RESEARCH ARTICLE OPEN ACCESS

Age-Dependent Effects of Loss of *Contactin-Associated Protein-Like 2*, an Autism-Associated Gene, on the Acquisition and Recall of Fear Memory

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Received: 8 November 2024 | **Revised:** 13 March 2025 | **Accepted:** 24 March 2025

Funding: This work was supported by startup funds from the Iowa Neuroscience Institute and the Department of Psychiatry at the University of Iowa.

Keywords: anxiety | autism spectrum disorder | CASPR2 | *Cntnap2* | development | fear | fear conditioning | memory

ABSTRACT

The *contactin-associated protein-like 2* (*Cntnap2*) gene is relevant to autism spectrum disorder (ASD), which is associated with age-specific structural alterations in limbic brain regions. The *Cntnap2* gene encodes for the contactin-associated protein-like 2 (CASPR2) protein, and CASPR2 protein levels are high in the amygdala, a limbic region that is essential for the processing of fear and anxiety. In humans, reduced levels of this protein arising from CNTNAP2 mutations could potentially account for the autism-associated increase in fear and anxiety. Here, we report the extent to which loss of CASPR2 in mice contributes to the development of fear- and anxiety-related behaviors. Pavlovian fear conditioning experiments revealed that loss of CASPR2 has age-dependent effects on the acquisition of fear memory, recall of both cue-evoked and context-related fear memory, and stability of cue-evoked fear memory. Additionally, data from the elevated zero maze suggest that CASPR2 deficiency contributes to anxiety-related behaviors, especially in juvenile (29-day old) mice. These are the first reports of age-dependent effects of CASPR2 deficiency on fear and anxiety-related behaviors, and they set the stage for a better understanding of developmental alterations of fear and anxiety in ASD.

1 | Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with early onset, and it is characterized by persistent deficits in social interactions and restricted repetitive behavior. In many individuals with ASD, increased levels of fear and anxiety cause significant impairments in their quality of life and daily functioning (Masi et al. 2017; Lai et al. 2014; Turner and Romanczyk 2012; Simonoff et al. 2008; Gangi et al. 2018; White et al. 2009). From developmental studies, it is known that the processing of fear in early life differs fundamentally from that in adulthood (Meyer and Lee 2019), and ASD is also associated with age-specific structural alterations in limbic brain regions (Avino et al. 2018; Lai et al. 2014; Schumann et al. 2004; Greimel et al. 2013) that are essential for the processing of fear and anxiety. A better understanding of these age-specific alterations and their underpinnings will be critical for developing more effective treatments and preventative strategies for fear and anxiety in patients with ASD.

Summary

- Autism spectrum disorder (ASD) is a neurodevelopmental disorder with early onset, and it is characterized by persistent deficits in social interactions and restricted repetitive behavior.
- Patients with ASD often also suffer from fear and anxiety, which significantly impairs their quality of life.
- To find better therapies, it is essential to better understand the underlying processes of fear and anxiety in ASD.
- A first step in this direction is to test if genes relevant to ASD have different effects on fear- and anxiety-related behaviors at different stages of development.
- The present study investigated the effect of loss of the *contactin-associated protein-like 2* (*Cntnap2*) gene, which is relevant to ASD, on fear- and anxiety-related behaviors by using Pavlovian fear conditioning, which tests the acquisition and recall of fear memory, and elevated zero maze, which tests anxiety-related behavior.
- We found, that *Cntnap2* deficiency has age-dependent effects on the acquisition and recall of fear memory.
- Our elevated zero maze results also suggest that *Cntnap2* deficiency contributes to anxiety-related behaviors, especially in juvenile (29-day old) mice.
- These are the first reports of age-dependent effects of *Cntnap2* deficiency on fear and anxiety-related behaviors and they set the stage for a better understanding of developmental alterations of fear and anxiety in ASD.

The *contactin-associated protein-like 2* (*Cntnap2*) gene encodes for the contactin-associated protein-like 2 (CASPR2) protein, a neurexin that has been associated with neuronal migration, dendritic arborization, dendritic spine development (Anderson et al. 2012; Gdalyahu et al. 2015; Penagarikano et al. 2011), and ASD (Williams et al. 2019; Uddin et al. 2021; Li et al. 2010; Alarcón et al. 2008). The *Cntnap2* knockout (*Cntnap2*$^{-/-}$) mouse model exhibits several key characteristics of ASD, including abnormal communication, restricted repetitive behavior, and abnormal social interactions (Penagarikano et al. 2011; Alarcón et al. 2008). Additionally, Stein et al. (2011) reported that a common genetic variant of *CNTNAP2* is associated with social anxiety traits in young adults. These findings and the high comorbidity of ASD with anxiety disorders (Masi et al. 2017; Lai et al. 2014; Turner and Romanczyk 2012; Simonoff et al. 2008; Gangi et al. 2018; White et al. 2009) link CASPR2 to ASD and suggest that fear processing might be altered in the context of CASPR2 deficiency. Thus, the *Cntnap2*$^{-/-}$ mouse model appeared to be an ideal system to probe for age-specific alterations in fear and anxiety relevant to ASD.

We tested the impact of CASPR2 deficiency on the development of fear-related behaviors using the well-established Pavlovian fear-conditioning model. In detail, we used five shock-tone pairings for training and assessed freezing throughout the experiment. For these experiments, *Cntnap2* knockout mice and age-matched wild-type mice were trained pre- and post-weaning, based on previous reports that the timing of training can result in different effects on the acquisition and recall of fear memory (Akers et al. 2012; Pattwell et al. 2011; Akers et al. 2014). Specifically, Pavlovian fear conditioning before postnatal day (P) 17 had been found not to induce stable context-evoked freezing when tested 24 h after training (Akers et al. 2012), and this early contextual fear memory was reported to decline significantly over time (Akers et al. 2014). In mice trained at P29–33, context-evoked freezing was found to be suppressed 24–48 h after training although cue-evoked freezing was strong (Pattwell et al. 2011). In adult mice, cue- and context-evoked freezing was reported to be strong after training and robust over time (Pattwell et al. 2011; Gale et al. 2004; Frankland et al. 2006; Akers et al. 2012). Based on these hallmarks of the development of fear conditioning, we probed the age-specific effects of loss of CASPR2 on Pavlovian fear conditioning in mice trained at P18, 30, and P61.

We additionally tested these mice for the effects of CASPR2 deficiency on the development of anxiety-related behaviors, using the elevated zero maze. To minimize the use of experimental animals, mice underwent zero maze testing the day before they underwent fear conditioning.

Given that CASPR2 is expressed in the mouse brain as early as embryonic Day 14, and its expression in cultured cortical neurons increases with time in culture (Penagarikano et al. 2011; Gao et al. 2018; Varea et al. 2015), we hypothesized that Pavlovian fear conditioning and anxiety-related behaviors might already be altered in 17/18-day-old CASPR2 deficient mice, but that the difference to wild-type animals would increase in mice trained during later stages of development.

2 | Methods

2.1 | Animals

Cntnap2$^{-/-}$ mice were purchased from The Jackson Laboratory, and heterozygous B6.129(Cg)-Cntnap2tm1Pele/J (Stock #017482) males and females were crossed to produce knockout (*Cntnap2*$^{-/-}$) and wild-type (*Cntnap2*$^{+/+}$) control mice. A total of 203 mice (95 knockout and 108 wild-type mice) were tested. Specific details on mouse number, litter number, and sex distribution for each age group and experiment are detailed in the figure captions. Mouse age at the start of the experiments ranged from P17 to P61. The following three developmental stages were tested: pre-weaning (P17-18), juvenile (P29-30), and young adult (P60-61). Distinct sets of mice were used for each age cohort. Mice of both sexes were used for experiments and, as detailed in the statistical analysis section, we controlled for potential sex effects and tested for sex-by-genotype interactions. For experiments initiated before weaning, only litters that included littermate controls were used; for experiments initiated post weaning, litters that did not include littermate controls were also used.

Mice were harem-bred, and dams were separated before giving birth. Mice were kept on a 12-h light–dark cycle in a temperature- and humidity-controlled facility and given ad libitum access to standard chow (7913, Teklad Standard Irradiated Diet, Inotiv Teklad Madison, WI, USA) and water. Animal care met the standards set by the National Institutes of Health, and all

experiments were approved by the University of Iowa Animal Care and Use Committee. Most mice that underwent fear conditioning to test fear-related behavior also underwent elevated zero maze the day before training to test anxiety-related behavior. Due to a late decision to perform zero maze experiments on the day before fear conditioning, five wild-type and six knockout mice in the P30 fear conditioning group did not undergo elevated zero maze the day before fear conditioning. Separate analyses with all P30 mice that were subjected to fear conditioning (Table 1) and with P30 mice that were subjected to the zero maze the day before fear conditioning did not yield statistically different results (Table S2). Therefore, the analysis of all P30 mice subjected to fear conditioning is reported in the results section of the manuscript.

At the beginning of the first experiment in which each mouse was used (zero maze or fear conditioning), each mouse was naive except for toe clipping and/or ear punching for genotyping purposes.

2.2 | Elevated Zero Maze

At P17, P29, and P60, mice underwent a 5-min elevated zero maze trial to measure anxiety-related behavior. Videos were obtained via an overhead camera and analyzed using the AnyMaze software (Version 6.32, Stoelting Co., Wood Dale, IL, USA). The maze was custom-made and consisted of a 5-cm wide white circular acrylic track that was 44.6 cm in diameter and elevated 42 cm above the floor. It was divided into four equally sized quadrants: two were closed and had 10-cm high white acrylic walls; and the other two were open and had white acrylic curbs that were 7-mm high and 5-mm wide to prevent falls. In the open arms, light intensity was 250 lx. Before each trial, the maze was cleaned with 70% ethanol. Parameters analyzed were the time spent in the closed and open arms, as well as the distance traveled.

