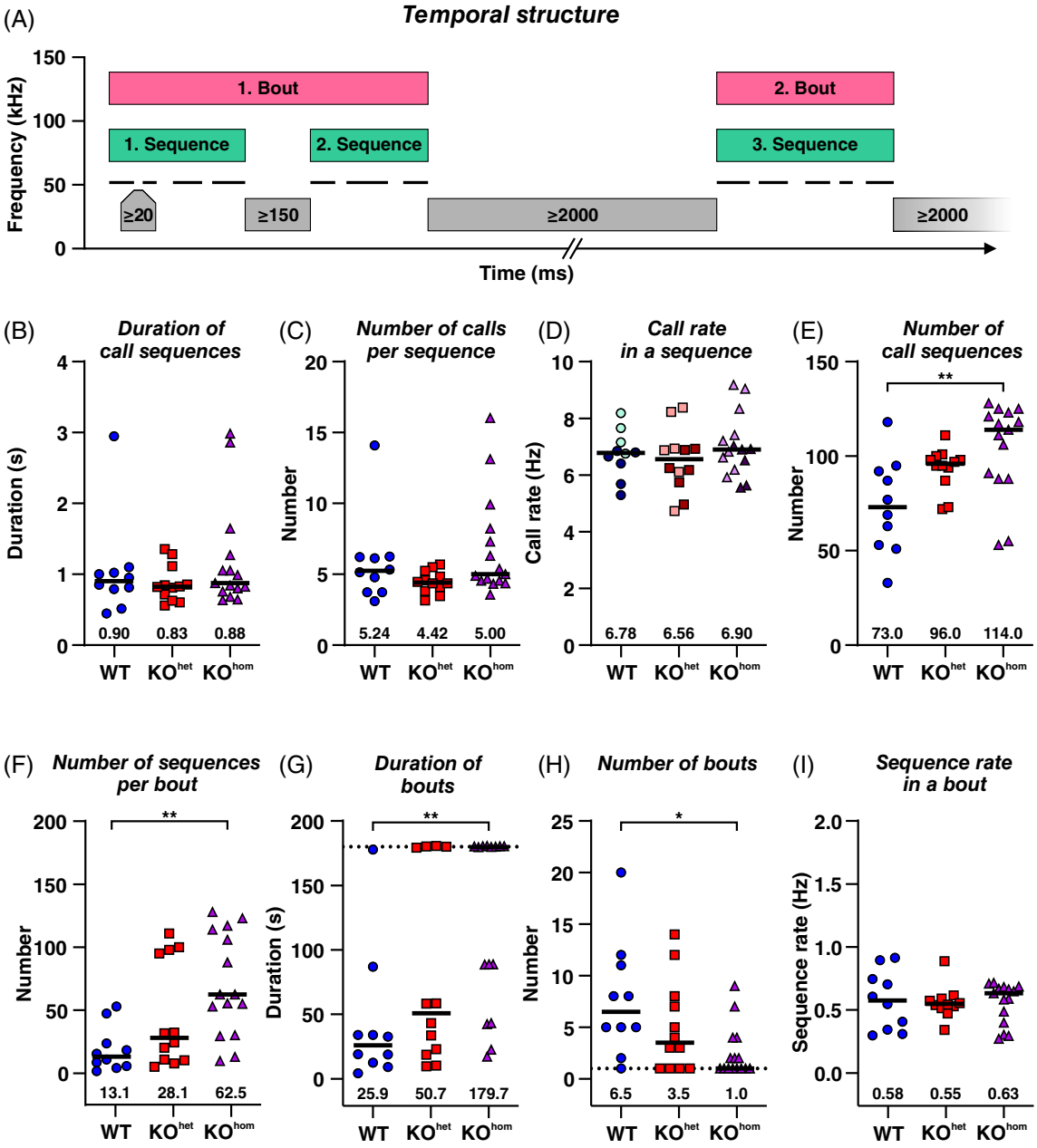

FIGURE 3 Temporal organization of *Cntnap2* WT, KO^{het} and KO^{hom} ultrasonic vocalizations (USVs) on PND12. (A) Schematic spectrogram of 50 kHz USVs (black horizontal lines). Three distinct ICI categories were defined as short duration ICIs separating individual USVs (≥20 ms), medium duration ICIs (≥150 ms) separating sequences of USVs, and long duration ICIs (≥2000 ms) separating bouts of USVs. (B) There were no significant differences between *Cntnap2* WT, KO^{het} and KO^{hom} pups for the duration of call sequences. (C) There were also no differences in number of calls per sequence. (D) Call rate within a sequence (number of calls per second) were also not different between groups. Note that females (light symbols) emitted calls at a higher rate than males (dark symbols), irrespective of genotype and rearing background. (E) USVs were arranged in a greater number of call sequences in *Cntnap2* KO^{hom} compared with WT pups. (F) The average number of sequences within a bout was increased in *Cntnap2* KO^{hom} compared with WTs. (G) *Cntnap2* KO^{hom} pups showed longer durations of USV bouts. Horizontal dotted line denotes total USV recording duration of 180 s. (H) *Cntnap2* KO^{hom} pups showed reduced numbers of bouts compared with WTs. Horizontal dotted line denotes minimum of 1 bout in a whole recording. (I) The temporal rate of USV sequences within bouts was similar between genotypes. For all figures: WT: $n = 10$, KO^{het}: $n = 12$, KO^{hom}: $n = 15$ female and male rat pups. Data expressed as individual animals (symbols) and median (horizontal black line and number insets). p values, $^{*}p < 0.05$, $^{**}p < 0.01$

TABLE 8 Post hoc pairwise comparisons of ultrasonic vocalization parameters number of call sequences, duration of bouts, number of bouts and number of call sequences per bout from *Cntnap2* WT, KO^{het} and KO^{hom} pups on PND12 (ART-C factor genotype)

Call parameter	Genotype comparison	Predicted (LS) mean diff.	95.00% CI of diff.	Summary	Adjusted *p* value
Number of call sequences	WT vs. KO^{het}	−7.000	−17.68 to 3.681	ns	0.2554
	WT vs. KO^{hom}	−13.50	−23.78 to −3.222	**	0.0080
	KO^{het} vs. KO^{hom}	−6.500	−16.14 to 3.142	ns	0.2368
Duration of bouts	WT vs. KO^{het}	−6.208	−16.77 to 4.350	ns	0.3298
	WT vs. KO^{hom}	−13.18	−23.34 to −3.021	**	0.0088
	KO^{het} vs. KO^{hom}	−6.972	−16.50 to 2.558	ns	0.1861
Number of bouts	WT vs. KO^{het}	5.375	−5.413 to 16.16	ns	0.4470
	WT vs. KO^{hom}	12.99	2.605 to 23.37	*	0.0117
	KO^{het} vs. KO^{hom}	7.611	−2.127 to 17.35	ns	0.1490
Number of sequences per bout	WT vs. KO^{het}	−8.083	−18.37 to 2.206	ns	0.1463
	WT vs. KO^{hom}	−14.69	−24.60 to −4.794	**	0.0027
	KO^{het} vs. KO^{hom}	−6.611	−15.90 to 2.677	ns	0.2025

Note: Tukey's multiple comparisons test, *p* values, $^{*}p < 0.05$, $^{**}p < 0.01$, ns not significant.

arrangement of calls within sequences was similar between *Cntnap2* WT, KO^{het} and KO^{hom} pups (Figure 3B–D).

In contrast, the total number of call sequences within the 3-minute recordings was significantly different between genotypes ($p = 0.0107$, $F(2, 31) = 5.273$, Figure 3E). Specifically, post hoc comparisons showed that an increase in number of call sequences was not yet statistically significant in *Cntnap2* KO^{het} (96.00), but significant in *Cntnap2* KO^{hom} pups (114.0), in comparison with WT pups (73.00, Figure 3E and Table 8). In a similar fashion, the number of call sequences per bout was not yet significantly increased in *Cntnap2* KO^{het} (28.08), however, they were significantly increased in *Cntnap2* KO^{hom} pups (62.50) compared with WT controls (13.14, $p = 0.0038$, $F(2, 31) = 6.695$, Figure 3F, for all post hoc comparison see Table 8). Along with the increase in number of call sequences per bout, the duration of call bouts was also significantly longer in *Cntnap2* KO^{hom}, but not in *Cntnap2* KO^{het} ($p = 0.0112$, $F(2, 31) = 5.216$, Figure 3G). In line with this, the number of bouts was significantly decreased in *Cntnap2* KO^{hom}, but not in *Cntnap2* KO^{het} ($p = 0.0134$, $F(2, 31) = 4.968$, Figure 3H). In fact, only 1 out of 10 WT pup continuously vocalized without any breaks ≥2 s, whereas 33.3% of *Cntnap2* KO^{het} and 53.3% of *Cntnap2* KO^{hom} animals produced calls that were arranged in a single, long bout close to the duration of the full 3-min recording. Interestingly, despite the altered total number of call sequences, number of sequences per bout, and duration and number of call bouts, the temporal spacing of call sequences within a bout remained similar between all three groups ($p = 0.9023$, Figure 3I).

Taken together, our results indicate that the homozygous *Cntnap2* KO maternal genotype exacerbates the alterations caused by *Cntnap2* knock-out in the temporal USV structure such that calls are arranged in less frequent, but longer bouts that comprise more call sequences. In contrast, the temporal spacing of sequences within bouts (sequence rate), and of USVs within the sequences (call rate), remained unchanged between genotypes and breeding backgrounds.

