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Heterozygosity of murine *Crkl* does not recapitulate behavioral dimensions of human 22q11.2 hemizygosity

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Abstract

Deletions in 22q11.2 human chromosome are known to be associated with psychiatric disorders, such as intellectual disability, schizophrenia, autism spectrum disorder, and anxiety disorders. This copy number variation includes a 3.0 Mb deletion and a nested proximal 1.5 Mb hemizygous deletion in the same region. Evidence indicates that the distal 22q11.2 region outside the nested 1.5 Mb deletion also might be contributory in humans. However, the precise genetic architecture within the distal region responsible for psychiatric disorders remains unclear, and this issue cannot be experimentally evaluated beyond the correlation in humans. As *CRKL* (CRK-like Proto-Oncogene, Adaptor Protein) is one of the genes encoded in the distal 22q11.2 segment and its homozygous deletion causes physical phenotypes of 22q11.2 hemizygous deletion, we tested the hypothesis that its murine homolog *Crkl* contributes to behavioral phenotypes relevant to psychiatric disorders in mice. Congenic *Crkl* heterozygosity reduced thigmotaxis, an anxiety-related behavior, in an inescapable open field, but had no apparent effect on social interaction, spontaneous alternation in a T-maze, anxiety-like behavior in an elevated plus maze, or motor activity in an open field. Our data indicate that the heterozygosity of murine *Crkl* does not recapitulate social deficits, working memory deficits, repetitive behavior traits or hyperactivity of human 22q11.2 hemizygous deletion. Moreover, while 22q11.2 hemizygous deletion is associated with high levels of phobia and anxiety in humans, our data suggest that *Crkl* heterozygosity rather acts as a protective factor for phobia-like behavior in an open field.

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AUTHOR CONTRIBUTIONS

Takahira Yamauchi performed the data analyses, interpreted the results, and wrote the manuscript. Gina Kang performed the experiments and collected the data. Noboru Hiroi designed and planned all experiments, analyzed the data, and wrote the manuscript. All authors provided input to the manuscript and approved the final version.

CONFLICT OF INTEREST

The authors declare that they have no financial conflicts of interest related to this work.

Keywords

22q11.2; anxiety; autism; CNV; Crkl; intellectual disability; motor activity; schizophrenia; social behavior; working memory

1 | INTRODUCTION

One of the major challenges in understanding the mechanisms underlying psychiatric disorders associated with copy number variants (CNVs) is how to determine the genuine functional contribution of encoded genes to mental illness. One view is that all the genes encoded in a CNV contribute to any given clinically defined psychiatric disorder (i.e., contiguous gene effect). In its extreme interpretation, all CNV-encoded genes are thought to contribute to phenotypic features only collectively, and single genes cannot affect phenotypes. Consistent with this interpretation, the severity of the phenotype increases with the size of large CNVs, and exome sequencing does not find variants of autism spectrum disorder (ASD)-associated genes encoded in such CNVs.[1]

Another view is that some driver genes present in large CNVs contribute more to mental illness than other encoded genes. Evidence indicates that driver genes, rather than the collective action of all CNV-encoded genes, are determinants for mental illness in a disease- and CNV-specific manner. The size of deletions is not correlated with autism severity; the size of duplications is not associated with nonverbal IQ.[2] The deletion of some CNV-encoded single genes alone at least partially recapitulates behavioral phenotypes of a large CNV deletion in mouse models (e.g., 17p11.2 and 22q11.2).[3-13]

As there are insufficient cases of nested small CNV deletions, single-gene deletions, and loss-of-function mutations, these contradictory views of CNVs cannot be critically tested in humans. Attempts have been made to determine biological pathways influenced by the CNV genes in humans, and a wealth of evidence indicates pathways for synaptic functions and immune systems as contributory. However, such analyses are based on databases of known biological functions of genes. Novel functions cannot be reliably identified in such an approach, and well-studied functions tend to be over-represented.