After the first 16 (5 knockout mice and 11 wild-type mice) P29 animals were tested, the behavior room had to be changed. Although the same experimental protocol and the same zero maze were used, a significant difference in total distance traveled was observed. Therefore, we analyzed total distance traveled for each room separately. Given the small number of knockout mice that were tested in Room 1, we report only the total distance traveled by mice tested in Room 2 in the main text. However, Table 2 includes the Room 1 data as well. Additionally, the total distance traveled was unavailable for 14 of the 47 P29 mice tested in the second behavior room due to technical difficulties. The percentage of time spent in the closed arm was in the same range in both rooms. Therefore, the time spent in the closed arm reported in the main text is based on datasets from both rooms, but we also ran a separate analysis for each room (Table 2). Zero maze experiments for P17 and P60 animals were all tested in the second behavior room.

Data for a mouse were excluded from the zero maze sample if the animal fell off or was not recognized by the software, which was a rare event. For the P17 sample, the litter was excluded if no littermate control completed the zero maze. These animals nevertheless underwent subsequent fear conditioning experiments.

2.3 | Fear Conditioning

Fear conditioning was performed using Med Associates (Fairfax, VT, USA) fear conditioning chambers. Parameters that were scored were freezing and the maximum motor activity following the first foot shock, as scored using the VideoFreeze software (Med Associates). Maximum motor activity following the first foot shock was reported in arbitrary units and used as a proxy to assess shock sensitivity. Motion thresholds of the software were optimized for mouse age based on hand scoring of a representative group of mice. Details of the motion threshold and freeze duration at each age of interest are summarized in Table S1. All experiments were performed during the light cycle. At P18, P30, and P61, separate groups of mice were subjected to fear conditioning. Five wild-type and six knockout mice of the P30 fear conditioning group did not undergo the elevated zero maze test the day before fear conditioning training. All other mice underwent the elevated zero maze the day before training but were otherwise naïve except for toe clipping and/or ear punching for genotyping purposes.

2.3.1 | Fear Conditioning Protocol

Training and the contextual memory test were performed in Context A (grid, white light, and 1% bleach as contextual odor). The cue-evoked memory test was performed in Context B (light off, smooth floor, triangle roof, peppermint odor).

2.3.1.1 | Training (Context A). Initial experiments (data not shown) with pre-weaned mice revealed nonspecific freezing during the first minutes of habituation to the chamber (data not shown). Therefore, mice that were trained pre-weaning (P18) were given 8 min to become habituated to the chamber before the first tone (20 s, 80 db, 3000 Hz) sounded, but counterparts that were trained post-weaning were only given 3 min. Freezing during the habituation period is referred to as baseline freezing in the analysis and discussion, and the period starting with the onset of the first tone and ending with the end of the experiment is referred to as acquisition or fear memory acquisition. Each tone co-terminated with a 1-s foot shock (0.75 mA). The mice received a total of 5 tone-shock presentations, with an inter-trial interval of 120 s, and the last tone-shock presentation was followed by 80 s without tone or shock.

2.3.1.2 | Tests of Cue-Evoked Fear Memory (Context B). In all animals, cue-evoked fear memory recall was tested 24 h after training. Maintenance of cue-evoked fear memory was tested at 7 and 14 days after training in animals trained at P18, and at 15 days after training in animals trained post-weaning (see Figure S1). Different timelines were tested because declines in contextual fear memory over time had been reported in animals trained pre-weaning (Akers et al. 2012; Akers et al. 2014). The same protocol was used to test cue-evoked fear memory and its maintenance. As in Context A, mice trained post-weaning were given 3 min before cue onset, and mice trained pre-weaning (P18) were given 8 min before cue onset, to become habituated to the chamber. Freezing during this habituation period is referred to as pre-cue freezing in the analysis and discussion. After habituation, a 3-min, 80 dB tone (3000 Hz) (the cue) was played. Freezing during this period of time is referred to as

TABLE 1 | Percent freezing following fear conditioning in mice trained pre-weaning (P18), as juvenile mice (P30), and as young adult mice (P61).

		Cntnap2$^{+/+}$ P18	*Cntnap2*$^{-/-}$P18	*p*	*Cntnap2*$^{+/+}$ P30	*Cntnap2*$^{-/-}$P30	*p*	*Cntnap2*$^{+/+}$ P61	*Cntnap2*$^{-/-}$P61	*p*
Training	Pre cue onset	3.0 ± 0.3[a]	2.1 ± 0.3[a]	0.024*[a]	0.8 ± 0.2[a]	0.4 ± 0.2[a]	0.144[a]	0.3 ± 0.1[a]	0.1 ± 0.2[a]	0.180[a]
	From 1st cue	57.3 ± 4.2	50.2 ± 4.3	0.069	53.6 ± 2.9	40.4 ± 2.7	< 0.001*	41.7 ± 2.9	25.6 ± 3.7	< 0.001*
Cue-evoked memory D1	Pre cue	34.9 ± 5.2	17.5 ± 5.4	0.003*	26.1 ± 4.9	24.5 ± 4.4	0.807	21.1 ± 3.4	10.7 ± 4.1	0.020*
	Cue	80.4 ± 7.7	60.3 ± 7.9	0.026*	79.3 ± 4.2	77.4 ± 3.9	0.722	60.9 ± 5.5	36.6 ± 6.3	< 0.001*
	Post cue	28.6 ± 4.9	11.9 ± 5.1	0.007*	25.8 ± 3.4	15.9 ± 3.1	0.032*	22.6 ± 2.6	7.4 ± 3.4	< 0.001*
Cue-evoked memory D7	Pre cue	1.5 ± 0.4[a]	1.0 ± 0.4[a]	0.204[a]	—	—	—	—	—	—
	Cue	56.2 ± 7.4	59.2 ± 7.7	0.722	—	—	—	—	—	—
	Post cue	2.3 ± 0.4[a]	1.6 ± 0.4[a]	0.064[a]	—	—	—	—	—	—
Cue-evoked memory D14 (P18-trained) or D15 (P30-, P61-trained)	Pre cue	2.5 ± 0.2[a]	1.3 ± 0.3[a]	0.001*[a]	16.6 ± 2.7	7.5 ± 2.5	0.010*	11.8 ± 1.8	6.4 ± 2.3	0.070
	Cue	48.1 ± 7.7	46.2 ± 8.0	0.816	71.3 ± 6.0	47.7 ± 5.6	0.005*	62.6 ± 4.1	41.5 ± 5.3	0.003*
	Post cue	2.2 ± 0.3[a]	2.2 ± 0.3[a]	0.982[a]	17.9 ± 2.1	6.3 ± 2.0	< 0.001*	14.9 ± 2.7	7.2 ± 3.4	0.052
Contextual memory D2		36.4 ± 5.1	21.9 ± 5.3	0.035*	39.3 ± 3.7	35.2 ± 3.4	0.377	32.0 ± 3.4	20.5 ± 4.2	0.015*
Contextual memory D8		3.1 ± 2.1	6.4 ± 2.2	0.233	—	—	—	—	—	—
Contextual memory D15 (P18-trained) or D16 (P30-, P61-trained)		9.0 ± 2.2	6.4 ± 2.3	0.363	20.1 ± 3.4	14.7 ± 3.2	0.219	21.6 ± 2.7	9.5 ± 3.5	0.006*

Note: Mean ± standard error was estimated using mixed effects linear regression analysis. Potential litter and sex effects were controlled for.
[a]Based on square root scores.
*Statistically significant: $p < 0.050$, trend $p < 0.100$.

TABLE 2 | Results of elevated zero maze experiments in pre-weaning, juvenile, and young adult mice.

	P17			P29			P60		
	Cntnap2$^{+/+}$	*Cntnap2*$^{-/-}$		*Cntnap2*$^{+/+}$	*Cntnap2*$^{-/-}$		*Cntnap2*$^{+/+}$	*Cntnap2*$^{-/-}$	
Percent time in closed arm (both rooms for P29)	73.1 ± 2.6%	68.2 ± 2.6%	$p = 0.124$ FDR = 0.188	74.2 ± 1.7%	68.6 ± 1.8%	$p = 0.015$* FDR = 0.044	74.2 ± 2.4%	69.3 ± 3.1%	$p = 0.208$ FDR = 0.271
Total distance traveled in meters (room 2 for P29)	0.4 ± 0.0	0.6 ± 0.0	$p = 0.011$* FDR = 0.036	0.7 ± 0.1	0.9 ± 0.1	$p = 0.016$* FDR = 0.045	0.7 ± 0.0	0.9 ± 0.0	$p = 0.048$* FDR = 0.096
Percent time in closed arm (room 1 P29)	—	—	—	74.9 ± 2.24%	66.5 ± 3.22%	$p = 0.026$* FDR = 0.058	—	—	—
Percent time in closed arm (room 2 P29)	—	—	—	73.9 ± 2.20%	68.8 ± 2.12 %	$p = 0.074$ FDR = 0.122	—	—	—
Total distance traveled in meters (room 1 P29)	—	—	—	11.1 ± 0.66	12.6 ± 0.82	$p = 0.068$ FDR = 0.119	—	—	—

Note: Mean ± standard error was estimated using mixed effects linear regression analysis. Potential litter and sex effects were controlled for.
*Statistically significant: $p < 0.050$, trend $p < 0.100$.

cue-evoked freezing or freezing during cue presentation and corresponds to cue-evoked fear memory. In animals trained at P18, this was followed by an additional 5 min without a tone and in animals trained post-weaning by an additional 4 min without a tone. This difference in treatment was based on initial experiments showing that freezing declined more slowly after the end of the tone in animals trained at P18 (data not shown). Freezing during this period following cue presentation is referred to as post-cue freezing.

