〈Regular Article〉

Effects of Social Isolation and Resocialization on Post-weaning Development in Mice

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ABSTRACT **Purpose**: Participation in social interactions throughout childhood leads to the development of social cognition and social skills essential for daily life. Abnormal social experiences during adolescence have been shown to have long-term effects on brain function and structure, and a critical period for social behavior may exist. This study investigated the effects of periods of social isolation during development and of subsequent resocialization on the sociability of mice after adulthood. Through this experiment, we attempted to identify the developmental period associated with the critical period of social behavior.

Method: Social isolation was carried out for one or two weeks during the developmental period, starting from the third week of life. Thereafter, the mice were housed in groups (resocialization) until they were 9 weeks old. The social behavior of the mice was examined after nine weeks of age.

Finding: Social isolation during the third week of life did not reduce social novelty in the mice while isolation during the fourth and fifth weeks of life resulted in a decline in social novelty that could not be recovered by subsequent resocialization.

Conclusion: This study suggests that a critical period for sociality in mice may exist at approximately 3 weeks after birth. The results of this study demonstrate the importance of early psychological interventions such as cognitive behavioral therapy.

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Key words：Behavior, Critical period, Resocialization, Mouse, Social isolation, Roden

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INTRODUCTION

Sociability begins with the mother-infant relationship and is nurtured and maintained within the social relationships between individuals and groups with family members and peers. Developing children typically exhibit a strong preference for social interactions from an early age[1, 2]. Participation in social interactions throughout childhood leads to the development of social cognition and skills that are essential for daily life[3, 4]. Moreover, participating in and interpreting social interactions are beneficial in everyday life and important for the survival of most species. Reproduction, avoidance of aggression, and mutual cooperation all depend on social competence. Social buffering and supportive social contact between highly social mammals (such as humans) can significantly benefit mental and physical health and reduce mortality risk in stressful situations[5, 6]. Therefore, the disruption of the social environment (i.e. social isolation or social instability) is associated with a range of physiological, neuroendocrine, and behavioral dysfunctions in both human and nonhuman species[7].

Postweaning and peripubertal (i.e. mild to late adolescence) social isolation in rodents has pronounced behavioral, emotional, and neurological effects that increase anxiety, aggression, and cognitive impairment in adulthood[7, 8]. Reduced sociability during this sensitive period of social behavior development contributes to impaired development of social reciprocity, cognition, and skills[9, 10]. Since sociability is critical in driving various aspects of social behavior development, there is a strong need to improve our understanding of the biological factors that influence sociability during development[11]. Many studies have shown that aberrant social experiences in youth have long-lasting effects on brain function and structure that extend into adulthood in rodents and humans[12–15]. Reactive Attachment Disorder (RAD) is caused by inappropriate parenting such as abuse or neglect. People with RADs have issues with their social and emotional responses and exhibit problematic behaviors in social and interpersonal relationships. This can be said to be one of the proofs that human sociability is developed through social experiences. Deficits in social affiliative behavior are a core symptom of neurodevelopmental and neuropsychiatric disorders such as autism spectrum disorder, social anxiety disorder, and schizophrenia[16–18].

Adolescence is a critical developmental period characterised by increased reward-seeking and impulsivity, as well as the establishment of appropriate social behaviors[19–22]. The quality and quantity of social interactions during adolescence are associated with later human behavioral outcomes, such as the rates of drug and alcohol use and the formation of healthy social relationships[23–25]. Moreover, adolescence is characterized by increased stress sensitivity, and chronic stress exposure during this period has been shown to alter brain structure and function[26, 27]. Since peer interactions are especially important during adolescence, exposure to social stress can have particularly negative effects on brain development and behavior[23, 28–30] Understanding how social stress in adolescence alters neurophysiology and behavior may prove important in treating stress-related disorders throughout adolescence and later in life.