Hemizygous deletion of human chromosome 22q11.2 has been extensively studied since it was found to be associated with high rates of schizophrenia, ASD, intellectual disability (ID), anxiety disorders, and phobia.[13-16] The majority of cases are ~3.0 Mb deletions of more than 50 protein-coding genes and nested, proximal 1.5 Mb deletion cases of more than 30 protein-coding genes[17] (Figure 1). A few reported cases of atypical deletions distal to the nested proximal 1.5 Mb region are associated with schizophrenia, ASD, and ID[13,15,18] (Figure 1, Distal deletion). Moreover, intelligence quotient (IQ) scores might be lower in carriers of the large 3.0 Mb deletion compared to those of the nested 1.5 Mb proximal deletion.[19] These sporadic cases suggest that the distal deletion might exacerbate the severity of ID or contribute to schizophrenia and ASD. However, as there are only a few reported cases of distal 22q11.2 deletions in humans, its statistical power remains weak and an independent means is required for validation.

CRKL, CRK Like Proto-Oncogene, Adaptor Protein, is a gene encoded in the distal deletion of 22q11.2 and expressed in the brain.[17] Evidence suggests its potential contribution to the physical phenotypes characteristic for 22q11.2 hemizygous deletion in humans. Homozygous deletion of this gene results in craniofacial, cardio-vascular, thymic, and parathymic phenotypes in mice similar to those seen in 22q11.2 hemizygous deletion cases in humans. However, no abnormality was found in migration and early expansion of neural crest cells in heterozygotes in mice.[20-22] More importantly, a few cases of developmental neuropsychiatric disorders associated with small deletions, including *CRKL*, have been reported (see distal deletion cases associated with schizophrenia, ASD, ID and developmental delay (see Figure 1).[22] The functional contribution of *CRKL* to psychiatric disorders has not been established, because there are only a few cases of the distal deletions and they include more than *CRKL* in humans, however.

We tested the hypothesis that heterozygosity of *Crkl* alone causes phenotypes characteristic of psychiatric disorders associated with 22q11.2 hemizygous deletion, using congenic *Crkl* heterozygous mice; *Crkl* homozygous mice mostly die before birth. We established gene-phenotype relationships focusing on traits that cut across clinical classifications of psychiatric disorders (i.e., dimensions),[23,24] including social interaction in a naturalistic environment, working memory and repetitive behavior in a T-maze, anxiety-related behaviors in an elevated plus maze and an inescapable open field as well as motor activity in an open field.[24] Our data do not support a major role for *Crkl* in these dimensions and rather suggest that it might act as a protective factor for phobia-evoked anxiety.

2 | MATERIAL AND METHODS

2.1 | *Crkl*$^{+/-}$ mouse

Due to the presence of high levels of interpretative ambiguity in non-congenic mice[24], we used a congenic strain with a C57BL/6J background. This mouse was originally developed by targeting exon 1 with a neomycin resistance cassette. The construct was electroporated into R1 embryonic stem (ES) cells (F1, 129X1/SvJ × 129S1/Sv). Targeted ES cells were microinjected into blastocysts, and chimeric offspring were bred to 129X1/SvJ mice. Heterozygous (+/−) mice were backcrossed to C57BL/6J for at least 12 generations (#006910, Jackson Laboratory). We maintained two pairs of male congenic +/− mice and female C57BL/6J mice to generate mice for testing. Tails were clipped around postnatal day 10 and genotype was determined with primers: forward GTA AAG GCT TCC CAA GCA AGA and reverse CCG GTT CGA TTA AGG TGG TAG for the wild-type (WT) genotype, and an additional reverse AGC TCA TTC CTC CCA CTC ATG for the heterozygous (HT) genotype. Male mice, derived from 7 litters, were tested starting at 2 months of age. This age was chosen because it falls between the beginning of puberty at 1 month of age[25] and mature adulthood at 3 months of age.[25,26] The chosen time is optimal for detecting all behavioral phenotypes relevant to developmental neuropsychiatric disorders.[27,28]

Animal handling and use followed protocols approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine and University of Texas Health Science Center at San Antonio in accordance with NIH guidelines.