2.3.1.3 | Test of Contextual Fear Memory (Context A). Contextual fear memory was assessed 48 h after training. In animals trained at P18, the maintenance of contextual fear memory was tested 8 and 15 days later, and in animals trained post-weaning, the maintenance of contextual fear memory was tested 16 days later (see Figure S1). The difference in timelines was due to the reported decline of contextual fear memory over time in animals trained pre-weaning (Akers et al. 2012; Akers et al. 2014). The same protocol was used to test contextual fear memory (48 h after training) and its maintenance. Contextual fear memory was tested by 5-min exposure to the training context, regardless of when (pre-weaning vs. post-weaning) the training took place because initial observations based on pilot data (data not shown) had suggested that the duration of context exposure did not affect the extent of context freezing.

2.4 | Statistical Analysis

To test for genotype-specific differences in behaviors within the same age group and for developmental effects on behavior, we used mixed effects linear regression analysis. In all mixed-effects models, we controlled for potential sibling correlations by including a random effect for litter. We controlled for potential sex effects by including fixed covariate effects for sex and for sex-by-genotype interactions. Data are presented as mean ± standard error. In the case of highly skewed distributions, group comparisons were performed using the square root of the scores. In this case, means ± standard errors of square root scores are reported in the tables. The means and SEM reported in the tables are estimates provided by the regression model. A p value < 0.050 was considered statistically significant, and a p value < 0.100 was regarded as a statistical trend. We addressed the multiplicity of statistical test results by estimating false discovery rates (FDR) for all task-related p values for overall group differences. p values from all tasks were pooled, and the corresponding estimated FDR values were derived using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). A p value of 0.016 translates to a FDR of approximately 5%, and therefore, a p value of below 0.016 provides strong evidence for a result. A p value of 0.05 would correspond to a FDR of approximately 10%. FDRs for each genotype comparison are listed along with the p values for each result throughout the manuscript.

Sex effects and sex-by-genotype interactions are reported if they were significant. Follow-up models tested for significant genotype differences in behavioral trends over time. As above, mixed-effect models with random effects were used. The fixed effect predictors were genotype, test day, their interaction (the main parameter of interest), and sex. Statistical analyses were done in R, version 4, using the libraries lmerTest (v3.1), lme4

(v1.1), and emmeans (v1.1). Graphs represent raw data. The error bars in the graphs represent standard errors of the means of the raw data.

3 | Results

3.1 | Fear Conditioning

3.1.1 | In Mice Trained Pre-Weaning (P18), CASPR2 Deficiency Affects Cue-Evoked Fear Memory Differently at 24 h After Training and at Later Timepoints

As shown in Figure 1A, for P18 mice, the difference in baseline freezing on the training day (Context A) between *Cntnap2* knockout (*Cntnap2*$^{-/-}$) and wild-type (*Cntnap2*$^{+/+}$) littermate mice was significant ($t(39.3) = 2.345\ 7$, $p = 0.024$, FDR = 0.058) (Figure 1A, Table 1). Given that *Cntnap2*$^{-/-}$ mice have been reported to be hypersensitive to some but not all painful stimuli (Dawes et al. 2018), we also assessed shock sensitivity, using motor activity in response to the first foot shock as a proxy. No significant genotype effect was observed ($t(41.1) = 1.230$, $p = 0.226$, FDR 0.281) (Figure S2A). We next tested whether *Cntnap2* knockout impacts the acquisition of fear memory. No significant genotype effect was observed, but a trend towards lower freezing in knockout mice ($t(37.1) = 1.873$, $p = 0.069$, FDR = 0.119) (Figure 1A; Table 1). Twenty-four hours after training, mice were exposed to a novel context (Context B) to test cue-evoked fear memory as well as pre-cue and post-cue freezing. Cue-evoked freezing, which corresponds to cue-evoked fear memory, was lower in knockout mice ($t(38.7) = 2.317$, $p = 0.026$, FDR = 0.058) (Figure 1B, Table 1). We found that both pre- and post-cue freezing was lower in the knockout versus wild-type

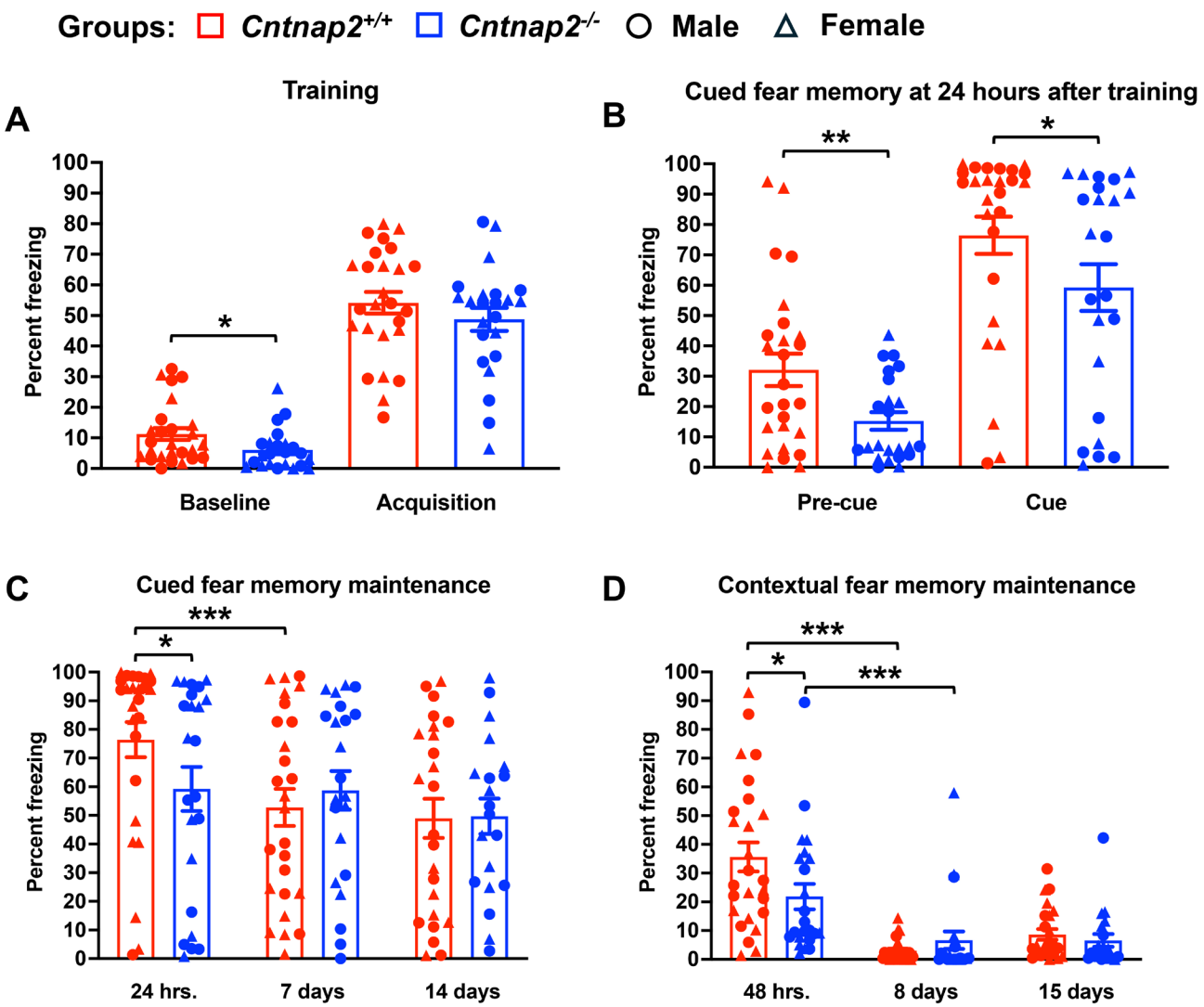

FIGURE 1 | Effects of *Cntnap2* deficiency on cue-evoked and contextual memory in mice trained at P18. Freezing in *Cntnap2* knockout ($^{-/-}$, blue) versus wild-type ($^{+/+}$, red) mice: (A) at baseline and during fear memory acquisition; (B) at 24 h after training, pre-cue and during cued fear memory recall; (C) over the 14 days following training, during cued fear memory recall (maintenance of cued fear memory); and (D) over the 15 days following training, during contextual fear memory recall (maintenance of contextual fear memory). N for training, 24 h and 48 h: 23 knockout (11 m, 12 f) and 26 wild-type (13 m, 13 f) mice, 13 litters. N for 7 days, 8 days: 22 knockout (10 m, 12 f) and 26 wild-type (13 m, 13 f), mice, 13 litters. N for 14 and 15 days: 20 knockout (9 m, 11 f) and 24 (13 m, 11 f) wild-type mice, 12 litters. Due to distribution skewness, baseline *p*-value in (A) is based on square root transformation. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

mice at 24 h after training (pre-cue freezing: $t(35.70) = 3.240$, $p = 0.003$, FDR = 0.017; post-cue freezing: $t(38.5) = 2.852$, $p = 0.007$, FDR = 0.028) (Figure 1B, Table 1). Post-cue freezing also showed a sex effect with males freezing more than females ($t(38.90) = 2.155$, $p = 0.037$).