Strategies to ameliorate or reverse the social deficits caused by social isolation are known as “resocialization” and constitute an experimental analogue of behavioral therapy[31, 32]. Many methods, including resocialization, have been reported to reduce behavioral abnormalities caused by social isolation[33]. It has been suggested that interventions such as resocialization after social isolation and enrichment following adverse experiences in adolescence can reverse brain abnormalities in rodents and humans[34–36]. The reason for the

recovery of behavioral abnormalities may be that adolescent mice have strong neuroplasticity and may be highly sensitive to resocialization[37]. However, the period required for recovery from abnormal social behavior due to social isolation and the period required for neuroplasticity to occur has not yet been clarified. Clarifying the timing of resocialization necessary to recover from behavioral abnormalities is important for elucidating the causes of human mental disorders and for early intervention treatment.

Mouse models are essential for studying the basic neurobiology of sociality because they provide experimental control and genetic resources[38]. Rodent models are widely used to investigate the neural circuitry underlying social behavior. Specifically, there are well-established assays to quantify sociability, such as the three-chamber social interaction test and reciprocal social interaction in the open field test[39, 40].

This study aimed to clarify the behavioral abnormalities that occur depending on the period of stress due to social isolation and the period of resocialization on the sociability of adult mice during the postweaning developmental period, which will lead to the discovery of the appropriate timing for psychological interventions such as cognitive behavioral therapy.

MATERIALS AND METHODS

Ethics statements

All animal experiments were performed in accordance with the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines) and the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised in 1996). This study was approved by the Committee for Animal Experiments at Kawasaki Medical School Advanced Research Centre (22-028). All efforts were made to minimise the number of animals used and their suffering. The number of animals was reduced via an experimental design, allowing statistically significant changes to be demonstrated, with the smallest number of animals per group and the smallest number of groups.

Animals

Only male mice (C57BL/6N) were used for these experiments. Since murine behavior is partially sex-dependent, and this study did not seek to compare sex differences, only male mice were included. All animal experiments were performed following the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines) and the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised in 1996), and were approved by the Committee for Animal Experiments at Kawasaki Medical School Advanced Research Centre. All efforts were made to minimise the number of animals used and their suffering. The required sample size was calculated using a power analysis. The animals were purchased from CLEA Japan (Tokyo, Japan). Transparent plastic cages (220 × 340 × 150 mm) with wire tops were used, and a nonwoven filter cap was attached to the top of the wire. The cages included the provision of nesting material with food (MF-R; ORIENTAL YEAST, Tokyo, Japan) and water *ad libitum*, under 12-h light/dark conditions (lights on at 8:00, lights off at 20:00), with a temperature maintained between 23℃ to 26℃.

Social isolation stress

The day of birth was defined as postnatal day 0 (P0). Male C57BL/6N mice were randomly divided into three groups: control, social isolation (3rd and 4th weeks of age), and social isolation (5th and 6th weeks of age) (https://www.randomizer.org). Mice in the control group were housed (5 mice/cage) in standard transparent plastic cages. Mice in the social

isolation group were housed separately in opaque plastic cages for two weeks. Thereafter, the animals were housed in groups (5 animals/cage) until 9 weeks after birth (resocialization) (Fig. 1A). Of the five mice caged for resocialization, two were mice that had experienced social isolation stress and three were group-housed.

Next, male C57BL/6N mice were randomly divided using a randomizer software (https://www.randomizer.org) into four groups: control, socially isolated (3 weeks old), socially isolated (4 weeks old), and socially isolated (5 weeks old). Mice in the control group were group-housed (5 mice/cage) in standard transparent plastic cages. Mice in the social isolation group were housed separately in opaque plastic cages for one week. Thereafter, the animals were housed in groups (5 animals/cage) until 9 weeks after birth (resocialization) (Fig. 1B). Of the five mice caged for resocialization, two were mice that had experienced social isolation stress and three