2.2 | Behavioral analysis

We used our published behavioral battery of assays that includes reciprocal social interaction, spontaneous alternation in the T-maze, behavior in the elevated plus maze (EPM), locomotor activity, and thigmotaxis in an inescapable open field.[5,7,8,27,28] Mice were group-housed as littermates. Testing was done between 1 PM and 4 PM during the light phase of the day. The order of behavioral assays was based on imposed stress levels. First, behaviors that occur in cage-like settings (i.e., social interaction) were tested. As the possibility of choice in an apparatus is assumed to impose less stress than the lack of choice, [29-31] T-maze and EPM tests were performed before the inescapable open field tests where no choice was available and stress levels were considerably high. Except for spontaneous alternation in the T-maze, where three delays were tested over a period of three consecutive days, one or two rest days were imposed between different tests to avoid carry-over effects. [32] The order of tested mice was randomized, in terms of genotypes, in tests of social interaction, spontaneous alternation and elevated plus maze. For locomotor activity and thigmotaxis in an open field, a litter of four mice or less was tested simultaneously in four sets of open field; a litter of more than four mice was tested randomly in terms of genotype.

2.2.1 | Reciprocal social interaction—A test subject and an unfamiliar C57BL/6J mouse, as a stimulus subject, were placed in a home-cage setting with new bedding material. Under these experimental conditions, there were no resident mice and mice did not exhibit aggressive behaviors.[5,7,8,28] The assay consisted of two 5 min sessions with a 30 min interval. The total amount of time mice spent in exhibiting affiliative nonaggressive behaviors was analyzed. One *Crkl*+/− mouse exhibited a social interaction score > 3SD, which deviated from the group average in the second session. This mouse was excluded from the analysis for this test only. *Crkl*+/+, n = 15; *Crkl*+/−, n = 13.

2.2.2 | T-maze—Spontaneous alternation in the T-maze measures working memory and memory-based repetitive behavioral tendencies.[33] Three sessions were conducted over a period of three consecutive days with intertrial intervals of 0, 15, and 30 s. The number of entries to the arm of the maze omitted in the previous trial was computed against the total numbers of entries to the maze arms, which were different from the previous trials, as an index of spontaneous alternation. *Crkl*+/+, n = 15; *Crkl*+/−, *n* = 14.

2.2.3 | Elevated plus maze—This task evaluated anxiety-related behavior[5,8] in a less stressful environment than the inescapable open field. The test consisted of a single 5 min session and the time spent in the open arms of the EPM relative to the total time spent in the open and closed arms of the EPM was calculated. In addition, a number of visits in the open arms of the EPM was analyzed against the total number of visits in the open and closed arms. Two out of 15+/+ mice and three out of 14+/− mice dropped from the maze within 1 min of testing and were not included in the analysis because those data disproportionally reflect behaviors at the beginning of testing. One *Crkl*+/+ and three *Crkl*+/− mice were excluded. *Crkl*+/+, *n* = 13; *Crkl*+/−, *n* = 11.

2.2.4 | Open field test—Horizontal locomotor activity and time spent in the margin of the open inescapable field (i.e., thigmotaxis) were measured to test motor activity and an

anxiety-related behavior under a higher level of stress, respectively.[29,31] Mice were tested for 30 min. The traveled distance (cm) and time spent in the margin area of the open field were analyzed. Three out of the 15 *Crkl+/+* mice and 1 out of the 14 *Crkl+/−* mice died before this testing. *Crkl*+/+, $n = 12$; *Crkl*+/−, $n = 13$.

3 | STATISTICAL ANALYSIS

We first evaluated the normality and homogeneity of variance using Shapiro–Wilk tests and Levene's tests, respectively. When neither assumption was found to be violated, two groups or multiple groups were evaluated by *t*-tests and analyses of variance (ANOVA), respectively. The ANOVA tests were followed by Newman–Keuls post-hoc tests, if interactions were significant. When either assumption was violated, nonparametric Mann–Whitney U tests were used. A probability of ≤0.05 was considered significant. When multiple tests were applied to a dataset, the significance level was adjusted using Benjamini-Hochberg's correction. All data will be provided upon request.