Analysis of the stability of cue-evoked fear memory revealed that the genotype effect we saw at 24 h after training was not present at 7 or 14 days after training (day 7 $t(37.1) = -0.359$, $p = 0.722$, FDR = 0.778; day 14 $t(32.4) = 0.234$, $p = 0.816$, FDR = 0.846) (Figure 1C, Table 1). Secondary analysis of the stability of fear memory revealed that the loss of this genotypic effect was due to a decline in cue-evoked freezing that occurs only in wild-type mice between the 24-h and 7-day timepoints (24 h vs. 7 days $t(88.2) = 3.53$, $p < 0.001$; 7 days vs. 14 days after training $t(89.3) = 0.25$, $p = 0.803$). In knockout mice, cue-evoked freezing was more stable over time (24 h vs. 7 days after training $t(89.7) = 0.276$, $p = 0.783$; 7 days vs. 14 days after training $t(89.5) = 0.984$, $p = 0.328$; 24 h vs. 14 days after training $t(90.9) = 1.257$, $p = 0.212$) (Figure 1C; Table 1). In contrast to the result at 24 h after training, we found that pre-cue and post-cue freezing at 7 days after training were similar in both genotypes (pre-cue freezing: $t(38.6) = 1.292$, $p = 0.204$, FDR = 0.271; post-cue freezing: $t(36.3) = 1.911$, $p = 0.064$, FDR = 0.119) (Table 1). At 14 days after training, however, pre-cue freezing was lower in knockout mice ($t(34.8) = 3.450$, $p = 0.001$, FDR = 0.007), but post-cue was similar in both genotypes ($t(34.7) = 0.023$, $p = 0.982$, FDR = 0.982) (Table 1).

To probe the effect of CASPR2 deficiency on context-evoked freezing, mice were re-exposed to the training context (Context A) 48 h after training and evaluated for freezing. In this context, freezing was lower in knockout vs. wild-type mice ($t(38.6) = 2.180$, $p = 0.035$, FDR = 0.073) (Figure 1D, Table 1). To assess the stability of this genotypic effect over time, mice were re-exposed to the training context 8 and 15 days after training and evaluated for freezing. This revealed a loss of the genotype effect seen 48 h after training (8 days after training: $t(40.2) = -1.210$, $p = 0.233$, FDR = 0.284; 15 days after training $t(34.32) = 0.922$, $p = 0.363$, FDR = 0.433). Comparison of the results for the knockout and wild-type mice revealed that this loss was due to the rapid return of context-associated freezing to baseline freezing levels within 8 days of training (wild-type: $t(88.4) = 8.291$, $p < 0.001$; knockout: $t(89.9) = 3.417$, $p = 0.001$) (Figure 1D, Table 1).

3.1.2 | CASPR2 Deficiency Impairs Fear Memory Acquisition in Mice Trained Post-Weaning

We next sought to determine whether mice that are trained post-weaning show the same phenotype as those trained pre-weaning. The fact that fear processing in early life fundamentally differs from that in adulthood (Meyer and Lee 2019) and the fact that CASPR2 is relevant to ASD, a neurodevelopmental disorder with developmentally regulated alterations in the fear circuit (Avino et al. 2018; Lai et al. 2014; Schumann et al. 2004; Greimel et al. 2013), suggests that the effects of CASPR2 deficiency on fear-related behaviors at various stages of development might be distinct.

In juvenile mice trained at P30, baseline freezing on training day (Context A) was similar between wild-type and knockout mice ($t(37.2) = 1.492$, $p = 0.144$, FDR = 0.212) (Figure 2A, Table 1). Also, shock sensitivity, as measured by motor activity in response to the first foot shock, did not differ significantly ($t(33.35) = -0.771$, $p = 0.446$, FDR = 0.500) (Figure S2B). In contrast to our result for mice trained at P18, the acquisition of fear memory was impaired in the knockout mice ($t(33.8) = 3.965$, $p < 0.001$, FDR < 0.007) (Figure 2B, Table 1). Despite this acquisition deficit in knockout mice, freezing during cue presentation at 24 h after training was similar for the two genotypes ($t(41.0) = 0.358$, $p = 0.722$, FDR = 0.778) (Figure 2C, Table 1). In contrast to our results for mice trained at P18, pre-cue freezing at 24 h after training was similar for the two genotypes ($t(41.0) = 0.246$, $p = 0.807$, FDR = 0.846), but post-cue freezing was lower in knockout mice ($t(41.0) = 2.227$, $p = 0.032$, FDR = 0.069) (Table 1). Knockout mice froze less upon cue presentation on day 15 ($t(36.0) = 2.967$, $p = 0.005$, FDR = 0.023) (Figure 2D; Table 1). While both wild-type and knockout mice showed a decrease in cue-evoked freezing from 24 h to 15 days after training (wild-type mice: $t(38.5) = 0.209$, $p = 0.043$; knockout mice: $t(38.8) = 6.743$, $p < 0.001$), the magnitude of the decrease was larger in the knockout animals ($t(38.6) = 3.05$, $p = 0.004$) (Figure 2E). In contrast to our results at 24 h after training, pre-cue and post-cue freezing at 15 days after training were both lower in knockout mice (pre-cue freezing: $t(32.6) = 2.737$, $p = 0.010$, FDR = 0.036; post cue freezing: $t(35.6) = 4.025$, $p < 0.001$, FDR < 0.007) (Table 1), with an evident sex effect for post-cue freezing showing lower freezing in males ($t(36.000) = -2.943$, $p = 0.006$).

In these mice trained at P30, contextual fear memory was tested at 48 h and 16 days after training. At both timepoints, freezing was similar for the wild-type and knockout mice (48 h after training: $t(36.9) = 0.894$, $p = 0.377$, FDR = 0.440; 16 days after training: $t(33.0) = 1.253$, $p = 0.219$, FDR = 0.279) (Figure 2F,G; Table 1). However, a sex effect with females freezing more than males was visible at 48 h after training ($t(36.667) = -2.358$, $p = 0.024$), but not at 16 days after training. Analysis of the stability of contextual fear memory revealed that freezing decreased in both the wild-type and knockout mice between 48 h and 16 days after training (wild-type: $t(39.0) = 4.895$, $p = 0.001$; knockout: $t(39.3) = 6.74$, $p = 0.001$) (Figure 2H, Table 1).

Given our discovery that *Cntnap2* deletion affects the acquisition and consolidation of fear memory in mice trained at P18 and P30, as well as the stability of cue-evoked fear memory over time, we tested its effects on fear conditioning in young adult mice (P61). Analysis of baseline freezing on the training day did not reveal a significant difference between the wild-type and knockout mice (Context A) ($t(40.0) = 1.364$, $p = 0.180$, FDR = 0.252). Very little freezing was observed in either group (Figure 2A, Table 1). Also, assessment of motor activity in response to the first foot shock revealed no difference in shock sensitivity ($t(40.0) = 0.107$, $p = 0.916$, FDR = 0.933) (Figure S2C), consistent with the lack of a genotype effect at earlier ages. Like knockout mice trained at P30, those trained at P61 froze less during the acquisition of fear memory than their wild-type counterparts ($t(33.0) = 4.00$, $p < 0.001$, FDR < 0.007) (Figure 2B). However, in contrast to those trained at P30, they also froze less than wild-type mice during cue presentation at 24 h after training ($t(30.6) = 4.446$, $p < 0.001$, FDR < 0.007) (Figure 2C), and this impairment of cue-evoked freezing persisted 15 days after training ($t(40.0) = 3.226$, $p = 0.003$, FDR = 0.017) (Figure 2D).