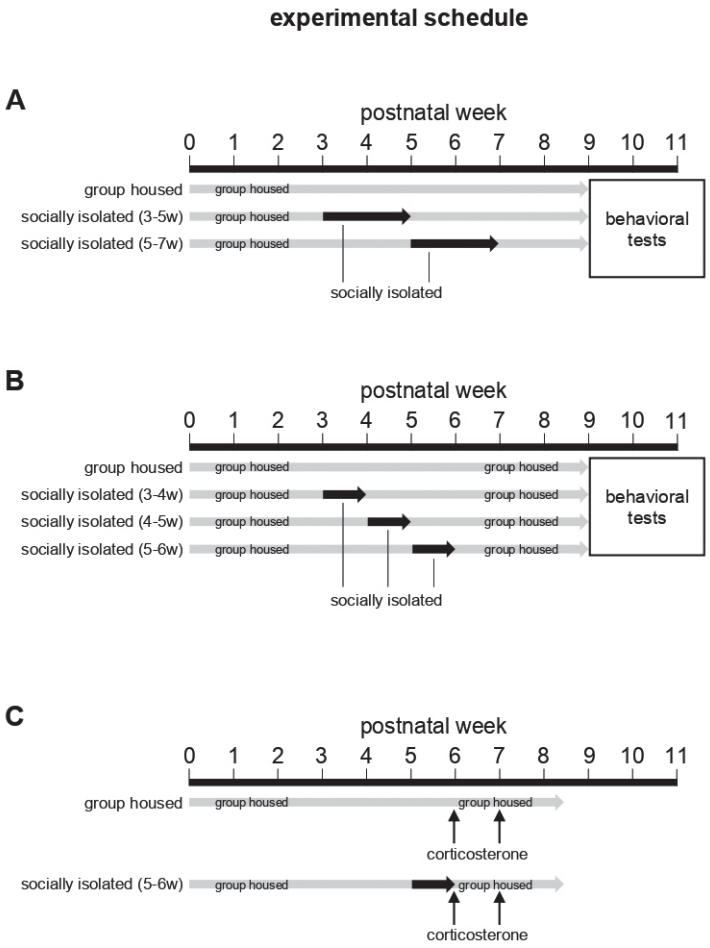

Fig. 1. Experimental Schedules

Two-week social isolation paradigm (A). The animals were kept in one cage per cage for 2 weeks during the developmental period after weaning. After 2 weeks of social isolation stress, the animals were housed in groups of 5 animals/cage. 1-week social isolation paradigm (B). The animals were kept in one cage per cage for 1 week during the developmental period after weaning. After one week of social isolation stress, the animals were housed in groups of 5 animals/cage. One-week social isolation paradigm and serum collection (C). Animals were housed singly per cage for 1 week after 5 weeks of age. After 1 week of social isolation stress, animals were housed in groups of 5 per cage. Serum was collected after 1 week of social isolation and 1 week after group housing.

were group-housed.

Behavioral tests

All behavioral tests were conducted in behavioral testing rooms between 09:00 and 16:00, during the light phase of the light/dark cycle. After these tests, the equipment and toys were cleaned with 70% ethanol and super-hypochlorous water to avoid artefacts caused by lingering olfactory cues. Behavioral tests were performed on naïve mice in accordance with the test order described below. The mice were randomly divided (http://www.randomizer.org) into two groups: demonstrator and observer (test mice). Cage mates were used as demonstration mice.

Wire Hang Test

A wire-hang test device (O'Hara & Co., Tokyo, Japan) was used for the wire-hang test. Each mouse was placed on top of a wire mesh which was turned over and gently shaken to encourage the mouse to grab the wire. Subsequently, the time until fall was recorded.

Grip strength test

Neuromuscular strength was examined using the grip strength test. Forelimb muscle strength was measured by using a grip dynamometer. Each mouse was lifted by its tail such that its front paws could grip the wire grid of the dynamometer. Subsequently, the mouse was slowly pulled back until the grid was released. The peak force exerted by the forelimbs was recorded in Newton (cN).

Hot Plate Test

A hot plate test was used to assess nociception. Mice were placed on a plate heated to 55.0 ± 0.3℃, and the latency to the first paw response was recorded. Valid responses included shaking and paw licking. A latency period of 30 s was defined as complete analgesia and was used as the cutoff time to prevent tissue damage.