4 | RESULTS

As body size and weight could nonspecifically alter behavioral phenotypes in mice, we measured the body weights at the beginning of behavioral testing at the age of 2 months. Mice did not differ in body weight at the beginning of behavioral testing (Table 1).

Next, we analyzed various behavioral data. The assumptions of normality and homogeneity of variance were not violated, except for the following cases. Normality was violated in spontaneous alternation in the T maze (Shapiro–Wilk, WT, 0 s, $p = 0.0013$; WT, 15 s, $p = 0.002$; WT, 30 s, $p = 0.0012$; HT, 0 s, $p = 0.0022$; HT, 15 s, $p = 0.0037$; HT, 30 s, $p = 0.0013$), % of time spent in the open arms of elevated plus maze (EPM) (Shapiro–Wilk, +/+, $p = 0.009$; +/−, $p = 0.0001$), and frequency of visits to the open arms of EPM (Shapiro–Wilk, +/+, $p = 0.0333$; +/−, $p = 0.0224$). These data were analyzed using nonparametric tests. In addition, data on locomotor activity violated the assumptions of normality at one time point (Shapiro–Wilk, WT, 15 min, $p = 0.0419$) as well as homogeneity of variance at one time point ($p = 0.0433$), but these particular cases did not survive the Benjamini-Hochberg correction for multiple comparisons.

Crkl+/+ and *Crkl*+/− mice were indistinguishable in terms of reciprocal social interaction (Genotype, $F(1, 26) = 0.004$, $p = 0.949$; Genotype x Session, $F(1, 26) = 0.005$, $p = 0.946$), spontaneous alternation in the T-maze (0 s, $U = 81$, $p = 0.2749$; 15 s, $U = 75$, $p = 0.1559$; 30 s, $U = 87$, $p = 0.4734$), anxiety-related behaviors in the EPM (% of time spent in the open arms of EPM, $U = 57.5$, $p = 0.4134$; % frequency of visits to the open arms of EPM, $U = 52.5$, $p = 0.2643$), and locomotor activity in the inescapable open field (Genotype, $F(1, 23) = 2.491$, $p = 0.128$; Genotype × Time, $F(5, 115) = 0.387$, $p = 0.857$). However, *Crkl*+/− mice spent less time in the margin area of the inescapable open field than *Crkl*+/+ mice (Genotype, $F(1, 23) = 5.985$, $p = 0.022$; Genotype × Time, $F(5, 115) = 0.577$, p = 0.717) (Figure 2).

5 | DISCUSSION

When placed in an inescapable open field, mice tend to explore mainly its marginal zone near the wall (i.e., thigmotaxis) and this trait is thought to reflect anxiety.[34,35] Our data showed that *Crkl*+/− mice exhibited a lower level of thigmotaxis than *Crkl*+/+ mice. In contrast, *Crkl*+/− mice were indistinguishable from *Crkl*+/+ mice in the elevated plus maze, another task that is designed to measure anxiety-related behaviors. While these two outcomes apparently contradict each other, the two tasks are thought to impose different stress levels on mice. Stress levels are generally much lower in the EPM than in the inescapable open field,[5,29,31] because there is a choice to escape from an anxiety-provoking area (i.e., open arms) to a safer zone (i.e., closed arms) in the EPM, but such a choice is absent in the inescapable open field where there is no clear divider between the center and marginal zones. In fact, blood corticosterone levels are lower when mice have a choice than when they do not.[29,30] Our data suggest that *Crkl* heterozygosity itself acts as a protective factor for anxiety or phobia-like behavior in an open field. By contrast, 22q11.2 hemizygosity carriers exhibit elevated rates of various anxiety disorders, including phobia.[14] These seemingly contradictory observations can be explained if it is assumed that other 22q11.2 genes have more robust pro-anxiety effects that override such a protective factor. This interpretation is consistent with the hypothesis that 22q11.2 CNV contains some genes whose deletion causes the opposite phenotypic effect to what is seen as a result of collective action of contributory genes.[15]