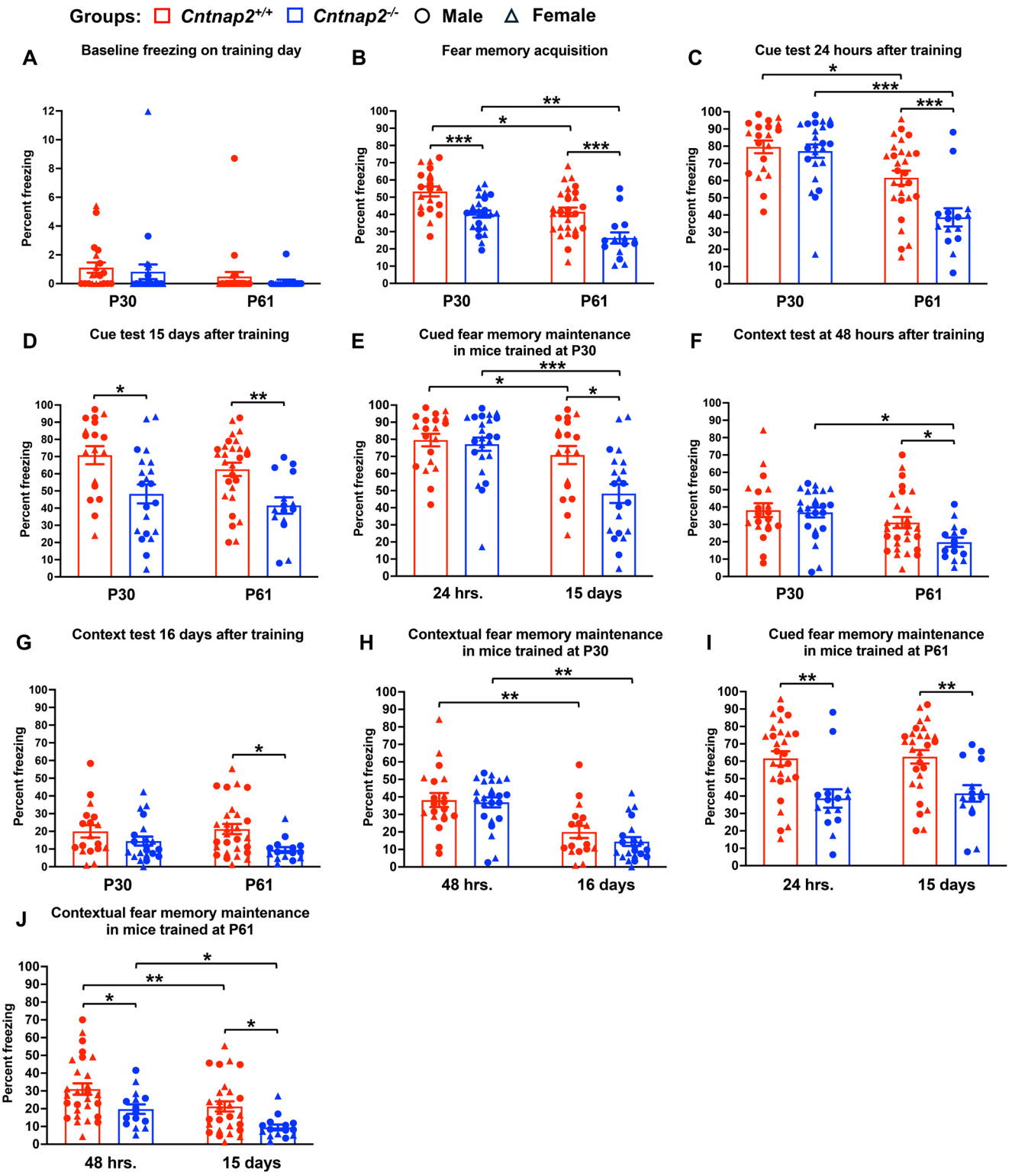

FIGURE 2 | Age-specific effects of *Cntnap2* deficiency on cue evoked and contextual fear memory. Freezing in *Cntnap2* knockout ($^{-/-}$, blue) versus wild-type ($^{+/+}$, red) mice: (A) at baseline and (B) during fear memory acquisition; during cued fear memory recall (cue test) (C) at 24 h after training and (D) at 15 days after training (maintenance of cued fear memory). (E) Stability of cued fear memory recall (cue test) in mice trained at P30. (F) Freezing during context text (contextual fear memory recall) at 48 h after training and (G) at 16 days after training (maintenance of contextual fear memory). (H) Stability of contextual fear memory recall (context test) in mice trained at P30. (I) Stability of cued fear memory recall (cue test) in mice trained at P61. (J) Stability of contextual fear memory recall (context test) in mice trained at P61. P30: N for training, 24 h and 48 h: 24 knockout mice (10 m, 14 f), 20 wild-type mice (12 m, 8 f), 14 litters. N for 15 and 16 days: 21 knockout (8 m, 13 f) and 18 wild-type (11 m, 7 f) mice, 12 litters. P61: N for all days: 15 knockout (9 m, 6 f) and 28 wild-type (11 m, 17 f) mice, 15 litters. Due to distribution skewness, baseline *p*-value in (A) for P30 and P61 is based on square root transformation. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

In mice of both genotypes, cue-evoked fear memory was stable over time (wild-type: $t(41.0) = -0.265$, $p = 0.792$; knockout: $t(41.0) = -0.591$, $p = 0.558$) (Figure 2I). At 24 h after training, the knockout mice trained at P61 showed lower pre- and post-cue freezing than wild-type mice (pre-cue: $t(33.6) = 2.447$, $p = 0.020$, FDR = 0.053, post-cue: $t(35.6) = 4.025$, $p < 0.001$, FDR < 0.007), a phenotype that was not observed at 15 days after training (pre-cue: $t(40.0) = 1.861$, $p = 0.070$, FDR = 0.119; post-cue: $t(35.3) = 2.016$, $p = 0.052$, FDR = 0.100) (Table 1).

Mice trained at P61 were also tested for the acquisition and stability of contextual fear memory. These knockout mice froze less in response to the training context than the wild-type mice at 48 h and at 16 days after training (48 h after training: $t(34.7) = 2.565$, $p = 0.015$, FDR = 0.044; 16 days after training: $t(36.5) = 2.923$, $p = 0.006$, FDR = 0.026) (Figure 2F,G; Table 1). Also, context-evoked freezing declined in both genotypes between 48 h and 16 days (wild-type: $t(41.0) = 3.374$, $p = 0.002$; knockout: $t(41.0) = 2.593$, $p = 0.013$) (Figure 2J, Table 1). The extent of this decline was similar for both genotypes ($t(41.0) = -0.1$; $p = 0.921$).

3.1.3 | The Effect of CASPR2 Deficiency on Fear Memory Acquisition Increases With Age

To identify age-related effects on Pavlovian fear learning, we compared the data for mice trained at P30 and at P61. Age had no significant effect on baseline freezing on the training day (Context A) in either genotype (knockout: $t(57.7) = -1.23$, $p = 0.225$; wild-type: $t(43.6) = -1.88$, $p = 0.066$) (Figure 2A) and no interaction was detected between genotype and age with respect to either baseline freezing ($t(73.787) = 0.122$, $p = 0.904$) or the acquisition of fear memory ($t(66.6) = 0.550$, $p = 0.584$). However, mice of both genotypes froze less during the acquisition of fear memory when trained at P61 versus P30 (knockout: $t(56.8) = -3.36$, $p = 0.001$; wild-type: $t(44.7) = -2.94$, $p = 0.005$) (Figure 2B). We found a genotype-by-age interaction with respect to cue-evoked freezing (Context B) at 24 h after training: Both genotypes froze less in response to cue presentation when trained at P61 (knockout: $t(56.4) = -4.85$, $p < 0.001$; wild-type: $t(45.7) = -2.13$, $p = 0.039$) (Figure 2C), but the decrease was significantly greater in the knockout group ($t(61.1) = 2.643$, $p = 0.010$). Fifteen days after training, however, neither an interaction between training age and genotype ($t(75.0) = -0.253$, $p = 0.801$) nor an effect of training age on cue-evoked freezing within the same genotype was observed (wild-type $t(40.2) = -1.267$, $p = 0.212$; knockout $t(55.0) = -0.807$, $p = 0.432$) (Figure 2D).

Although age at the time of training did not significantly affect context-evoked freezing 48 h after training in wild-type mice ($t(44.8) = -1.50$, $p = 0.140$), knockout mice trained at P61 froze less than those trained P30 ($t(56.7) = -2.69$, $p = 0.009$) (Figure 2F). Despite of this difference, the interaction test of training age and genotype was nonsignificant ($t(70.256) = 1.110$, $p = 0.271$). Training age also had no effect on context-evoked freezing 16 days after training (Context A) (knockout: $t(54.9) = -0.881$, $p = 0.382$; wild-type: $t(41.6) = 0.514$, $p = 0.610$; age × genotype: $t(68.7) = 1.086$, $p = 0.281$) (Figure 2G).

3.2 | Elevated Zero Maze

3.2.1 | *Cntnap2* Knockout Mice Tested at P29 Show Less Anxiety-Related Behavior

Given that the limbic circuitry is essential for regulating not only fear-related but also anxiety-related behaviors, we assessed the developmental effect of *Cntnap2* loss on anxiety-related behavior using the elevated zero maze. Time spent in the closed arm of the elevated zero maze was used as a proxy for anxiety-related behavior.

Neither 17- nor 60-day-old mice exhibited evidence of a statistically significant effect of genotype on the time spent in the closed arm of the elevated zero maze (P17: $t(63.0) = 1.560$, $p = 0.124$, FDR = 0.188 (Figure 3A); P60: $t(45.0) = 1.277$, $p = 0.208$, FDR = 0.271 (Figure 3C)). In 29-day-old mice, however, a statistically significant genotypic effect on time spent in the closed arm was observed; knockout mice spent less time in the closed arm ($t(64.0) = 2.494$, $p = 0.015$, FDR = 0.044) (Figure 3B). No sex effects or sex-by-genotype interactions were significant. For separate analysis of P29 animals per behavior room, means, and SEM see Table 2.

3.2.2 | Hyperactivity in *Cntnap2* Knockout Mice Increases With Age

Given that *Cntnap2* knockout mice have been reported to exhibit hyperactivity, we measured the total distance traveled (in meters) in the elevated zero maze. In all age groups, the knockout mice traveled further than wild-type mice (P17: $t(63.0) = -2.615$, $p = 0.011$, FDR = 0.036 (Figure 3D); P29: $t(34.5) = -2.537$, $p = 0.016$, FDR = 0.045 (Figure 3E); P60: $t(40.8) = -2.039$, $p = 0.048$, FDR = 0.096 (Figure 3F)). For mice tested at P29 and at P60, we found a sex effect in opposite directions. At P29, females traveled a longer distance ($t(34.2) = -2.521$, $p = 0.017$), while at P60, males traveled a longer distance ($t(39.0) = 2.346$, $p = 0.024$). At P60, we also saw a sex-by-genotype interaction for total distance traveled ($t(40.5) = -2.922$, $p = 0.006$), indicating that the genotype difference is greater in males than in females. There was also a trend for an age-by-genotype interaction for total distance traveled ($t(132.3) = -1.764$, $p = 0.080$), and both knockout ($t(102.0) = 4.63$, $p < 0.001$), and wild-type ($t(73.9) = 3.10$, $p = 0.003$) mice traveled longer distances with increasing age. For means and SEM, see Table 2.