3.3 | Call type profile is altered in *Cntnap2* KO^{het} and *Cntnap2* KO^{hom} pups

Rodent USVs can be categorized into different subtypes based on their frequency modulation, temporal continuity and duration.[43] These subtypes have been proposed to coordinate context-dependent social interactions such as maternal retrieval behavior,[22,43,55,56] and their utilization is malleable including by selective breeding and drugs.[22,23,43,57] In order to investigate the influence of ASD risk gene–environment interactions on call type utilization, we classified USV call types of *Cntnap2* WT, KO^{het} and KO^{hom} pups across PND3, 5, 8, 12, 15 and 21 (Figure 4). We used the MATLAB software suite DeepSqueak[41] to train an artificial neural network for automated USV call classification. Our classifier is based on >8500 calls, manually classified into one of 13 different call types (Figure S1, modified from Wright et al,[43] Riede[44]). *Cntnap2* WT pups showed a distinct ontogenetic postnatal profile of call type usage (Figure 4A). The most common call type until including PND8 was "Flat." Its usage first decreased with age from 80.5% at PND3 to 17.5% at PND12, before rising again to 44.6% at PND21. Compared with the "Flat" call type, the "Step down" call type showed an inverted development, being low in usage at PND3 (2.1%), peaking at PND12 at 40.3%, and then declining to 7.5% at PND21 (Figure 4A). In more detail, on PND8 the proportion of "Flat" calls was significantly higher than that of the second most common call type "Step down" ($p = 0.0005$, Friedman test $p = 0.0005$, $Fr = 17.64$, Dunn's multiple comparisons test, $p = 0.0040$, $Z = 3.291$ Figure 4B). From PND8 to 12 the usage of "Flat" calls significantly decreased ($p = 0.0073$, $Z = 3.118$), whereas that of "Step down" significantly increased ($p = 0.0223$, $Z = 2.771$). As a result, on PND12 the proportion of "Step down" USVs was significantly higher compared with "Flat" ($p = 0.0375$, $Z = 2.598$; Figure 4B).

In both *Cntnap2* KO^{het} (Figure 4C) and KO^{hom} pups (Figure 4E) the "Flat" call type profile appeared to be similar to WTs with its

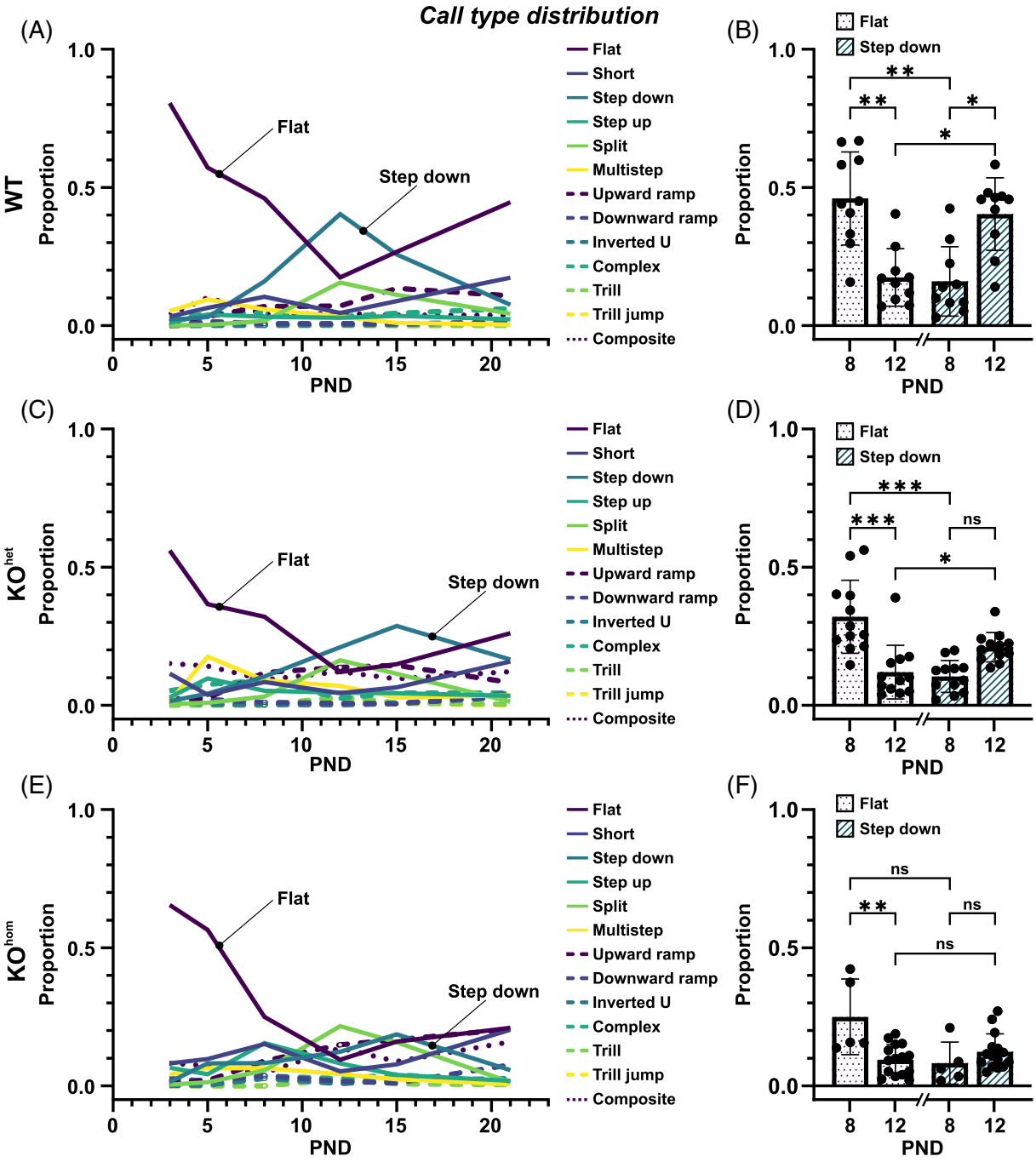

FIGURE 4 Ultrasonic vocalization (USV) call type utilization in (A, B) *Cntnap2* WT, (C, D) KO^{het} and (E, F) KO^{hom} pups across PND3, 5, 8, 12, 15 and 21. (A) Proportion of frequency modulated call types in *Cntnap2* WT pups during development. (B) On PND8, the proportion of "Flat" calls was significantly higher than "Step down" calls. Between PND8 and 12 the proportion of "Flat" calls significantly decreased, and the proportion of "Step down" calls increased. On PND12, the proportion of "Flat" calls was significantly lower than "Step down" calls. (C) In both *Cntnap2* KO^{het} and (E) KO^{hom}, the proportion of "Flat" calls decreased with age until its minimum on PND12, but the peak of "Step down" usage was delayed from PND12 to PND15. In *Cntnap2* KO^{hom}, the most common call type on PND12 was "Split." (D) In *Cntnap2* KO^{het}, the proportion of "Flat" calls was significantly higher than "Step down" calls on PND8, and significantly lower on PND12. Between PND8 and 12 the proportion of "Flat" calls significantly decreased, but the increase of "Step down" calls was not statistically significant. (F) In *Cntnap2* KO^{hom}, the "Flat" call type usage was significantly lower on PND12 than PND8 (PND8 median = 0.1581, $n = 5$; PND12 median = 0.09887, $n = 15$), whereas "Step down" usage was not significantly higher on PND12 than PND8 (PND8 median = 0.06279, $n = 5$; PND12 median = 0.1082, $n = 15$). There was no statistically significant difference between the proportion of "Flat" compared with "Step down" calls on PND8 or PND12. For all panels: The number of USVs within call type was normalized to the total amount of calls from all types for individual pups and then averaged across animals within genotype and rearing background. WT: PND3 $n = 5$, PND5 $n = 8$, PND8, 12, 15, 21 $n = 10$, KO^{het}: PND3 $n = 9$, PND5 $n = 10$, PND8, 12, 15, 21 $n = 12$, KO^{hom}: PND3, 5, 8 $n = 5$, PND12, 15, 21 $n = 15$ female and male rat pups. Data expressed as (A, C, E) mean and (B, D, F) mean ± SD (bars and error bars) and individual animals (symbols). *p* values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns not significant

minimum on PND12. Indeed, on PND12, the amount of "Flat" calls was not different, whereas on PND8, 15 and 21 the number was higher in *Cntnap2* WT pups than KO^{het} or KO^{hom} pups (Figure S5A and Tables S2 and S3). In terms of the "Step down" call, however, its peak usage was delayed to PND15 in KO^{het} or KO^{hom} pups (Figure 4C–E). Consequently, *Cntnap2* KO^{het} and KO^{hom} pups emitted less "Step down" calls than WT pups on PND12 (Figure S5B and Tables S2 and S3). In more detail: in line with WT controls, there was a significant decrease of "Flat" calls from PND8 to 12 in both *Cntnap2* KO^{het} ($p < 0.0001$, $F_r = 22.60$, Figure 4D) and KO^{hom} pups (Mann Whitney test $p = 0.0077$, $U = 8$, Figure 4F), whereas different from WTs, there was no simultaneous increase of "Step down" calls (*Cntnap2* KO^{het} $p = 0.1074$, Figure 4D; *Cntnap2* KO^{hom} $p = 0.1418$, Figure 4F). Furthermore, while the amounts of "Flat" and "Step down" calls were significantly different on either PND8 or PND12 in *Cntnap2* KO^{het} (PND8: $p = 0.0006$, $Z = 3.795$; PND12: $p = 0.0456$, $Z = 2.530$; Figure 4D), they were similar on either PND in *Cntnap2* KO^{hom} pups (PND8 $p = 0.1250$; PND12 $p = 0.2293$, Figure 4F).