Our results show that no other behaviors differed between *Crkl*+/− and *Crkl*+/+ mice. We used a battery of behavioral assays to recapitulate various species-specific dimensional aspects of schizophrenia, ASD, ID, and anxiety disorders.[24] Reciprocal social interaction is a measure of social capacity that is defective in schizophrenia and ASD. Working memory and repetitive behavioral traits can be measured in spontaneous alternation in a T-maze. Working memory deficits are seen in patients with schizophrenia, ASD, and ID while repetitive behavior is seen in schizophrenia and ASD. Abnormal motor activities are also observed in patients with schizophrenia, ADHD and ASD. All of these dimensions deviate in 22q11.2 hemizygosity carriers, compared to noncarriers.[24] Our data do not support the idea that deficiency of *Crkl* alone in the distal region of the 3.0 Mb hemizygous deletion is a risk factor for these dimensions of psychiatric disorders.

In humans, defective PPI is seen in patients with schizophrenia and 22q11.2 deletion carriers; however, it is normal in ASD and ID patients under the standard paradigm.[24] Within the atypical distal deletion cases, several genes have been deleted and screened for some anxiety-related behaviors and PPI (see underlined genes in Figure 1). Homozygous deletion of *Scarf2, Aifm3, or P2rk6* does not alter PPI.[36] Anxiety-like behaviors are increased in mice deleted for *Scarf2*, but unaffected in mice deficient for *Klhl22, Snap29*, or *P2rx6*.[36] These effects are inconsistent with the strict interpretation of the contiguous gene effect, which states that a phenotype is caused by the collective action of all encoded genes, but individual genes alone do not have any phenotypic impact. The presence of individual driver genes, together with genes with no phenotypic consequence, suggests the existence of a mosaic-like landscape of driver genes.[15] More work is needed to fully characterize the

genetic architecture responsible for the association of atypical distal 22q11.2 deletion cases and dimensions of psychiatric disorders.

Interestingly, homozygous deletion of *Crkl* in mice recapitulates many physical abnormalities of 22q11.2 hemizygosity in humans, including craniofacial, cardiovascular, thymic, and parathymic phenotypes; however, the heterozygous deletion of this gene induced none of these phenotypes in mice.[20] A parsimonious interpretation of this observation is that *Crkl* hemizygosity does not contribute to the physical phenotypes seen in carriers of 22q11.2 hemizygosity. Another possibility is that a gene deletion might not be tolerated to the same degree in mice and humans. A higher gene dose reduction (e.g., homozygous deletion) might be needed to cause corresponding phenotypes in mice than in humans. Similarly, the homozygous deletion of *Tbx1*, another 22q11.2-encoded gene, causes a wide range of abnormalities including cardiovascular malformations, thymic aplasia, and cleft palate in mice, but its heterozygosity has no such effects.[37] Moreover, it takes the homozygous deletion of murine *Sept5*, a 22q11.2 gene encoded in the 1.5 Mb proximal region, to impair social interaction while its heterozygous deletion does not cause such an effect.[7,8]

It should be cautioned, however, that a species-specific gene dose sensitivity might not be applicable to all 22q11.2 genes and all phenotypes. The heterozygous deletion of *Tbx1* is sufficient to impair social communication, social interaction, and working memory in mice.[4-6] Such a discrepancy between physical and behavioral phenotypes questions the premise of screening genes relevant to psychiatric disorders using physical abnormalities in model organisms (e.g., *zebrafish* and *Drosophila*). This also refers to monitoring behavioral phenotypes using readouts that do not have any predictive validity for mammalian social interaction, working memory, and cognitive flexibility. Genes identified in the screening of lower species require validation in mice in order to correlate them with behavioral dimensions more relevant to dimensions of human psychiatric disorders.