4 | Discussion

Our experiments provide the first evidence that CASPR2 deficiency has developmentally regulated effects on fear- and anxiety-related behaviors. Specifically, CASPR2 deficiency affects fear learning and fear memory in an age-specific manner, and its age-specific effects on cued and contextual fear memory differ. The finding that in knockout mice, the acquisition of fear memory was significantly impaired only when they were trained post-weaning suggests that the effect of CASPR2 deficiency on the acquisition of fear memory increases during development. Given the trend towards lower acquisition in knockout mice trained pre-weaning, it is unlikely that the effect appears

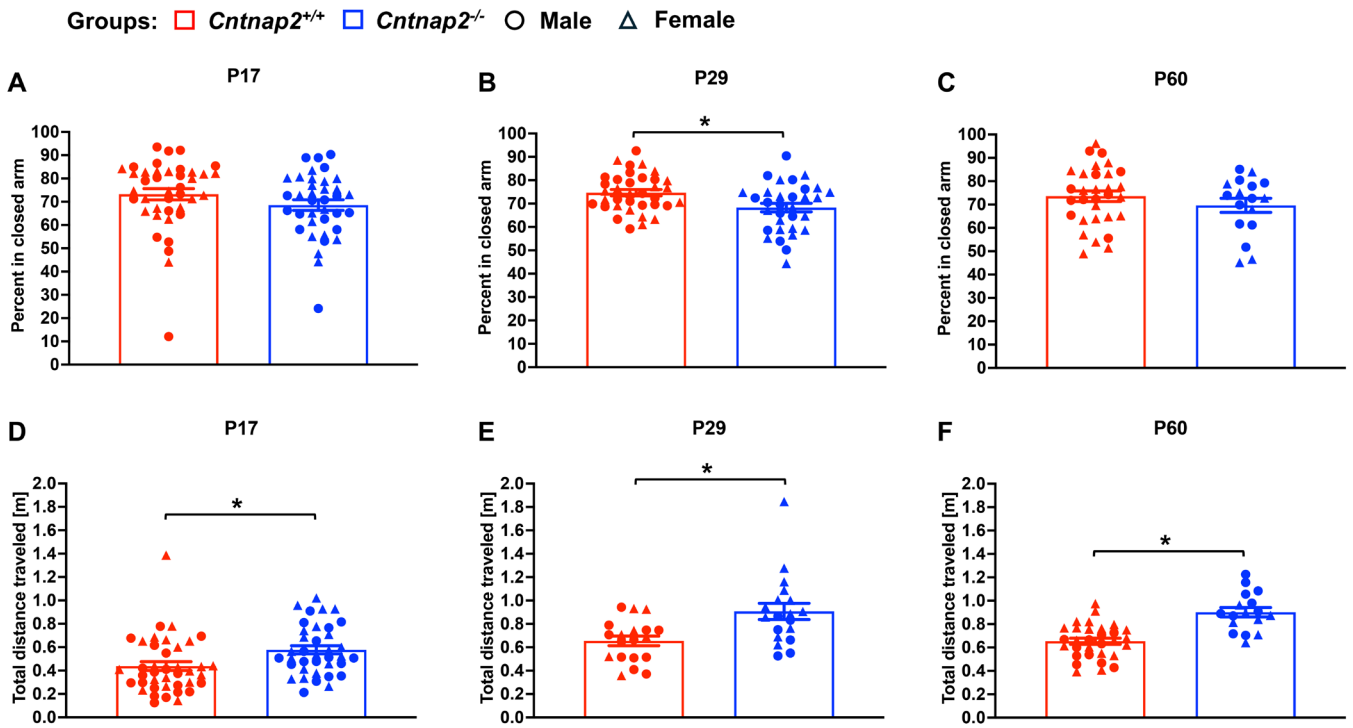

FIGURE 3 | *Cntnap2* knockout mice travel longer distances at all ages. Percent time in closed arm in *Cntnap2* knockout ($^{-/-}$, blue) versus wild-type ($^{+/+}$, red) mice: (A) at P17 (37 knockout (17 m, 20 f) and 40 wild-type (20 m, 20 f) mice, 20 litters), (B) at P29 (32 knockout (13 m, 19 f) and 36 wild-type (20 m, 16 f) mice, 23 litters) and, (C) at P60 (17 knockout (10 m, 7 f), 31 wild-type (11 m, 20 f) mice, 16 litters). Total distance traveled in meters [m] in *Cntnap2* knockout ($^{-/-}$, blue) versus wild-type ($^{+/+}$, red) mice: (D) at P17 (37 knockout (17 m, 20 f) and 40 wild-type (20 m, 20 f) mice, 20 litters), (E) at P29 (19 knockout (6 m, 13 f) and 19 wild-type (12 m, 7 f) mice, 14 litters), and (F) at P60 (17 knockout (10 m, 7 f), 31 wild-type (20 m, 20 f) mice, 20 litters). $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

after weaning. Our results also suggest that CASPR2 deficiency plays a developmentally regulated role in the consolidation of fear memory. Our findings that knockout mice trained at P18 showed impaired cue-evoked freezing 24 h after training but no deficit in acquisition, whereas counterparts trained at P30 showed a deficit in acquisition but not cue-evoked freezing 24 h after training, suggest that CASPR2 deficiency impairs the consolidation of fear memory in mice trained at P18 but not in mice trained at P30. Although loss of CASPR2 seems to stabilize cued fear memory maintenance (24 h vs. 7 days after training) in mice trained at P18, it seems to impair cued fear memory maintenance in mice trained at P30 (24 h vs. 15 days). It also seems to impair the acquisition and recall of both cued and contextual fear memory without destabilizing its maintenance in mice trained at P61.

One potential explanation for the lack of significant impairment in the acquisition of fear memory in knockout mice trained at P18 might be high expression or phosphorylation of certain glutamate receptor subunits in the basolateral amygdala (BLA) at this stage of development (Bessieres et al. 2019). The glutamate receptor subunit NR2B is essential for the acquisition of fear memory (Zhao et al. 2005; Zhang et al. 2008; Rodrigues et al. 2001). At P17, NR2B expression in the BLA is almost double than that in adulthood (Bessieres et al. 2019), and in *Cntnap2*-deficient mice, whole-brain expression of NR2B is reduced by about 50% at P28 (Kim et al. 2019). Therefore, it is possible that in our P18 mice, the developmentally high BLA NR2B expression compensated for any NR2B reduction caused by *Cntnap2* loss, so that the difference in the acquisition of fear memory between wild-type and knockout mice was not statistically significant. Given that NR2B expression in the BLA declines during development (Bessieres et al. 2019), it is possible that such compensation did not occur in the older mice, accounting for the observed deficits in the acquisition of fear memory in those groups.

A second glutamate receptor subunit that could have contributed to the observed effects is GluA1, which is essential for the acquisition of fear memory. Its phosphorylation at Ser831 contributes to the formation of short-term memory by promoting GluA1 insertion into the synaptic membrane (Johansen et al. 2011). In the BLA, the fraction of GluA1 receptors that is phosphorylated at Ser831 decreases by more than 80% from P17 to adulthood (Bessieres et al. 2019). Also, *Cntnap2* deficiency has been reported to reduce either the expression (Kim et al. 2019) or trafficking (Varea et al. 2015) of GluA1. Thus, the large population of GluA1 phosphorylated at Ser831 in specifically those animals trained at P18 might compensate for *Cntnap2* deficiency-induced deficits in GluA1 expression or trafficking. Given the marked decline in GluA1 phosphorylated at Ser831 during development, such compensation might not be possible at P30 and P61, accounting for the observed deficits in the acquisition of fear memory in the mice trained at these times.

Although our knockout mice trained at P18 showed no significant impairment in the acquisition of fear memory, the significant impairment of their recall of cued (at 24 h) and contextual (at 48 h) fear memory suggests that they were deficient in the

consolidation of fear memory. An essential step in memory consolidation is the stabilization of new dendritic spines (Holtmaat et al. 2008; Muñoz-Cuevas et al. 2013), a process with which *Cntnap2* has been associated (Gdalyahu et al. 2015). Thus, it is possible that the impaired recall of cued and contextual fear memory in these mice despite their intact ability to acquire such memory is due to defects in the stabilization of new dendritic spines, and hence to impairment of memory consolidation. In knockout mice trained at P61, the impairment of both memory acquisition and consolidation (due to impaired stabilization of new spines) might contribute to the observed impairment of cued and contextual fear memory recall.

The observation that knockout mice trained at P30 show impaired acquisition but intact recall of both cued and contextual fear memory (at 24 and 48 h after training, respectively) was surprising because an impairment in freezing during acquisition suggests impaired fear learning. If *Cntnap2* deficiency impairs the stabilization of new spines as early as P18, an additional strong physiological mechanism that impairs memory consolidation in wild-type mice must be active in mice trained at P30. A mechanism that is active at this time of development and could potentially account for this effect is synaptic pruning. In 16–25-day old mice, 8-day spine stability has been reported to be as low as 35% due to high rates of pruning at this age (Holtmaat et al. 2005; Runge et al. 2020). If the timing of pruning is significantly delayed in knockout mice, the developmentally regulated excessive pruning may be ongoing in wild-type mice trained at P30, but not yet in same-age knockout mice. Therefore, wild-type mice trained at P30 might lose more newly formed spines than same-age knockout mice, despite their deficiency in spine stabilization. This could explain the similar levels of cue- and context-evoked freezing in knockout and wild-type mice trained at P30 (at 24 and 48 h after training, respectively) despite the impairment of fear memory acquisition in the former.