Sociability and social novelty preference tests

The apparatus had a rectangular shape (45 × 45 × 40 cm). Two transparent cages (7.5 × 7.5 × 10 cm, with several holes with a diameter of 1 cm) were placed at both ends of the rectangular apparatus (Fig. 3A, 5A). Each mouse was placed in a box for 6 min and allowed to freely explore the habituation. For the sociability test, an unfamiliar C57BL/6N male (stranger), that had had no prior contact with the subject mice, was placed inside the cage. The subject mouse was placed in the centre and allowed to explore the experimental apparatus for 6 min. the number of entries and time spent by subject mice around each cage were measured to quantify the initial sociability of strangers compared to empty cages. In the next session, another unfamiliar mouse (stranger 2) was placed in a cage that was empty for the first 6-min session. The subject mouse was placed in the centre and allowed to explore the experimental apparatus for 6 min. They were given a choice between the first, previously-explored stranger mouse (stranger 1) and a new stranger mouse (stranger 2). We quantified the social novelty preference by measuring the number of entries and the time spent by the subject mice around each cage. The apparatus was cleaned after each test phase. Data was recorded on video and analysed using ANY-MAZE software.

Corticosterone measurements

The mice were anaesthetised with a lethal dose of sodium pentobarbital (120 mg/kg, intraperitoneally) and immediately sacrificed by decapitation under isoflurane anaesthesia. Trunk blood was collected in tubes and centrifuged at 0.8 × g for 10 min. Serum was collected and frozen at -80℃ until analysis. Serum corticosterone concentrations were measured using an enzyme-linked immunoassay (Cat. # K014-H5, Ann Arbor, Michigan, USA) according to

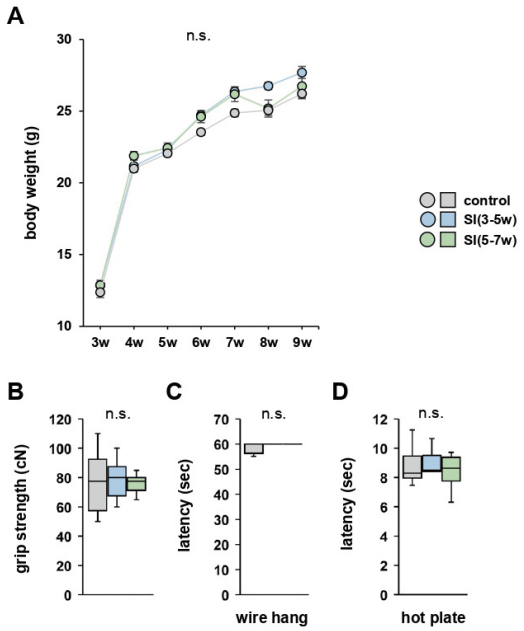

Fig. 2. Effects of two weeks of social isolation and subsequent group housing on weight gain.
(A) Measure the weight of mice weekly starting from 3 weeks of age. (B) Grip strength. (C) Latency to fall in the wire hang test. (D) Hot plate test. Data are presented as mean ± standard error (A) or box plots (B-D). control: n = 10, social isolated stress (3-5w): n = 10, social isolated stress (5-7w): n = 10. $^{*}p < 0.05$. The *p*-values were calculated using two-way repeated-measures analysis of variance (ANOVA) in (A), and ANOVA in (B-D). *SEM* standard error of the mean.

the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Normal distribution for all samples was assessed using the Shapiro-Wilk normality test prior to group analysis. Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's test, or two-way repeated-measures ANOVA followed by Fisher's least significant difference test. Data are presented as the mean ± standard error or box plots. Statistical significance was defined as $^{*}p < 0.05$, and $+ p < 0.1$.

RESULTS

Effects of social isolation stress and resocialization on body weight during two weeks of development

The weights of the mice were recorded weekly starting from the third week after birth (Fig. 2A; $F_{6, 12} = 3.335$, $p = 0.3010$, cont vs. 3-5w: $p = 0.106$; cont vs. 5-7w: $p = 0.329$). There was no significant difference in body weight between the social isolation stress group (3-5 weeks old) and the social isolation stress group (5-7 weeks old) as compared to the control mice (Fig. 2A). Neuromuscular strength (grip strength test) was compared among the control, social isolation group (3-5 weeks old), and social isolation group (5-7 weeks old) groups. There were no significant differences between the three groups (Fig. 2B; $F_{2, 28} = 0.072$, $p = 0.931$, cont vs. 3-5w: $p = 0.816$; cont vs. 5-7w: $p = 0.893$). The wire hang test was compared between the control group, the social isolation group (3-5 weeks old), and the social isolation group (5-7 weeks old). There were no significant differences in the test results between the three groups (Fig. 2C; $F_{2, 28} = 2.180$, $p = 0.132$, cont vs. 3-5w: $p = 0.067$; cont vs. 5-7w:

$p = 0.098$). Mice in the control, social isolation group (3-5 weeks old), and social isolation group (5-7 weeks old) groups were placed on a hot plate to evaluate nociception and chronic suppression of aggressive behavior due to heat pain. No significant differences in pain thresholds were observed among the three groups (Fig. 2D; $F_{2, 28} = 0.025$, $p = 0.976$, cont vs. 3-5w: $p = 0.938$; cont vs. 5-7w: $p = 0.887$).

Effects of social isolation stress and resocialization on social behavior during two weeks of development

Mice were also subjected to Crawley's sociability and social novelty preference tests, which consisted of a sociability test and a social novelty preference test. We investigated whether interest in strange mice differed between socially isolated and control groups. First, an unfamiliar mouse was placed in the cage at the end of the experimental setup (Fig. 3A). There was no significant difference in the total distance travelled among the three groups (Fig. 3B; $F_{2, 54} = 0.046$, $p = 0.955$, cont vs. 3-5w: $p = 0.759$; cont vs. 5-7w: $p = 0.848$). In the control group, there were no significant differences in the number of entries into the empty cage or around the unfamiliar mouse cage (Fig. 3C; $F_{2, 54} = 2.252$, $p = 0.115$, cont, empty vs. stranger: $p = 0.261$; 3-5w, empty vs. stranger: $p = 0.001$; 5-7w, empty vs. stranger: $p = 0.010$). The social isolation groups (3 and 4 weeks old) and the social isolation groups (4 and 5 weeks old) increased the number of times stranger mice entered the cage surroundings compared to the empty cage surroundings (Fig. 3C). The three groups spent more time around the stranger's cage than around the empty cage (Fig. 3D; $F_{2, 54} = 1.035$, $p = 0.362$, cont, empty vs. stranger: $p = 0.049$; 3-5w, empty vs. stranger: $p = 0.003$; 5-7w, empty vs. stranger: $p < 0.001$). There were no significant differences in sociability index among the three groups (Fig. 3E; $F_{2, 27} = 1.610$, $p = 0.218$, cont vs. 3-5w: $p = 0.103$; cont vs. 5-7w: $p = 0.183$).

Subsequently, a new, unfamiliar mouse was placed in the cage at one end of the experimental apparatus (Fig. 3A). There was no significant difference in the total distance travelled among the three groups in the social novelty preference test (Fig. 3B; $F_{2, 54} = 0.179$, $p = 0.837$, cont vs. 3-5w: $p = 0.588$; cont vs. 5-7w: $p = 0.978$). Furthermore, there were no significant differences in the number of entries into the familiar mouse cage and surrounding the stranger mouse cage among the three groups (Fig. 3F; $F_{2, 54} = 1.031$, $p = 0.363$, cont, familiar vs. stranger: $p = 0.946$; 3-5w, familiar vs. stranger: $p = 0.285$; 5-7w, familiar vs. stranger: $p = 0.095$). The control group spent more time in the stranger mouse cage than in the familiar mouse cage (Fig. 3G; $F_{2, 54} = 2.904$, $p = 0.063$, cont, familiar vs. stranger: $p = 0.013$; 3-5w, familiar vs. stranger: $p = 0.389$; 5-7w, familiar vs. stranger: $p = 0.536$). There was no significant difference in the time spent around the familiar and stranger mouse cages in the social isolation groups (3 and 4 weeks old) and the social isolation groups (5 and 6 weeks old) (Fig. 3G). There were no significant differences in the sociability novelty index between the three groups (Fig. 3H; $F_{2, 26} = 1.124$, $p = 0.340$, cont vs. 3-5w: $p = 0.152$; cont vs. 5-7w: $p = 0.589$).