Crkl is involved in the molecular pathways of FGF, FGFR, Reelin, and other related proteins.[21,38-41] It is thought to participate in dendritogenesis,[41,42] cortical, and hippocampal layer formation,[41] cerebellar development[41] and neural migration during brain development.[38,40,41] An analysis of molecular pathways in cell, organoid, and mouse models of any CNVs exhibiting correlation with psychiatric disorders suggests these biological processes as being relevant. However, the analysis using databases of molecular networks requires caution, as they might be confounded by biological pathways irrelevant to specific dimensions of mental illness and could simply aggregate known functions of all genes physically present in a given CNV. Such data may not provide genuine insights into the mechanistic basis of mental illness and could be misleading in the context of the development of novel therapeutic strategies. The literature includes many such instances where apparent anatomical and molecular hallmarks of a disorder turned out to be irrelevant.

Our data provide evidence that *Crkl* heterozygosity alone does not contribute to various dimensions of psychiatric disorders and might rather act as a protective factor for anxiety-related behavior. A corollary of this conclusion is that neural, cellular and molecular alterations caused by *Crkl* heterozygosity are unlikely to contribute to behavioral dimensions

of developmental neuropsychiatric disorders seen in 22q11.2 hemizygous deletion cases. By excluding this gene as a singly contributory one, our negative data, nevertheless, improve our understanding of the 22q11.2 CNV genetic architecture known to affect phenotypic traits in psychiatric disorders.

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DATA AVAILABILITY STATEMENT

All raw data are available upon request.

Abbreviations:

ADHD	attention deficit hyperactivity disorder
AIFM3	apoptosis inducing factor mitochondria associated 3
ASD	autism spectrum disorder
CNVs	copy number variants
CRKL	CRK-like proto-oncogene, adaptor protein
EPM	elevated plus maz
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
ID	intellectual disability
IQ	intelligence quotient
KLHL22	Kelch like family member 22
P2RX6	purinergic receptor P2X 6
PI4KA	phosphatidylinositol 4-kinase alpha
PPI	prepulse inhibition
SCARFf2	Scavenger receptor class F member 2
SEPT5	septin 5

SLC7A4	solute carrier family 7 member 4
SNAP29	synaptosome associated protein 29
ZNF74	zinc finger protein 74

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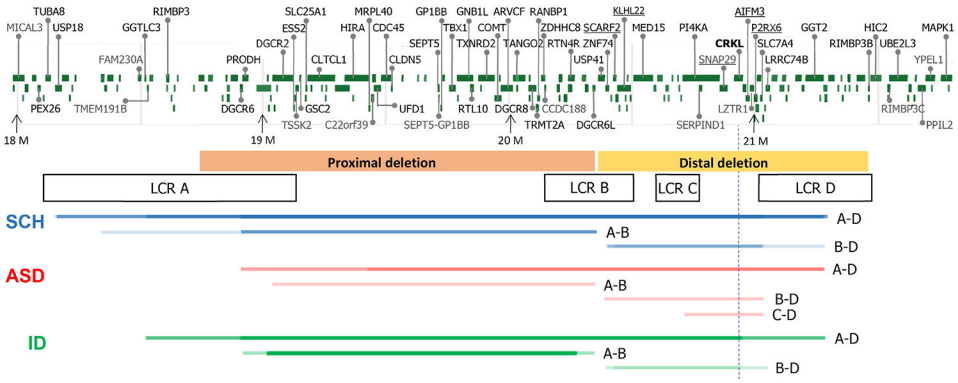

FIGURE 1.

Deletion sizes and locations of 22q11.2 CNVs. Various sizes and loci of 22q11.2 hemizygous deletion associated with schizophrenia (blue), autism spectrum disorder (red), and intellectual disability/developmental delay (green). Individual deletions are based on published data[18,22,43-53] but converted to GRCh38. The intensity of each color represents the relative frequency of variable deletion sizes. Note that *CRKL* (bold) is located in the distal end of the ~3.0 Mb deletion outside the proximal 1.5 Mb region. Low copy repeat (LCR) loci are designated as A, B, C, and D. Prepulse inhibition and anxiety-related behaviors have been variably tested in co-isogenic mice homozygous (*Scarf2, Klhl22, Aifm3, and P2rx6*) or heterozygous (*Snap29*) for the underlined distal murine homolog genes; no phenotypic abnormality is found in those models except for increased thigmotaxis in *Scarf2* homozygous mice (International Mouse Phenotyping Consortium online database as of October 15, 2020)[36]