The preservation of spines is also important for the maintenance of memory after initial consolidation (Basu and Lamprecht 2018). A delay in the timing of pruning in knockout mice might result in higher spine stability following acquisition in mice trained at P18, and this could account for our finding that such mice have more stable cue-evoked fear memory during the week after training than their wild-type counterparts. Although Gdalyahu et al. (2015) did not observe an effect of *Cntnap2* loss on pruning in adult mice in the barrel cortex, such an effect might be age- and possibly also region-dependent. Our hypothesis is supported by human postmortem data on spine density in the amygdala of ASD cases. Specifically, Weir et al. (2018) reported that spine density was increased in the lateral nucleus of the amygdala of young, but not older, ASD cases. This would also be consistent with a delay in pruning in the context of ASD.

In wild-type mice, pruning has been reported to slow with age (Runge et al. 2020). Specifically, data reported by Holtmaat et al. (2005) suggest that the rate of pruning is slower at P35 than at earlier ages. If the developmental trajectory of pruning is in fact delayed in knockout mice, it might still be excessive between P31 (cue recall at 24 h) and P45 (cue recall at 15 days), whereas pruning slows down in their wild-type counterparts. This could explain the more pronounced decrease in cue-evoked freezing in P30-trained knockout versus wild-type mice. Our observation that young adult mice of both genotypes showed stable cue-evoked freezing over time is consistent with the hypothesis that *Cntnanp2* deficiency might play a role in the developmental trajectory of pruning.

Potential explanations for the observed loss of contextual memory in our study relate to training age, the timing of hippocampal neurogenesis, and the fear-learning paradigm used. Previous studies examining the stability of contextual fear memory acquired before weaning (postnatal days 15–17) reported that context-evoked freezing declines substantially within a week of training (Akers et al. 2014; Akers et al. 2012) and that this is at least partly due to high levels of hippocampal neurogenesis at that age (Akers et al. 2014). Our finding that P18-trained mice of both genotypes lost the contextual fear memory that was observed 48 h after training within 8 days of training is consistent with these earlier observations. One possible explanation for the loss of contextual fear memory over time (48 h vs. 16 days after training) in mice trained at P30 and P61 could be the use of a dual training paradigm for cue and context. This approach might favor the acquisition and consolidation of cued over contextual fear memory.

Our zero maze experiments revealing that 29-day-old wild-type mice spent significantly more time in the closed arm than their knockout counterparts suggest that CASPR2 deficiency has a greater influence on anxiety-related behavior at that age than at others. The mean genotype differences were only slightly smaller at the other ages. However, the within-genotype variation was greater at those ages. This resulted in a lack of statistical significance for P17 and P60. A larger experimental sample size could clarify evidence for mean genotype differences at those ages. Contrasting our result with mice tested at P29, Binder and Bordey (2023) did not observe altered anxiety-related behavior in 4–5 week-old *Cntnap2* knockout mice. This could be due to the slightly older age of their mice, experimental conditions (different room/lighting) or due to different proxies used to measure anxiety-related behaviors (time spent in the closed arm of the elevated zero maze versus perimeter preference in the locomotor box). In line with our result for young adults, Scott et al. 2020 could also not detect altered anxiety-related behavior in their experiments with adult *Cntnap2* knockout rats. Our discovery that distance traveled was greater for knockout versus control mice at all ages is consistent with the hyperactivity previously reported for *Cntnap2* knockout mice and rats (Penagarikano et al. 2011; Scott et al. 2020; Binder and Bordey 2023). Penagarikano and colleagues (Penagarikano et al. 2011) reported that this hyperactivity was reduced by treatment with the second-generation antipsychotic risperidone, which is consistent with it being caused by a nigrostriatal dopaminergic dysfunction. Our finding of a longer total distance traveled in P17 mice suggests that this dysfunction might be present as early as P17.

It is unclear if the increased total distance traveled in the zero maze has implications for our fear conditioning results. Increased distance traveled does not necessarily translate to a reduction in freezing. In knockout mice trained P18, we observed a reduction in baseline freezing during acquisition, which may reflect baseline hyperactivity. Given the lack of consensus on

how baseline freezing influences freezing during acquisition and memory testing (Jacobs et al. 2010), it remains uncertain if this altered baseline behavior impacts freezing during acquisition and memory testing. In contrast, in mice trained P30 and P61, there was no genotype effect in baseline freezing, suggesting no impact of the potential locomotor phenotype on freezing behaviors at these ages.

5 | Conclusion

Our findings are the first demonstration that *Cntnap2* loss has complex, age-dependent effects on fear learning, fear memory recall (both cued and contextual), and the stability of cued fear memory. Our findings are congruent with an overall delayed development in these mice. Future studies of the developmental and pathophysiological effects of *Cntnap2* loss on anxiety, fear learning, and fear memory hold promise for developing better treatments for fear and anxiety in individuals with ASD.

Acknowledgments

This research was supported by startup funds from the Iowa Neuroscience Institute and the Department of Psychiatry at the University of Iowa. We want to thank Drs. John A Wemmie, Hanna E Stevens, and Ted Abel, as well as the Neural Circuits and Behavior Core at the University of Iowa, for their advice and for generously letting us use their behavioral equipment.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets generated and analyzed for this study are available from the corresponding author upon reasonable request.

References

Akers, K. G., M. Arruda-Carvalho, S. A. Josselyn, and P. W. Frankland. 2012. "Ontogeny of Contextual Fear Memory Formation, Specificity, and Persistence in Mice." *Learning & Memory* 19, no. 12: 598–604. https://doi.org/10.1101/lm.027581.112.

Akers, K. G., A. Martinez-Canabal, L. Restivo, et al. 2014. "Hippocampal Neurogenesis Regulates Forgetting During Adulthood and Infancy." *Science* 344, no. 6184: 598–602. https://doi.org/10.1126/science.1248903.

Alarcón, M., B. S. Abrahams, J. L. Stone, et al. 2008. "Linkage, Association, and Gene-Expression Analyses Identify CNTNAP2 as an Autism-Susceptibility Gene." *American Journal of Human Genetics* 82, no. 1: 150–159. https://doi.org/10.1016/j.ajhg.2007.09.005.

Anderson, G. R., T. Galfin, W. Xu, J. Aoto, R. C. Malenka, and T. C. Südhof. 2012. "Candidate Autism Gene Screen Identifies Critical Role for Cell-Adhesion Molecule CASPR2 in Dendritic Arborization and Spine Development." *Proceedings of the National Academy of Sciences of the United States of America* 109, no. 44: 18120–18125. https://doi.org/10.1073/pnas.1216398109.

Avino, T. A., N. Barger, M. V. Vargas, et al. 2018. "Neuron Numbers Increase in the Human Amygdala From Birth to Adulthood, but Not in Autism." *Proceedings of the National Academy of Sciences of the United States of America* 115, no. 14: 3710–3715. https://doi.org/10.1073/pnas.1801912115.

Basu, S., and R. Lamprecht. 2018. "The Role of Actin Cytoskeleton in Dendritic Spines in the Maintenance of Long-Term Memory." *Frontiers in Molecular Neuroscience* 11: 143. https://doi.org/10.3389/fnmol.2018.00143.

Benjamini, Y., and Y. Hochberg. 1995. "Approach to Multiple Testing." *Journal of the Royal Statistical Society: Series B: Methodological* 57: 289–300.

Bessieres, B., M. Jia, A. Travaglia, and C. M. Alberini. 2019. "Developmental Changes in Plasticity, Synaptic, Glia, and Connectivity Protein Levels in Rat Basolateral Amygdala." *Learning & Memory* 26, no. 11: 436–448. https://doi.org/10.1101/lm.049866.119.

Binder, M. S., and A. Bordey. 2023. "The Novel Somatosensory Nose-Poke Adapted Paradigm (SNAP) is an Effective Tool to Assess Differences in Tactile Sensory Preferences in Autistic-Like Mice." *eNeuro* 10, no. 8: ENEURO.0478-22.2023. https://doi.org/10.1523/eneuro.0478-22.2023.

Dawes, J. M., G. A. Weir, S. J. Middleton, et al. 2018. "Immune or Genetic-Mediated Disruption of CASPR2 Causes Pain Hypersensitivity due to Enhanced Primary Afferent Excitability." *Neuron* 97, no. 4: 806–822. https://doi.org/10.1016/j.neuron.2018.01.033.

Frankland, P. W., H. K. Ding, E. Takahashi, A. Suzuki, S. Kida, and A. J. Silva. 2006. "Stability of Recent and Remote Contextual Fear Memory." *Learning & Memory* 13, no. 4: 451–457. https://doi.org/10.1101/lm.183406.

Gale, G. D., S. G. Anagnostaras, B. P. Godsil, et al. 2004. "Role of the Basolateral Amygdala in the Storage of Fear Memories Across the Adult Lifetime of Rats." *Journal of Neuroscience* 24, no. 15: 3810–3815. https://doi.org/10.1523/jneurosci.4100-03.2004.