The results of this experiment showed that two weeks of social isolation stress at the 3rd and 4th weeks of age and the 5th and 6th weeks of age decreased social novelty in mice. However, two weeks of social isolation did not reveal a critical period for sociability. Following this, we attempted to clarify the critical period of sociability by subjecting the participants to social isolation stress for one week. Social isolation was performed for one week at 3, 4, and 5 weeks after birth, followed by group housing (resocialization) until 9 weeks after birth.

Effects of social isolation stress and resocialization on body weight during one week of development

The weights of the mice were recorded weekly

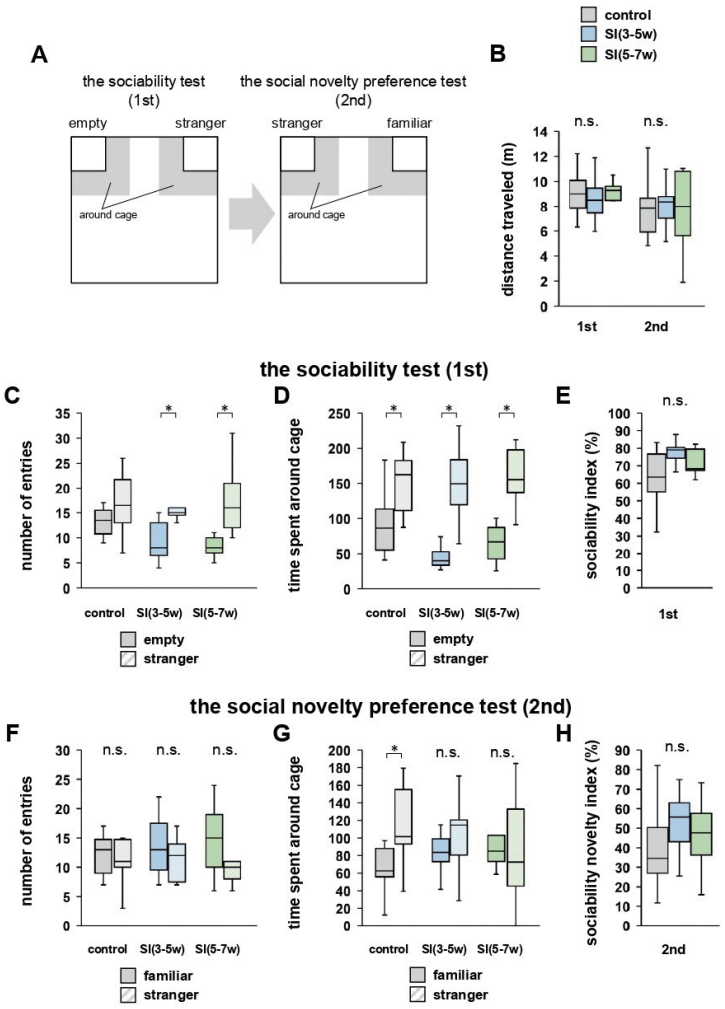

Fig. 3. Effects of two weeks of social isolation and subsequent group housing on mouse sociability.
(A) Schematic representation of Crawley's sociability and social novelty preference test. In the first session, one cage was empty and the other cage contained an unfamiliar mouse. In the second session, one cage contains a familiar mouse (an unfamiliar mouse in the first session) and the other cage contains a new unfamiliar mouse. (B) Total distance traveled in the sociability test and total distance traveled in the social novelty preference test. (C) Number of times entering around the cage in the sociability test. (D) Time spent around the cage in the sociability test. (E) Sociability index is calculated as the ratio of time spent around strange cages to time spent around all cages. (F) Number of entries into the cage surroundings in the social novelty preference test. (G) Time spent around the cage in the social novelty preference test. (H) Social novelty index is calculated as the ratio of time spent around strange cages to time spent around all cages. Data are presented as box plots (B-H). control: n = 10, social isolated stress (3-5w): n = 10, social isolated stress (5-7w): n = 10. $^{*}p < 0.05$. The *p*-values were calculated using one-way analysis of variance (ANOVA) in (B-H).