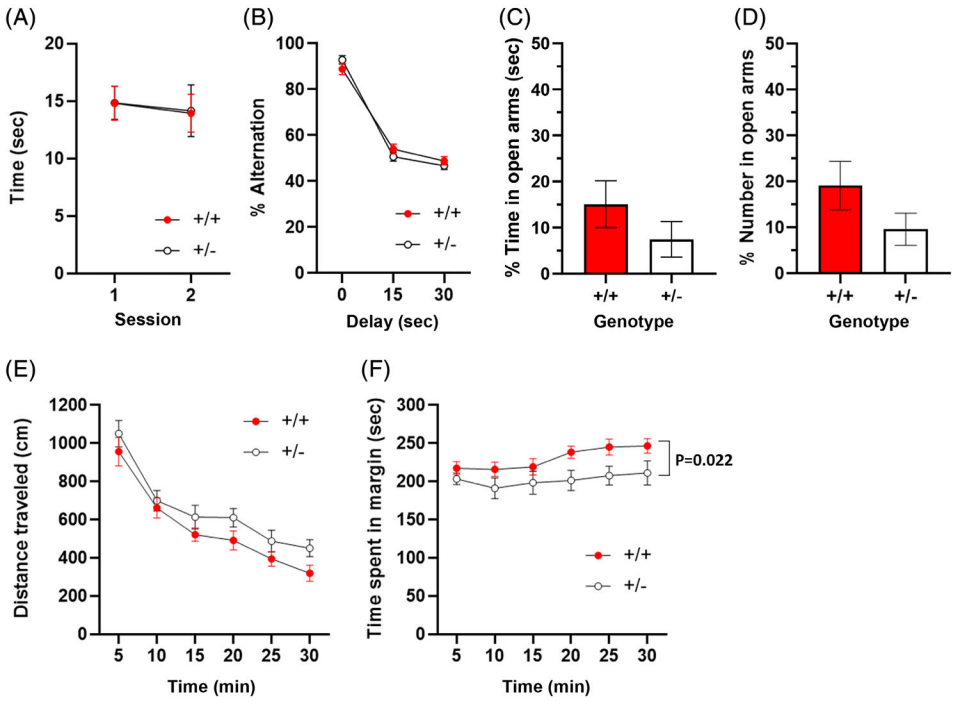

FIGURE 2.

Behavioral phenotypes. *Crkl*+/+ and *Crkl*+/− mice were tested at 2 months of age in reciprocal social interaction in a novel home cage setting (A, *Crkl*+/+, *n* = 15; *Crkl*+/−, *n* = 13), spontaneous alternation in the T-maze (B, *Crkl*+/+, *n* = 15; *Crkl*+/−, *n* = 14), % of time spent in the open arms of the EPM (C, *Crkl*+/+, *n* = 13; *Crkl*+/−, *n* = 11) and frequency of visits to the open arms of the EPM (D, *Crkl*+/+, *n* = 13; *Crkl*+/−, *n* = 11), locomotor activity (horizontal distance in cm) (E, *Crkl*+/+, *n* = 12; *Crkl*+/−, *n* = 13), and thigmotaxis (time spent in the margin areas) in the inescapable open field (F, *Crkl*+/+, *n* = 12; *Crkl*+/−, *n* = 13)

TABLE 1

Effect of *Crkl* heterozygosity on body weight

Genotype	***Crkl*+/+**	***Crkl*+/−**
Average body weight	23.03 (SEM 0.35)	22.69 (SEM 0.464)
Sample size	$N = 15$	$N = 14$

Note: $t(27) = 0.60387$, $p = 0.551$. Neither normality (+/+, W = 0.902, $p = 0.103$; +/−, W = 0.963, $p = 0.777$, $p > 0.05$) and homogeneity of variance. ($F(1, 27) = 1.1664$, $p = 0.283116$) of data was violated.