Taken together, our results indicate a call type-specific developmental delay in USV utilization through *Cntnap2* mutation that is exacerbated by the homozygous maternal genotype. This suggests that the specificity of the ontogenetic call type profile is diminished in a genotype- and breeding background-dependent manner.

3.4 | Transitions between call types are moderately altered in *Cntnap2* KO^{het} and severely altered in *Cntnap2* KO^{hom} pups

It has been shown that rodent USV subtypes are not selected in a random order, but rather display a characteristic syllabic structure and are organized into phrases and motifs.[58] We therefore analyzed how *Cntnap2* mutation and breeding background affected the USVs call repertoire and syntax flow. On PND12, both *Cntnap2* KO^{het} and KO^{hom} pups employed a higher number of individual call types than WT controls, even though only *Cntnap2* KO^{hom} had a higher total number of calls within the three-minute recordings (see Figure 1, Table 6). Interestingly, of the different call types identified by our classifier, only *Cntnap2* KO^{hom} pups used on average calls of all 13 individual types (Figure 5, see also Figure S4, Table 7). Comparisons of the call type distribution showed genotypic differences in those USVs containing frequency modulations, instantaneous frequency jumps and breaks and combinations of call types, whereas frequency unmodulated USVs were similar between *Cntnap2* WT, KO^{het} and KO^{hom} pups on PND12 (Figure S5C, for post hoc tests see Tables S1 and S4–S6). In particular, both *Cntnap2* KO^{het} and KO^{hom} showed a higher proportion of call types "Upward ramp," "Trill," "Trill jump" and "Composite" compared with WTs. Furthermore, *Cntnap2* KO^{hom} had a higher proportion of "Inverted U" calls compared with either WT or KO^{het} pups, as well as more "Step up" calls than WT controls and a higher proportion of "Downward ramp" USVs than *Cntnap2* KO^{het}. In contrast, "Step down" call type usage was significantly reduced in both *Cntnap2* KO^{het} and KO^{hom} pups, the former compared with WT controls, and the latter compared with either *Cntnap2* WT or KO^{het} pups (Figure S5C, for post hoc tests see Tables S1 and S4–S6). This indicated that *Cntnap2* KO^{het}, and even more so, KO^{hom} pups had an atypically diversified USV repertoire, where call type distribution is shifted from the normally most common type "Step down" to other call types. Given this unusual call type utilization, we used DeepSqueak's syntax analysis tool[41] to investigate how the ASD risk gene–environment interaction affected the orderly transitioning between individual call types within USV bouts (maximum bout separation 2 s). For this analysis, USVs from males and females were pooled, since there were no main effects or interactions of sex on call type distribution within any of the three groups (Table S7). The transitioning between call types in *Cntnap2* WT, KO^{het} and KO^{hom} pups on PND12 is displayed in a syntax flow path that represents the conditional probability of the USVs changing from one call type to another (Figure 5). Within call bouts, *Cntnap2* WT pups transitioned between 9 different call types (Figure 5A and Figure S6A), whereas *Cntnap2* KO^{het} and KO^{hom} pups transitioned between 11 and 12 call types, respectively, within call bouts (Figure 5B,C and Figure S6B,C), which goes along with the higher absolute numbers of different call types (Figure S4). A more detailed analysis of the transitioning probabilities between specific call types can be found in the Supporting Information and Figures S6 and S7. Taken together, our results suggest that *Cntnap2* mutation led to a more diversified call syntax. This increase in syntax variability was intensified by the homozygous breeding background, suggesting compounding effects of *Cntnap2* gene mutation and breeding conditions on call type usage and syntax.

In summary, *Cntnap2* knockout within a heterozygous breeding scheme resulted in normal absolute numbers of calls and largely similar temporal structures of USVs. In contrast, both USV number and temporal structure were affected in KOs bred from homozygous dams. Moreover, *Cntnap2* KOs from heterozygous breeding showed altered call pitch, call types and transitioning repertoire, all of which were further exacerbated through the homozygous maternal genotype.

3.5 | No influence of MIA on basic acoustic call characteristics and USV temporal organization on heterozygously bred *Cntnap2* pups on PND8

In the following, we explored the interaction of *Cntnap2* mutation with a different environmental factor, namely with maternal immune activation during fetal development. We used poly I:C administration in the *Cntnap2* rat model to characterize potential compounding effects of ASD risk gene mutation and MIA on early vocal communication in the offspring. Male and female heterozygous *Cntnap2* rats were mated and on GD9.5, pregnant dams were injected with either poly I:C or vehicle (saline). Offspring's USVs were recorded on PND8. Isolation-induced USVs from *Cntnap2* WTs and KOs from saline and poly I:C offspring produced similar numbers of USVs within the 3-min recordings, irrespective of genotype and maternal treatment (WT

+ saline: 185, WT + poly I:C: 137, KO + saline: 180, KO + poly I:C: 225, Figure 6A, for statistical results see Table 9). In contrast, call length, pitch and bandwidth were significantly increased in *Cntnap2* KO pups on PND8, however there were no effects of the maternal exposure to poly I:C in either the WT or KO offspring (Figure 6B–D, for statistical results see Table 9). None of the parameters used to

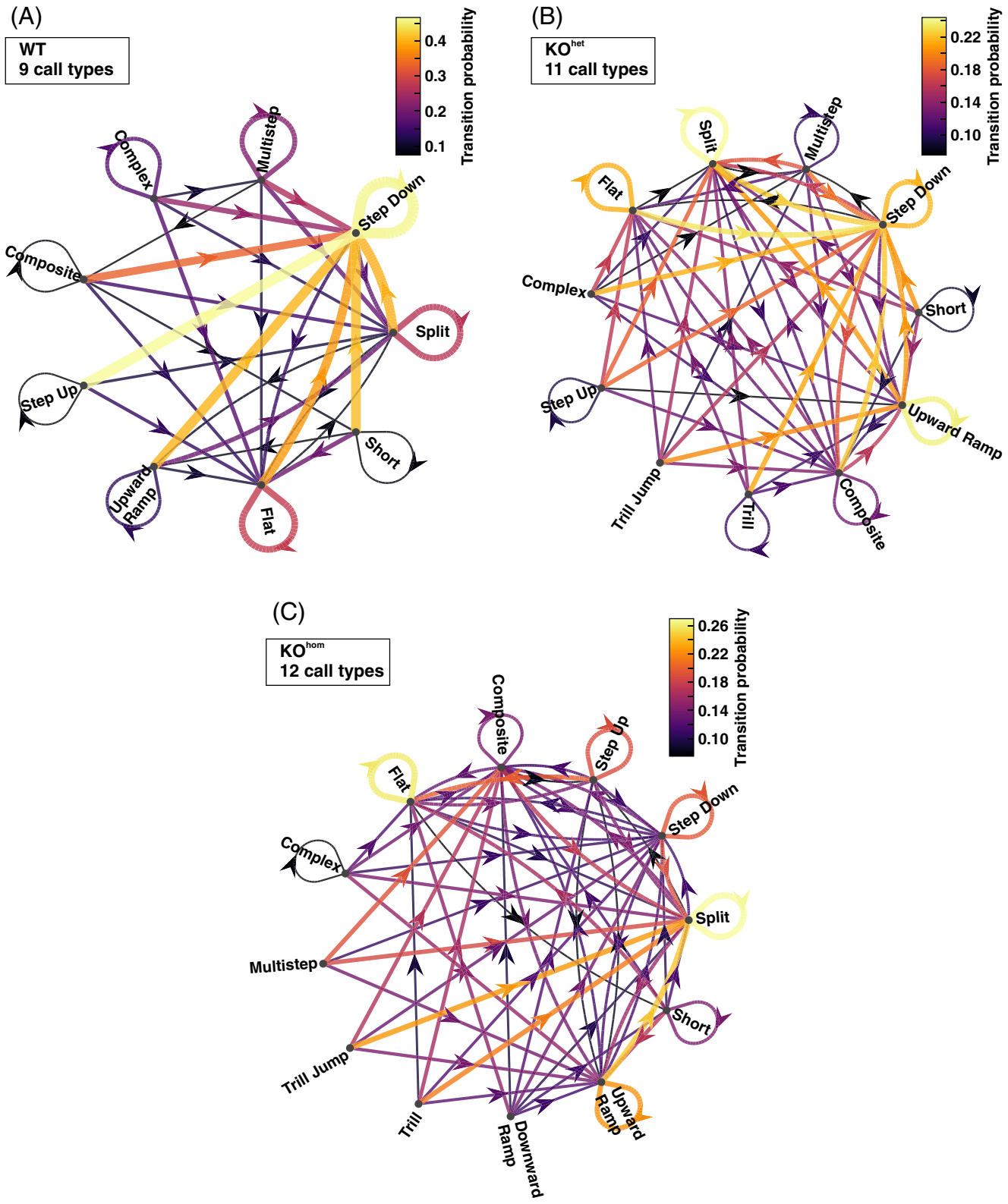

FIGURE 5 Legend on next page.

quantify the USV temporal organization showed any effects of maternal poly I:C treatment, including the duration of call sequences, number of calls within a sequence, the resulting call rate within sequences (calls per second), the total number of call sequences within a 3-min recording, number of sequences per call bout, duration of bouts, total number of bouts and the sequence rate in a bout (Figure S8 and Table S8). Taken together, in our hands, there were neither main effects of MIA nor interaction effects of *Cntnap2* gene mutation and MIA on basic categorical, acoustic or temporal call characteristics on PND8.