Gangi, D. N., A. J. Schwichtenberg, A. M. Iosif, G. S. Young, F. Baguio, and S. Ozonoff. 2018. "Gaze to Faces Across Interactive Contexts in Infants at Heightened Risk for Autism." *Autism* 22, no. 6: 763–768. https://doi.org/10.1177/1362361317704421.

Gao, R., N. H. Piguel, A. E. Melendez-Zaidi, et al. 2018. "CNTNAP2 Stabilizes Interneuron Dendritic Arbors Through CASK." *Molecular Psychiatry* 23, no. 9: 1832–1850. https://doi.org/10.1038/s41380-018-0027-3.

Gdalyahu, A., M. Lazaro, O. Penagarikano, P. Golshani, J. T. Trachtenberg, and D. H. Geschwind. 2015. "The Autism Related Protein Contactin-Associated Protein-Like 2 (CNTNAP2) Stabilizes New Spines: An In Vivo Mouse Study." *PLoS One* 10, no. 5: e0125633. https://doi.org/10.1371/journal.pone.0125633.

Greimel, E., B. Nehrkorn, M. Schulte-Rüther, et al. 2013. "Changes in Grey Matter Development in Autism Spectrum Disorder." *Brain Structure & Function* 218, no. 4: 929–942. https://doi.org/10.1007/s00429-012-0439-9.

Holtmaat, A., V. De Paola, L. Wilbrecht, and G. W. Knott. 2008. "Imaging of Experience-Dependent Structural Plasticity in the Mouse Neocortex In Vivo." *Behavioural Brain Research* 192, no. 1: 20–25. https://doi.org/10.1016/j.bbr.2008.04.005.

Holtmaat, A. J., J. T. Trachtenberg, L. Wilbrecht, et al. 2005. "Transient and Persistent Dendritic Spines in the Neocortex In Vivo." *Neuron* 45, no. 2: 279–291. https://doi.org/10.1016/j.neuron.2005.01.003.

Jacobs, N. S., J. D. Cushman, and M. S. Fanselow. 2010. "The Accurate Measurement of Fear Memory in Pavlovian Conditioning: Resolving the Baseline Issue." *Journal of Neuroscience Methods* 190, no. 2: 235–239. https://doi.org/10.1016/j.jneumeth.2010.04.029.

Johansen, J. P., C. K. Cain, L. E. Ostroff, and J. E. LeDoux. 2011. "Molecular Mechanisms of Fear Learning and Memory." *Cell* 147, no. 3: 509–524. https://doi.org/10.1016/j.cell.2011.10.009.

Kim, J. W., K. Park, R. J. Kang, et al. 2019. "Pharmacological Modulation of AMPA Receptor Rescues Social Impairments in Animal Models of Autism." *Neuropsychopharmacology* 44, no. 2: 314–323. https://doi.org/10.1038/s41386-018-0098-5.

Lai, M. C., M. V. Lombardo, and S. Baron-Cohen. 2014. "Autism." *Lancet* 383, no. 9920: 896–910. https://doi.org/10.1016/s0140-6736(13)61539-1.

Li, X., Z. Hu, Y. He, et al. 2010. "Association Analysis of CNTNAP2 Polymorphisms With Autism in the Chinese Han Population." *Psychiatric Genetics* 20, no. 3: 113–117. https://doi.org/10.1097/YPG.0b013e32833a216f.

Masi, A., M. M. DeMayo, N. Glozier, and A. J. Guastella. 2017. "An Overview of Autism Spectrum Disorder, Heterogeneity and Treatment Options." *Neuroscience Bulletin* 33, no. 2: 183–193. https://doi.org/10.1007/s12264-017-0100-y.

Meyer, H. C., and F. S. Lee. 2019. "Translating Developmental Neuroscience to Understand Risk for Psychiatric Disorders." *American Journal of Psychiatry* 176, no. 3: 179–185. https://doi.org/10.1176/appi.ajp.2019.19010091.

Muñoz-Cuevas, F. J., J. Athilingam, D. Piscopo, and L. Wilbrecht. 2013. "Cocaine-Induced Structural Plasticity in Frontal Cortex Correlates With Conditioned Place Preference." *Nature Neuroscience* 16, no. 10: 1367–1369. https://doi.org/10.1038/nn.3498.

Pattwell, S. S., K. G. Bath, B. J. Casey, I. Ninan, and F. S. Lee. 2011. "Selective Early-Acquired Fear Memories Undergo Temporary Suppression During Adolescence." *Proceedings of the National Academy of Sciences of the United States of America* 108, no. 3: 1182–1187. https://doi.org/10.1073/pnas.1012975108.

Penagarikano, O., B. S. Abrahams, E. I. Herman, et al. 2011. "Absence of CNTNAP2 Leads to Epilepsy, Neuronal Migration Abnormalities, and Core Autism-Related Deficits." *Cell* 147, no. 1: 235–246. https://doi.org/10.1016/j.cell.2011.08.040.

Rodrigues, S. M., G. E. Schafe, and J. E. LeDoux. 2001. "Intra-Amygdala Blockade of the NR2B Subunit of the NMDA Receptor Disrupts the Acquisition but Not the Expression of Fear Conditioning." *Journal of Neuroscience* 21, no. 17: 6889–6896. https://doi.org/10.1523/jneurosci.21-17-06889.2001.

Runge, K., C. Cardoso, and A. de Chevigny. 2020. "Dendritic Spine Plasticity: Function and Mechanisms." *Frontiers in Synaptic Neuroscience* 12: 36. https://doi.org/10.3389/fnsyn.2020.00036.

Schumann, C. M., J. Hamstra, B. L. Goodlin-Jones, et al. 2004. "The Amygdala Is Enlarged in Children but Not Adolescents With Autism; the Hippocampus Is Enlarged at all Ages." *Journal of Neuroscience* 24, no. 28: 6392–6401. https://doi.org/10.1523/jneurosci.1297-04.2004.

Scott, K. E., K. Kazazian, R. S. Mann, et al. 2020. "Loss of Cntnap2 in the Rat Causes Autism-Related Alterations in Social Interactions, Stereotypic Behavior, and Sensory Processing." *Autism Research* 13, no. 10: 1698–1717. https://doi.org/10.1002/aur.2364.

Simonoff, E., A. Pickles, T. Charman, S. Chandler, T. Loucas, and G. Baird. 2008. "Psychiatric Disorders in Children With Autism Spectrum Disorders: Prevalence, Comorbidity, and Associated Factors in a Population-Derived Sample." *Journal of the American Academy of Child and Adolescent Psychiatry* 47, no. 8: 921–929. https://doi.org/10.1097/CHI.0b013e318179964f.

Stein, M. B., B. Z. Yang, D. A. Chavira, et al. 2011. "A Common Genetic Variant in the Neurexin Superfamily Member CNTNAP2 Is Associated With Increased Risk for Selective Mutism and Social Anxiety-Related Traits." *Biological Psychiatry* 69, no. 9: 825–831. https://doi.org/10.1016/j.biopsych.2010.11.008.

Turner, L. B., and R. G. Romanczyk. 2012. "Assessment of Fear in Children With an Autism Spectrum Disorder." *Research in Autism Spectrum Disorders* 6, no. 3: 1203–1210.

Uddin, M. S., A. Azima, M. A. Aziz, et al. 2021. "CNTNAP2 Gene Polymorphisms in Autism Spectrum Disorder and Language Impairment Among Bangladeshi Children: A Case-Control Study Combined With a Meta-Analysis." *Human Cell* 34, no. 5: 1410–1423. https://doi.org/10.1007/s13577-021-00546-8.

Varea, O., M. D. Martin-de-Saavedra, K. J. Kopeikina, et al. 2015. "Synaptic Abnormalities and Cytoplasmic Glutamate Receptor Aggregates in Contactin Associated Protein-Like 2/Caspr2 Knockout Neurons." *Proceedings of the National Academy of Sciences of the United States of America* 112, no. 19: 6176–6181. https://doi.org/10.1073/pnas.1423205112.

Weir, R. K., M. D. Bauman, B. Jacobs, and C. M. Schumann. 2018. "Protracted Dendritic Growth in the Typically Developing Human Amygdala and Increased Spine Density in Young ASD Brains." *Journal of Comparative Neurology* 526, no. 2: 262–274. https://doi.org/10.1002/cne.24332.

White, S. W., D. Oswald, T. Ollendick, and L. Scahill. 2009. "Anxiety in Children and Adolescents With Autism Spectrum Disorders." *Clinical Psychology Review* 29, no. 3: 216–229. https://doi.org/10.1016/j.cpr.2009.01.003.

Williams, S. M., J. Y. An, J. Edson, et al. 2019. "An Integrative Analysis of Non-Coding Regulatory DNA Variations Associated With Autism Spectrum Disorder." *Molecular Psychiatry* 24, no. 11: 1707–1719. https://doi.org/10.1038/s41380-018-0049-x.

Zhang, X. H., F. Liu, Q. Chen, et al. 2008. "Conditioning-Strength Dependent Involvement of NMDA NR2B Subtype Receptor in the Basolateral Nucleus of Amygdala in Acquisition of Auditory Fear Memory." *Neuropharmacology* 55, no. 2: 238–246. https://doi.org/10.1016/j.neuropharm.2008.05.030.

Zhao, M. G., H. Toyoda, Y. S. Lee, et al. 2005. "Roles of NMDA NR2B Subtype Receptor in Prefrontal Long-Term Potentiation and Contextual Fear Memory." *Neuron* 47, no. 6: 859–872. https://doi.org/10.1016/j.neuron.2005.08.014.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.