3.6 | Compounding effects of MIA and *Cntnap2* mutation on call type repertoire and transitions

Poly I:C-induced MIA has previously been described to alter call type transition probability in male adult Sprague Dawley WT rats.[59] Despite similar total numbers of calls in *Cntnap2* WT and KO rats from poly I:C and saline offspring within our 3-min recordings, we found that a higher number of different call types was utilized in *Cntnap2* KO on PND8 (KO + saline: 11, KO + poly I:C: 10) than WT pups (WT + saline: 9, WT + poly I:C: 9), irrespective of MIA (Figure 7A, see also Table 9). In-depth analysis of the call subtype distribution revealed a significant interaction between call type, genotype and treatment (see Table S9). Specifically, a higher proportion of calls was used by *Cntnap2* KO pups for subtypes "Step up," "Composite," "Downward ramp" and "Trill" (Figure 7B–E, for statistical results see Table S10). Maternal exposure to poly I:C reduced the proportion of "Trill" USVs in the offspring in both genotypes (Figure 7E, Table S10). Interestingly, utilization of the most common call type on PND8—"Flat"—was decreased in *Cntnap2* KO pups. This genotypic difference was intensified by maternal poly I:C treatment that further decreased "Flat" call usage in KO pups compared with increased usage in WT poly I:C littermates, and compared with WT controls from saline offspring (Figure 7F, Tables S10 and S11). Furthermore, Poly I:C KO offspring showed higher "Multistep" USVs compared with WT offspring, and compared with WTs from saline offspring, MIA again aggravating genotypic differences (Figure 7F, Tables S10 and S11). Further interaction effects of genotype and maternal poly I:C treatment became apparent in call types "Split" and "Step down" (Figure 7H,I, Table S10). Both "Split" and "Step down" usage was increased in KOs from poly I:C offspring compared with WT littermates (Figure 7H,I, Table S11), and "Step down" USVs were decreased in the latter compared with WT controls from saline offspring (Figure 7I, Table S11). No genotype or treatment effects were found for "Short," "Upward ramp," "Complex," "Inverted U" and "Trill jump" USVs (Figure S9F–I and Table S10).

Taken together, compounding effects of MIA through poly I:C and *Cntnap2* mutation were found for four out of 13 call types ("Flat," "Multistep," "Split," "Step down"). The effect of poly I:C was call type-dependent: The usage of the most common call type on PND8, "Flat," was increased in WT and decreased in KO offspring by poly I:C, whereas "Composite," "Split" and "Step down" usage was decreased in WT and increased in KO offspring by poly I:C. Post hoc pairwise comparisons of USV call type proportions between male and female *Cntnap2* pups from saline and poly I:C offspring on PND8 indicated similar treatment effects in either sex (Table S12).

Our results indicate that MIA causes an atypically diversified USV repertoire in *Cntnap2* KO offspring, where call type distribution is already shifted from the normally most common type "Flat" to other call types ("Multistep," "Split," "Step down"). This notion was also confirmed by analysis of call type transition probability within 2-s bouts (Figure 8). For this analysis, USVs from males and females were pooled, since there were no main effects or interactions of sex and treatment on call type distribution within genotype (see Table S13). Within a 2-s bout, *Cntnap2* WT pups from saline offspring transitioned between the 9 different call types "Flat," "Complex," Composite," "Short," "Upward ramp," "Step up," "Step down," "Multistep" and "Split" (Figure 8A and Figure S10A), whereas KO littermates transitioned between 10 call types (plus "Downward ramp," Figure 8B and Figure S10B). Even though there was no effect of treatment on the number of different call types used (Figure 7A, see also Table 9), *Cntnap2* WT pups from poly I:C offspring showed only 8 call types in their transition repertoire (lack

FIGURE 5 Conditional probabilities for call type transitions within ultrasonic vocalization (USV) bouts in *Cntnap2* WT, KO^{het} and KO^{hom} pups on PND12. (A) *Cntnap2* WT pups transitioned between 9 call types. "Step down" had the highest probability to be the next call type in the call bout including repeated use. The most probable transitions to "Step down" (in descending order) were from "Step up," repeated use of "Step down" and from "Short," "Upward ramp," "Split," "Flat," "Composite," "Multistep" or "Complex" to "Step down." "Flat" and "Split" USVs were the second and third most transitioned to, respectively, most of which was because of repeated use. (B) *Cntnap2* KO^{het} pups transitioned between 11 call types. "Step down" was still most frequently transitioned to, but transition probability to "Step down" was decreased for all call types (i.e., "Flat," "Split," "Composite," repeated "Step down," "Complex," "Upward ramp," "Short," "Multistep," "Step up") compared with WTs. Instead, "Trill" and "Trill jump" USVs additionally appeared in the *Cntnap2* KO^{het} transition repertoire. Other call types had high transition probabilities driven by repeated use ("Split," "Upward ramp," "Flat"), but also from "Step down" to "Composite." (C) *Cntnap2* KO^{hom} transitioned between 12 call types, with additional transitioning from "Trill," "Trill jump" and "Downward ramp" USVs in their call bouts. "Split" was the most transitioned to, through repeated use of "Split," or from "Upward ramp," "Trill Jump," "Trill," "Step down," "Multistep," "Composite," "Step up," "Complex," "Short," "Flat," "Downward ramp" in descending order. Transitions to "Composite" USVs had the second highest overall probability, mostly from "Multistep," "Trill," "Trill jump" USVs. "Upward ramp," "Step down" and "Flat" USVs were the third, fourth and fifth most transitioned to, respectively, most of which was because of repeated use. WT: $n = 10$, KO^{het}: $n = 12$, KO^{hom}: $n = 15$ female and male rat pups. Arrows represent directions of transitions. Thicker arrows and brighter colors denote higher transition probability.

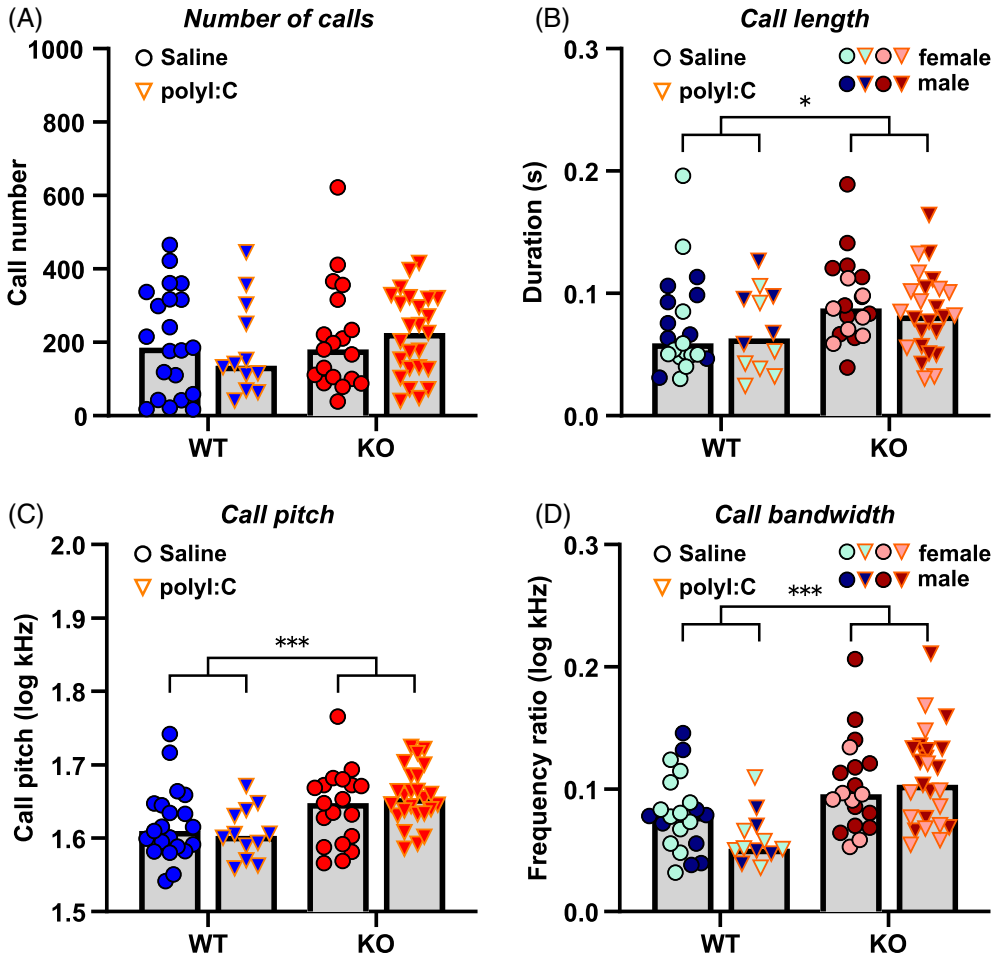

FIGURE 6 Acoustic call characteristics in *Cntnap2* WTs and KOs from saline and poly I:C offspring on PND8. (A) The number of calls emitted by *Cntnap2* WTs and KOs from saline and poly I:C offspring were similar (ART 3-way ANOVA see Table 9). (B) *Cntnap2* KO pups had longer call durations than WTs, irrespective of maternal treatment with poly I:C or saline. Note that calls from females (light symbols) were shorter than calls from males (dark symbols, ART 3-way ANOVA see Table 9). (C) Calls from *Cntnap2* KO pups had higher frequencies than WT pups, irrespective of treatment (ART 3-way ANOVA see Table 9). (D) *Cntnap2* KO pups showed increased call bandwidth compared with WTs, irrespective of treatment. Call bandwidth was greater in males (dark symbols) than females (light symbols, ART 3-way ANOVA see Table 9). WT + saline: $n = 21$, KO + Saline: $n = 19$, WT + poly I:C: $n = 12$, KO + poly I:C: $n = 25$ female and male rat pups. Data expressed as individual animals (symbols) and median (bars). p values, $^{*}p < 0.05$, $^{***}p < 0.001$

of "Split" calls, Figure 8C and Figure S10C), whereas KO littermates showed 10 call types (plus "Downward ramp," Figure 8D and Figure S10D). For a more detailed analysis of call transition probabilities please see Supplementary analysis and Figures S10 and S11.

In summary, poly I:C-induced MIA had no effect on categorical, spectral and temporal features of USVs in *Cntnap2* WT or KO offspring, including call numbers, pitch, bandwidth, call length and call sequence and bout temporal characteristics. In contrast, interactions of *Cntnap2* mutation and MIA became apparent in call type distribution and call transition repertoire, where maternal poly I:C exposure further diverged the WT and KO phenotype: the already diversified call syntax in *Cntnap2* KO pups compared with WT was further exacerbated by poly I:C MIA, whereas the less diverse call type distribution and call transition repertoire in WT littermates demonstrated an even more simplified call transition syntax after Poly I:C MIA. These results indicate compounding effects of *Cntnap2* gene mutation and MIA on call syntax.

3.7 | Comparison between cohorts confirms robustness of data

Comparisons within genotype and between cohorts showed no differences in heterozygously bred *Cntnap2* WT or KO pups from the first cohort (*Cntnap2* mutation × breeding background) and second cohort (*Cntnap2* mutation × MIA, saline offspring) for any parameters analyzed on PND8 including number of calls in the 3-min recordings, call duration, number of sequences per bout and call type transitions (Figure S12A–D). Like for PND12, on PND8 *Cntnap2* KO^{hom} pups from the first cohort had consistently higher numbers of calls than *Cntnap2* WT or KO^{het} pups from heterozygous parents pooled for cohort 1 and 2, Figure S12A. Similar to PND12, on PND8 *Cntnap2* KO^{het} pups pooled for cohort 1 and 2 also had longer call durations compared with *Cntnap2* KO^{hom} pups and WTs (Figure S12B). In contrast, call durations in *Cntnap2* KO^{hom} pups and *Cntnap2* WTs were

TABLE 9 Statistical comparisons of ultrasonic vocalization call numbers, duration, pitch and bandwidth for effects of genotype, treatment, sex, genotype × treatment, genotype × sex, treatment × sex, treatment × genotype × sex in *Cntnap2* WT and KO from saline and poly I:C offspring on PND8 (ART ANOVA)

Call characteristic	Source of variation	*p* Value	*p* Value summary	*F* (DFn, DFd)
Number	Genotype	0.5863	ns	$F(1, 69) = 0.2990$
	Treatment	0.9469	ns	$F(1, 69) = 0.0045$
	Sex	0.0790	ns	$F(1, 69) = 3.1777$
	Genotype × treatment	0.4926	ns	$F(1, 69) = 0.4759$
	Genotype × sex	0.4630	ns	$F(1, 69) = 0.5448$
	Treatment × sex	0.1627	ns	$F(1, 69) = 1.9908$
	Treatment × genotype × sex	0.9758	ns	$F(1, 69) = 0.0009$
Length	Genotype	0.0204	*	$F(1, 69) = 5.6308$
	Treatment	0.8040	ns	$F(1, 69) = 0.06209$
	Sex	0.0458	*	$F(1, 69) = 4.1364$
	Genotype × treatment	0.5842	ns	$F(1, 69) = 0.3024$
	Genotype × sex	0.2184	ns	$F(1, 69) = 1.5430$
	Treatment × sex	0.5259	ns	$F(1, 69) = 0.4064$
	Treatment × genotype × sex	0.1803	ns	$F(1, 69) = 1.8320$
Pitch	Genotype	0.0008	***	$F(1, 69) = 12.3419$
	Treatment	0.2224	ns	$F(1, 69) = 1.5158$
	Sex	0.4925	ns	$F(1, 69) = 0.4762$
	Genotype × treatment	0.2864	ns	$F(1, 69) = 1.1540$
	Genotype × sex	0.1443	ns	$F(1, 69) = 2.1807$
	Treatment × sex	0.8790	ns	$F(1, 69) = 0.02337$
	Treatment × genotype × sex	0.6980	ns	$F(1, 69) = 0.1518$
Bandwidth	Genotype	0.0001	***	$F(1, 69) = 18.5702$
	Treatment	0.7103	ns	$F(1, 69) = 0.1391$
	Sex	0.0187	*	$F(1, 69) = 5.8029$
	Genotype × treatment	0.0699	ns	$F(1, 69) = 3.3886$
	Genotype × sex	0.1032	ns	$F(1, 69) = 2.7274$
	Treatment × sex	0.2849	ns	$F(1, 69) = 1.1615$
	Treatment × genotype × sex	0.7428	ns	$F(1, 69) = 0.1086$
Number of call types	Genotype	0.0023	**	$F(1, 69) = 10.0449$
	Treatment	0.3570	ns	$F(1, 69) = 0.8598$
	Sex	0.0541	ns	$F(1, 69) = 3.8381$
	Genotype × treatment	0.9510	ns	$F(1, 69) = 0.0038$
	Genotype × sex	0.8220	ns	$F(1, 69) = 0.0510$
	Treatment × sex	0.1677	ns	$F(1, 69) = 1.9439$
	Treatment × genotype × sex	0.0522	ns	$F(1, 69) = 3.9018$

Note: 3-way ANOVA, *p* values, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns not significant.

similar (Figure S12B). The number of sequences per bout on PND8 was significantly increased in *Cntnap2* KO^{hom} pups compared with *Cntnap2* WT or KO^{het} pups pooled from cohort 1 and 2 (Figure S12C), similar to results in cohort 1 on PND12 (see Figure 3F). Call types that always occurred in the transition repertoire irrespective of PND, genotype, breeding condition and MIA were "Flat," "Short," "Step down," "Step up," "Upward ramp," "Complex" and "Composite" USVs (Figure S12D). Of the remaining 6 call types, 4 had a general PND-dependent profile (in WT controls: "Split": PND8-21, "Multistep": PND3-12, "Downward ramp": PND5 &21, "Inverted U": PND21, Figure S12D). The remaining 2 call types ("Trill," "Trill jump") were dependent on both the PND and genotype/breeding condition, and were only present in the transition repertoire on PND12 in *Cntnap2* KO^{het}, and on PND12 and 15 in *Cntnap2* KO^{hom} pups. Importantly, *Cntnap2* KO^{het} and, even more so, *Cntnap2* KO^{hom} pups showed an increasingly more diversified transition repertoire than WTs not only on PND12, but also on PND8 and 15, when USVs were recorded from different subgroups/litters of pups (Figure S12D,

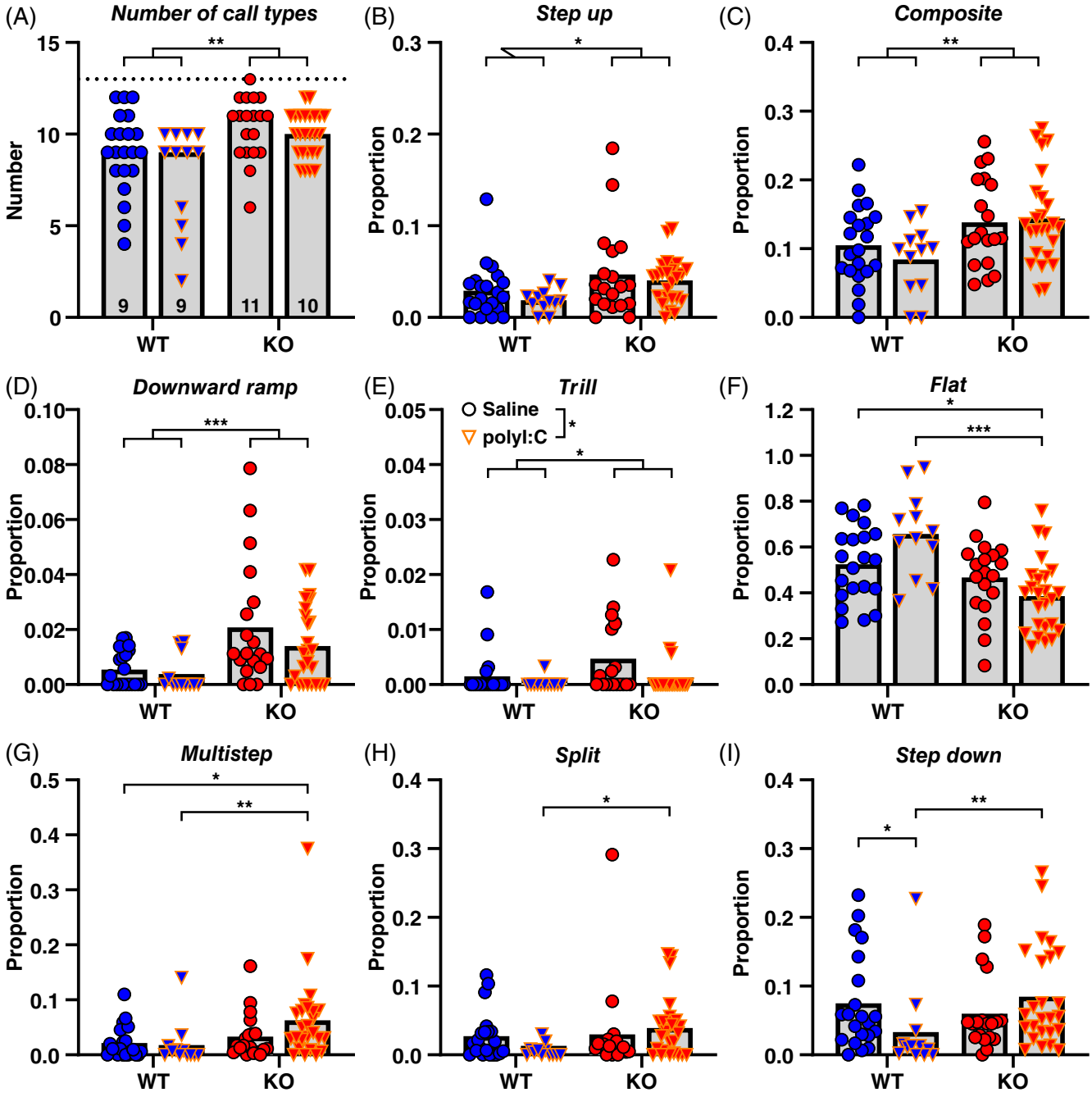

FIGURE 7 Call type usage in *Cntnap2* WT and KO pups from saline and poly I:C offspring on PND8. (A) The number of different call types utilized was significantly increased in *Cntnap2* KO compared with WT pups (ART 3-way ANOVA see Table 9). Horizontal dotted line at 13 denotes maximum possible call type number. (B–D) *Cntnap2* KO pups had higher proportions of call types (B) "Step up," (C) "Composite" and (D) "Downward ramp," irrespective of maternal treatment with poly I:C or saline (Table S10 ART 3-way ANOVA). (E) Proportions of the "Trill" call type was greater in *Cntnap2* KO pups than WTs, and decreased through maternal exposure to poly I:C in both genotypes (Table S10 ART 3-way ANOVA). (F–I) There were genotype × treatment interactions for call types "Flat," "Multistep," "Split" and "Step down" (Table S10 ART 3-way ANOVA). (F) Maternal poly I:C treatment led to decreased "Flat" call usage in KO pups compared with increased usage in WT littermates, and compared with WT controls from saline offspring (Table S11 ART-C for factor genotype × treatment). (G) Proportion of "Multistep" calls were increased in *Cntnap2* KO pups from poly I:C offspring compared with WT littermates and controls (Table S11 ART-C for factor genotype × treatment). (H) "Split" call usage was significantly increased in KOs from poly I:C offspring compared with WT littermates (Table S11 ART-C for factor genotype × treatment). (I) "Step down" ultrasonic vocalization (USV) usage was increased in *Cntnap2* KOs from poly I:C offspring compared with WT littermates, whereas it was decreased in WT pups from poly I:C offspring compared with from saline offspring (Table S11 ART-C for factor genotype × treatment). The number of USVs within call type was normalized to the total amount of calls from all types for individual pups and then averaged across animals within genotype and maternal treatment. WT + saline: $n = 21$, KO + Saline: $n = 19$, WT + poly I:C: $n = 12$, KO + poly I:C: $n = 25$ female and male rat pups. Data expressed as individual animals (symbols) and (A) median (bars) or (B–I) mean (bars). p values, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Table 1 and 2). On PND8, *Cntnap2* WT and KO^{het} from the first cohort transitioned between the same 9 and 10 call types, respectively, as respective groups in the second cohort (Figure S12D). Interestingly, WT pups from poly I:C offspring from the second cohort transitioned between the same call types as WT pups on PND3 from the first cohort, indicating that the simplification of their transition repertoire through MIA corresponded to a developmental delay in USV repertoire (Figure S12D). This consistency between results from different subgroups and cohorts of animals speaks to the reliability and feasibility of pup USVs—and their

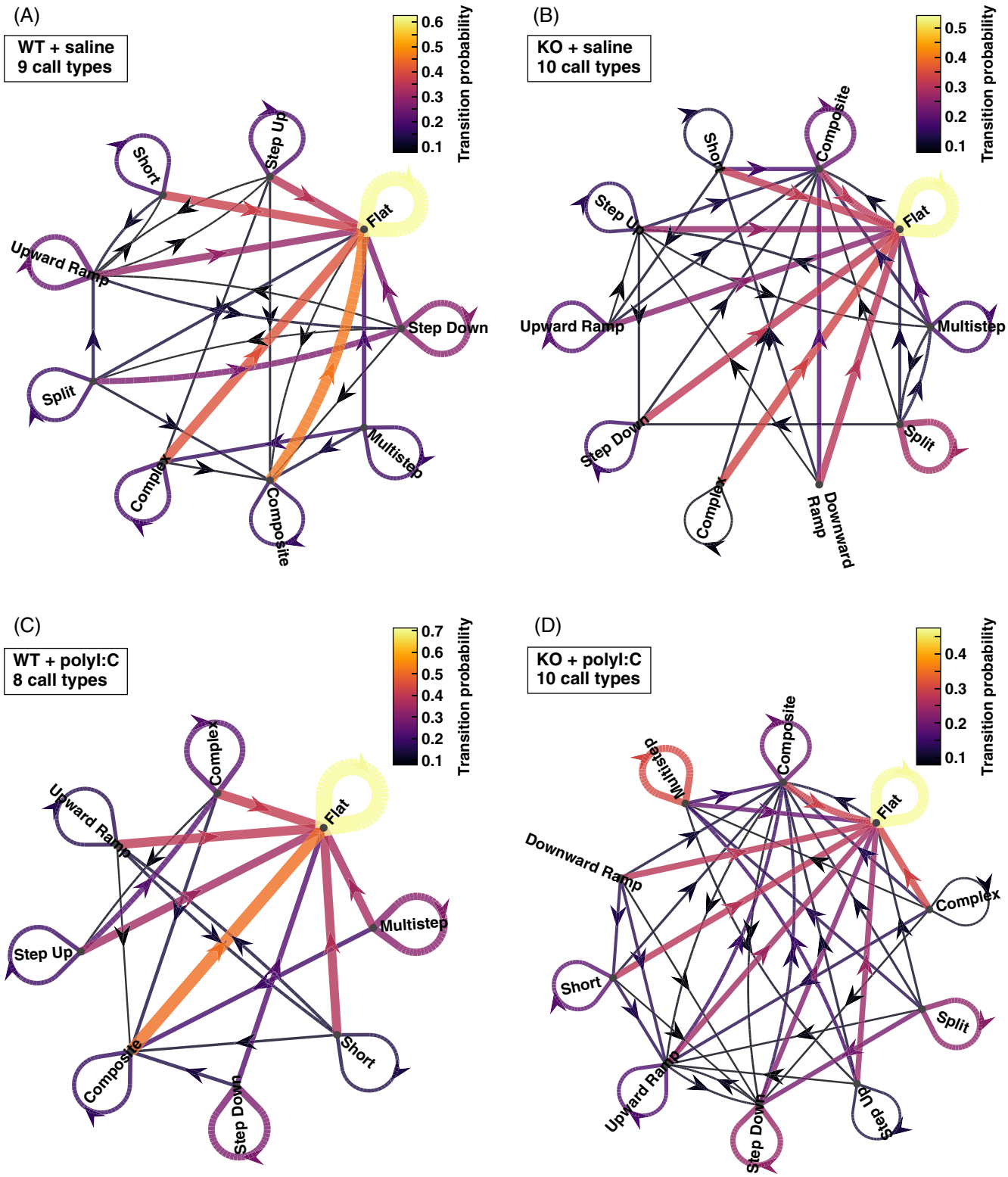

FIGURE 8 Legend on next page.

complex features in particular—as diagnostic tools for autistic-like alterations in early vocal communication.

4 | DISCUSSION

4.1 | Summary of findings

The present study sought to characterize the interplay of ASD risk gene mutation and environmental challenges including the maternal genotype and rearing environment, as well as MIA during pregnancy, on early vocal communication in the offspring. To do this, we first analyzed maternal isolation-induced USVs in *Cntnap2* KO rat pups bred from either heterozygous or homozygous KO parents and compared them with WT littermates from heterozygous breeding as controls. In a second cohort, USVs were recorded from *Cntnap2* WT or KO offspring from heterozygous dams injected with either vehicle (saline) or poly I:C during pregnancy. We show that *Cntnap2* KO rats from heterozygous parents (1) produced the same number of USVs as WTs, but the calls were (2) slightly longer in call duration, (3) higher in pitch, (4) had a largely similar temporal organization including the call rate and the duration of call sequences and bouts, and (5) had a more diverse call type repertoire and call syntax. The breeding background intensified these *Cntnap2*-related alterations such that KO pups from homozygous parents (6) produced more USV calls (7) at an even higher pitch, (8) that were arranged in fewer, but longer call bouts that comprised more call sequences, and (9) an even more variable call type repertoire and call syntax. Interestingly, the call length was rectified in KO pups from homozygous parents, indicating that the effects of *Cntnap2* mutation and breeding condition were rather interactive than simply additive. In contrast to the influence of breeding background, (10) MIA left all categorical, spectral and temporal USV characteristics intact, but (11) MIA altered the call type repertoire and call syntax with opposing effects in WT pups (more simplified) and KO pups (more diversified). The call rate was unaltered, indicating that short duration ICIs separating calls and the temporal call arrangement within call sequences was robust to the influence of *Cntnap2* mutation, breeding condition and/or MIA. Taken together, our results provide evidence that the interaction of ASD risk gene mutation and environmental challenges can exacerbate certain aspects of early vocal communication, with emphasis on more complex call features for identification of autistic-like alterations in the rodent model.

4.2 | Developmental trajectory of vocalizations

Neonatal USVs in rodents are assumed to be a form of social communication that can reflect similar functions of babies' cries, that is, to elicit the parents' attention and care.[60] In rodents, pups that are separated from their nest emit USVs for the dams to locate and retrieve the pups.[53,61–63] Alterations in neonatal USV have been found in a variety of rodent models of neurodevelopmental disorders (e.g., genetic,[64–66] MIA[30,67]), paralleling findings in infants at risk for ASD or diagnosed with ASD.[68–72] Studies from our lab have demonstrated that the *Cntnap2* KO rat model has considerable face validity for ASD-related alterations in social and stereotypic behaviors,[49] in sensory processing and filtering,[49,73,74] and for auditory processing impairments seen in language-related human disorders.[75] While the results were generally consistent in mimicking many human symptoms[76] independently from heterozygous[49,74] or homozygous breeding,[73,75] to our knowledge, no study has directly compared the effect of the two breeding schemes on the severity of *Cntnap2*-linked phenotypes. The ontogenetic profile of USV numbers in *Cntnap2* WT and KO pups followed an inverted U-shape, corroborating earlier findings in rats and mice (e.g., References 40,60,64,65,77,78). In rat pups, the USV trajectory before weaning is related to the pups' thermoregulatory and locomotor capabilities. The peak number of USVs on PND12 coincides with first spontaneous walking (∼PND11) and opening of the ear canals (PND12-13[79,80]). At ∼PND21, when the USV emission approaches zero, rat pups typically reach homeothermy.[80] Neither *Cntnap2* KO^{het} nor KO^{hom} pups presented with a developmental delay of USV numbers, which is different from some mouse models with autism-linked gene mutations (Setd5,[65] Shank1[81]).

FIGURE 8 Conditional probabilities for call type transitions within ultrasonic vocalization (USV) bouts in *Cntnap2* WTs and KOs from saline and poly I:C offspring on PND8. (A) *Cntnap2* WTs from saline offspring transitioned between 9 call types. "Flat" had the highest probability to be the next call type in the call bout, most of which was repeated use. The subsequent most probable transitions to "Flat" were from "Composite," "Complex," "Short," "Step up," "Upward ramp," "Step down," "Multistep" and "Split." "Step down" and "Composite" USVs were the second and third most transitioned to, most of which was because of repeated use. (B) *Cntnap2* KO pups from saline offspring transitioned between 10 call types, with additional transitioning from "Downward ramp." "Flat" was still most frequently transitioned to, through repeated use, and from "Composite," "Upward ramp," "Step up," "Step down," "Short," Split," Multistep," "Complex" or Downward ramp in descending order of probability. The second most transitioned to call type was "Composite" with an overall probability that was about half as much as for "Flat." (C) Poly I:C decreased transitions between call types to 8 in *Cntnap2* WTs offspring. "Split" USVs did not appear in the transition matrix. The probability to transition to "Flat" USVs was increased, that is, from preceding "Flat," "Composite," "Complex," "Upward ramp," "Short" and "Multistep." The second most transitioned to call type was "Composite" with a probability that was less than one-third than for "Flat." (D) Transitions between 10 call types in *Cntnap2* KO pups from poly I:C offspring were more diversified. The transition probability to "Flat" was decreased from all call types (i.e., from preceding "Flat," "Complex," "Composite," "Downward ramp," "Short," "Upward ramp," "Step up," "Step down," "Multistep" or "Split") and probability to transition to other call types was increased (i.e., "Step down," "Upward ramp," "Multistep," "Short"). WT + saline: $n = 21$, KO + Saline: $n = 19$, WT + poly I:C: $n = 12$, KO + poly I:C: $n = 25$ female and male rat pups. Arrows represent directions of transitions. Thicker arrows and brighter colors denote higher transition probability.

4.3 | Changes in basic USV features: Number of calls

Cntnap2 mutations led to an increased number of calls, which has also been identified in some other models of neurodevelopmental disorders, for example in NF-κB p50 KO mouse pups,[64] as well as in BTBR T+tf/J and BALB/c pups, two inbred strains predisposed to show ASD-like behaviors.[61,67] It has been suggested that the amount of isolation-induced USVs might be an indication of the pups' affective state, as stress- and anxiety-inducing conditions—such as low maternal care—increase USV emissions.[61,67,82,83] Adult *Cntnap2* KO rats exhibit deficits in sociability behavior, whereas *Cntnap2* heterozygotes present similar to WTs.[49] Therefore, *Cntnap2* KO dams, but not heterozygous dams, might display reduced maternal responsiveness, causing greater number of USVs in *Cntnap2* KO^{hom} offspring related with heightened anxiety-like state. In the human ASD population, anxiety is a common comorbidity,[84] in particular increased separation anxiety disorder in young children.[85] Altered amounts of infant USVs can be predictive of adult emotionality.[22,24,86,87] Indeed, in adulthood, both NF-κB p50 KO mice[88] and *Cntnap2* KO^{hom} rats display decreased anxiety-like behavior, whereas *Cntnap2* KO^{het} rats are similar to WTs (Figure S13A and Reference 49). In contrast to anxiety-like behavior, exploratory and stereotypic behavior was equally increased in adult *Cntnap2* KO^{hom} and KO^{het}. (Figure S13B,C). This supports the notion that the interactive effect of *Cntnap2* mutation and parental genotype on pup USVs is likely related with altered emotionality traits that affect anxiety-like behaviors in adulthood (although not straightforward), rather than abnormal motor or stereotypic behavior.

It should be noted that there is some variability in the number of pup USVs between this study and previous studies in *Cntnap2* KO mice. Like our findings in *Cntnap2* KO^{het} rats, heterozygously bred KO mice presented either similarly to WTs (PND4, 7, 15[19]) or vocalized less often than WT littermates (PND6,[6] PND3[16]). While the reason for this variability remains to be explored, there appears to be a developmental pattern in *Cntnap2* KO rodents where—relatively speaking—a lower number of pup USVs along a higher number of ASD "hits" is accompanied by normal anxiety-like behavior later in life,[6,16] and a higher number of pup USVs tends to go along with decreased behavioral read-outs of anxiety in adulthood ([19] and the present study). In contrast to the breeding condition, MIA had no compounding effects on USV numbers in the present study. In line with our results, MIA induced by lipopolysaccharide had no interaction effects on neither pup USV production nor anxiety-like behavior at 6 weeks of age in a multiple-hit *Cntnap2* KO mouse model.[16] One reason for the lack of effect on USV numbers could be the timepoint of MIA induction. Another study that also induced MIA on GD9.5 saw also no change in USV numbers,[38] whereas other studies with MIA induction on a later GD found decreased USV numbers (GD15-16[35–37,39]).

4.4 | Changes in Basic USV features: Frequency changes

Since the number of USV calls might be related with anxiety-like behavior, it may not be the most representative indicator of the animals' communication abilities.[89,90] The acoustic quality of cry or speech sounds is crucial to communication and measured by variations in pitch as well as duration and time intervals between cries and spoken words.[71,91] The mean fundamental frequency of infant cries is one of the strongest predictors for the caregiver's perception of urgency and distress.[92] Infants at risk for ASD produce cries with higher fundamental frequency, and high-risk infants later classified with ASD had higher fundamental frequency than those who were not.[68,72] It has been suggested that such differences in acoustic features of babies' cries may be an early manifestation of an atypical affective state that might hamper the development of social communication.[71] In line with this, the average principal USV frequency in both *Cntnap2* KO^{het} and KO^{hom} pups was increased in comparison to WTs in a "gene × environment" dose-dependent manner (Figure S14A).

Calls from rat pups can be categorized into a lower frequency class with a peak around 30–40 kHz, and a higher frequency class at 50–66 kHz.[82,93,94] In rodent infants both frequency call classes have negative valence,[95] in contrast to juvenile and adult rodents. The low frequency component is assumed to be associated with stronger, painful, aversive stimuli and the high frequency component with a milder aversive state.[82,96–98] The frequency distribution of USVs is sensitive to changes in negative affective state.[82,94] In particular, the average power of the high frequency component in the USV spectrum can be increased by stressful environmental stimuli, and it is sensitive to modulations of the glutamatergic/GABAergic system.[94,99] The higher average call pitch in *Cntnap2* KO^{het} and KO^{hom} pups might therefore indicate a categorical shift in call frequency distributions because of an increasingly altered affective state, which might play a role in the development of ASD-related social interaction deficits seen in *Cntnap2* KO adults.[49] Spectrographic deviations have also been found in other rodent models of ASD, including more USVs in the high-frequency cluster and call type-specific shifts to higher carrier frequencies.[66,100–102] It has also been shown that higher pitched autistic children's cries are perceived more negatively by caregivers as to how to appropriately respond[103]; and altered USVs in the NF-κB p50 mouse model go along with reduced maternal care for KO pups.[64] Interestingly, it has been hypothesized that gene mutation-induced atypical vocalizations in both human and rodent infants may not only represent an early biomarker for ASD, but that their negative impact on maternal care might act as a self-generated environmental factor.[71,104] It remains to be determined how the altered USVs in *Cntnap2* KO^{het} and KO^{hom} pups interferes with maternal care behavior. Testing maternal responsiveness of *Cntnap2* Het and KO dams to WT and KO pups, possibly including cross-fostered pups, is an exciting outlook for future studies that will help disentangle the genetic and environmental contributions exacerbating the phenotypic expression of ASD-related USVs in the present study.

4.5 | Changes in temporal USV aspects

Call duration, inter-call intervals and the resulting call rate have been shown essential for the elicitation of maternal retrieval in

rodents.[54,105] The average call duration in rat pups increases with age (PND10 to 15[106]). It has been shown that dams prefer calls with a duration of 80 ms over such with 15 ms,[107] and that maternal responsiveness requires a minimum duration of more than 25 ms.[105] In rat pups, the call duration is sensitive to stress-level, and longer USVs are affectively different than shorter ones.[108] In the present study, *Cntnap2* KO^{hom} pups showed no differences in call durations compared with WTs (close to 80 ms on PND12), whereas KO^{het} pups from heterozygous breeding had longer call durations on both PND8 and 12. It might be possible that—as discussed above—the *Cntnap2* KO maternal environment induces an altered affective state in KO^{hom} pups that expresses itself in shortening of the call durations back to WT level. In contrast, KO^{het} pups reared by a heterozygous dam might show longer call durations because of their slightly accelerated postnatal development which is not reverted by an altered affective state. Some other models for neurodevelopmental disorders have demonstrated longer pup call durations and an influence of maternal genotype, whereas others have not (BTBR,[60] NF-κB p50,[64] Fmr1,[66] TSC2[109]). For comparison, the first utterances of high-risk ASD toddlers had longer cry durations than low risk toddlers.[68] Prenatal poly I:C exposure had no influence on acoustic parameters including duration, bandwidth and peak frequency in the present and a previous study in rat pups.[35]

In human adults, the perception of distress is also influenced by the duration of pauses in a crying episode, with shorter pause durations signaling more distress.[110,111] Despite differences in call durations, most ICIs from *Cntnap2* WT, KO^{het} and KO^{hom} pups in our study occurred with highest probability between 100 to 150 ms, which is quite similar to previous findings in mouse pups (100 to 200 ms[54]). Indeed, it has previously been shown in mice that the ICI does not depend on call duration.[112] Pup USV distress calls are typically temporally organized at 3–8 Hz.[48,62,113,114] Neurons in the auditory cortex of lactating dams are temporally tuned to the most common call rate (~5 Hz), which might reflect the behavioral salience of pup call ICIs.[46] Interestingly, *Cntnap2* KO rats show impaired temporal auditory processing, including a reduced ability of cortical neurons to consistently respond to a pulse noise burst train at rates of ~5 Hz or higher.[75] This might lead to reduced maternal responsiveness and put pups reared by a KO dam at a disadvantage in eliciting maternal care despite similar call rates. We also found that female pups had consistently shorter call duration and faster call rates than males, independent of genotype and environmental challenge. This might be as a result of a higher stress resistance to certain adverse effects of maternal separation.[115,116]

While there was no effect on call sequences, we found a "gene × environment" effect in *Cntnap2* KO^{het} and KO^{hom} pups on the number of sequences per bout, and the duration and number of bouts (Figure S14B–D). Young et al[109] also found an effect of maternal genotype on the offspring's USV temporal organization in the TSC2 mouse model. They raised the question if longer bursts of calls elicit faster retrieval by the dams because of the possibly greater display of distress or urgency, as the USV bout duration and persistence is a crucial element in the communication of the caller's affect and arousal.

4.6 | Effects on USV syntax

Age-dependent changes in USV emission are tightly linked to maturation of laryngeal function and innervation, the development of ultrasound representation in the auditory cortex, and to the brain circuitries that regulate respiration and arousal—processes that are associated with developmental milestones that can be dysfunctional in ASD.[93,95,117–119] The usage of simple, frequency unmodulated calls typically decreases during development,[40] whereas the usage of frequency modulated calls increases.[40,120] Specifically, the percentage of "Trill type" calls is low in rat pups during early development, and increases gradually with age[40] . In contrast, *Cntnap2* KO^{het} and KO^{hom} pups showed increased proportions of, and transitioning from, "Trill" and "Trill jump" USVs on PND12 and 15. Furthermore, *Cntnap2* KO^{hom}, but no other group, demonstrated frequency modulated "Downward ramp" USVs. Interestingly, a high proportion of pup "Downward ramp" calls has previously been described in a MIA mouse model of ASD.[121] The generally higher presence of frequency modulated USVs was accompanied by the earlier occurrence of developmental milestones, especially in KO^{hom} pups, and both are possibly expressions of an aberrant neurodevelopmental profile. Accelerated developmental milestones and growth rates along with an unusual pattern of USV types, have previously been described in BTBR mice.[60] In infants with ASD, early generalized overgrowth (including head circumference, height and weight measurements), possibly reflects underlying atypical brain development and is predictive of lower social, verbal and nonverbal skills.[122]

Even though none of the categorical, acoustic and temporal parameters investigated were affected by maternal treatment with poly I:C only, the USV call type and transition repertoire showed a characteristic exacerbation of the *Cntnap2* phenotype in the KO offspring: In all *Cntnap2* KO groups the proportion of and transitioning to the most common call type on a given PND was reduced, and this alteration was intensified by both MIA and the maternal KO environment. In contrast to the increasingly diversified call transition profile in *Cntnap2* KO pups, *Cntnap2* WT pups from poly I:C offspring showed a simplification of their call transition profile with increased usage of nonfrequency-modulated calls. This was more reminiscent of the reduced vocal repertoire and invariable call sequences with less complicated call types seen in other autism models (Tbx1,[123] *Cntnap2* mice[124]) and higher "Flat" call type emission in poly I:C exposed rat pups.[35] In order to better assess the developmental trajectory and possibly subtle effects of a second hit on rat pup USVs, future studies should include several early postnatal time points including PND12 when USV emission peaks.

4.7 | Potential underlying neural mechanisms

At this point, we can only speculate about the brain mechanisms underlying the altered USVs in *Cntnap2* KO rat pups. In mice, the bout duration is flexibly scaled through a two-step, di-synaptic disinhibition motif: Long-range inhibitory neurons of the hypothalamic lateral

preoptic area (LPOA) relieve a clamp of local periaqueductal gray (PAG) inhibition, enabling excitatory PAG USV-gating neurons to trigger vocalizations.[47] Increasing activity of LPOA neurons induces disinhibition, with the delayed recovery of the PAG inhibition clamp prolonging USV bouts.[47] We have previously identified a brain region-specific altered balance of excitatory/inhibitory neurotransmitters underlying sensory processing disruptions in *Cntnap2* KO rats, in particular a pronounced increase in GABA level in the acoustic startle response-mediating nucleus reticularis pontis caudalis.[74] Thus, it might be possible that the increased USV bout duration in *Cntnap2* KO^{het}, and even more so in *Cntnap2* KO^{hom}, pups in the present study involves prolonged or increased inhibitory input from the LPOA neurons on the PAG neuron inhibition clamp; and/or a more delayed recovery of the local PAG neuron inhibition clamp. Interestingly, in mice activating LPOA neurons evoked a rich USV repertoire that was similar to natural calls during adult male–female interactions, and less similar to infant USVs.[47]

It has been demonstrated that *Cntnap2* KO mice have decreased numbers of oxytocin immunoreactive neurons in the hypothalamus and an overall decrease in brain oxytocin levels.[125] Early postnatal treatment with oxytocin is able to rescue social deficits in *Cntnap2* KO mice,[125] as well as to ameliorate USV disruptions in the valproic acid MIA mouse model of ASD.[121,126] Thus, it might also be possible that a distinct dysfunction in the oxytocin system underlies the USV phenotype in *Cntnap2* KO rat pups from heterozygous and saline offspring. The postnatal development of the oxytocin system and production appears to be particularly vulnerable to early life manipulations.[125,127,128] Postnatal early-life stress because of altered maternal care can induce alterations in the oxytocin system, hypothalamic protein expression and neuronal hyperexcitability[129–132] and can lead to increased numbers of USVs and likely contribute to long-term negative outcomes.[83,120] The homozygous *Cntnap2* KO rearing environment might constitute early life stress for *Cntnap2* KO pups and therefore intensify the USV phenotype. In contrast, we do not expect poly I:C injected dams to have altered maternal behaviors as Schaafsma et al[16] found no effects of MIA on a measure for maternal behavior in *Cntnap2* heterozygous mouse dams. Therefore, MIA-related effects on USVs would have been limited to prenatal effects on the brain, as has been described in the hypothalamus and USVs in a valproic acid MIA mouse model.[126] The lack of early life stress in poly I:C offspring might explain why basic categorical, acoustic and temporal USV features remained intact, and changes were limited to more subtle alterations in USV subtype proportions and transitions.

4.8 | Limitations

One major limitation in our study is that we did not test maternal responsiveness to the altered USV profiles, therefore their impact on mother-pup social interactions remain speculative. Based on the present data it is therefore impossible to determine if differences in maternal care or other postnatal environmental interaction (e.g., with littermates) are responsible for the differences between *Cntnap2* WT, KO^{het} and KO^{hom} pups, or whether there are prenatal influences. The recording of USVs simultaneous with maternal retrieval, or maternal responsiveness to USV playback of *Cntnap2* WT, KO^{het} and KO^{hom} pup USVs might help to elucidate the functionality and efficacy of certain call types in the mother-pup communication and thereby to narrow down call types or categories of interest. While understanding the correlation between specific USV types or categories and social behaviors is an intriguing outlook, it should be mentioned that rodent vocal communication is only suitable to a certain extent as a model for studying human speech. The communicative purpose of isolation-induced calls in rodent pups serves a self-preservation function through the elicitation of maternal care, and they are simple signals that are not translatable into human language, grammar or word meaning.[91,95,133]

5 | CONCLUSION

Taken together, our results support the "double-hit hypothesis" of ASD risk gene–environment interactions affecting vocal communication, with effects of parental genotype and rearing environment outweighing MIA. In-depth analysis of call type repertoire and sequential structure of pup USVs can be a sensitive tool to quantify the extent of ASD-like traits in rodent models during early development, and to validate treatment strategies for their individual therapeutic success not only acutely in infants, but possibly also in longitudinal studies comparing the postnatal phenotype to the severity of autistic-like manifestations in adolescence and adulthood. Future studies will have to determine what the exact environmental factors are that impact the *Cntnap2* KO phenotype, whether these are prenatal effects on fetal development or indeed postnatal effects through differences in behavior of the dam and/or littermates.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data that support the findings of this study are available from the corresponding author upon reasonable request.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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