starting from the third week after birth (Fig. 4A; $F_{6, 18} = 1.135$, $p = 0.321$, cont vs. 3-4w: $p = 0.987$; cont vs. 4-5w: $p = 0.968$; cont vs. 4-5w: $p = 0.969$). There was no significant difference in body weight between the social isolation stress group (3-4 weeks old), social isolation stress group (4-5 weeks old), and social isolation stress group (5-6 weeks old) as compared to control mice (Fig. 4A). Neuromuscular strength (grip strength test) was compared among the control, social isolation (3-4 weeks old), social isolation (4-5 weeks old), and social isolation (5-6 weeks old) groups. No significant differences were

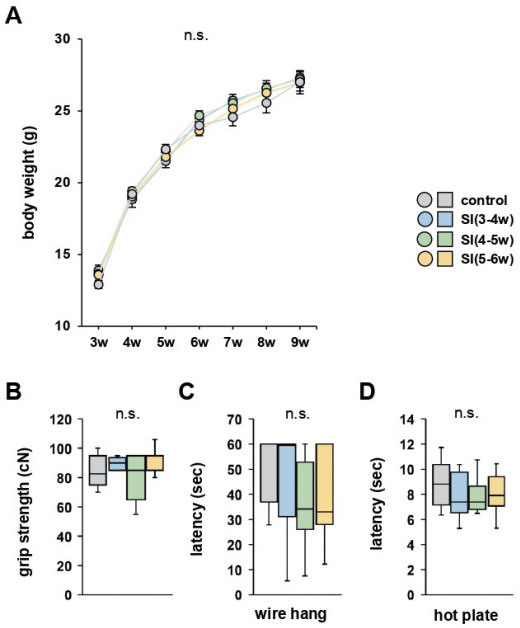

Fig. 4. Effects of one week of social isolation and subsequent group housing on weight gain.
(A) Measure the weight of mice weekly starting from 3 weeks of age. (B) Grip strength. (C) Latency to fall in the wire hang test. (D) Hot plate test. Data are presented as mean ± standard error (A) or box plots (B-D). control: n = 10, social isolated stress (3-4w): n = 10, social isolated stress (4-5w), n = 10, social isolated stress (5-6w): n = 10. $^{*}p < 0.05$. The *p*-values were calculated using two-way repeated-measures analysis of variance (ANOVA) in (A), and ANOVA in (B-D). SEM standard error of the mean.

observed between the four groups (Fig. 4B; $F_{3, 32} = 0.780$, $p = 0.514$, cont vs. 3-4w: $p = 0.289$; cont vs. 4-5w: $p = 0.688$; cont vs. 4-5w: $p = 0.642$). The wire hang test results were compared among the control, social isolation (3-4 weeks old), social isolation (4-5 weeks old), and social isolation (5-6 weeks old) groups. No significant differences were observed between the four groups (Fig. 4C; $F_{3, 32} = 0.801$, $p = 0.502$, cont vs. 3-4w: $p = 0.319$; cont vs. 4-5w: $p = 0.543$; cont vs. 4-5w: $p = 0.635$). Mice in the control, social isolation (3-4 weeks old), social isolation (4-5 weeks old), and social isolation (5-7 weeks old) groups were placed on a hot plate to evaluate nociception and chronic suppression of aggressive behavior due to heat pain. No significant differences in pain thresholds were observed among the four groups (Fig. 4D; $F_{3, 32} = 0.537$, $p = 0.661$, cont vs. 3-4w: $p = 0.271$; cont vs. 4-5w: $p = 0.287$; cont vs. 4-5w: $p = 0.389$).

Effects of social isolation stress and resocialization on social behavior during one week of development

Mice were also subjected to Crawley's sociability and social novelty preference tests, which consisted of a sociability test and a social novelty preference test. We investigated whether interest in strange mice differed between socially isolated and control groups. First, an unfamiliar mouse was placed in the cage at the end of the experiment (Fig. 5A). The total distance travelled increased in the social isolation group (5 weeks old) as compared to the control group (Fig. 5B; $F_{3, 64} = 3.861$, $p = 0.013$, cont vs. 3-4w: $p = 0.274$; cont vs. 4-5w: $p = 0.075$; cont vs. 4-5w: $p = 0.002$). The four groups had an increased number of entries into the surrounding stranger mice compared to the surrounding empty cage (Fig. 5C; cont, empty vs. stranger: $p = 0.007$; 3-4w, empty vs. stranger: $p = 0.001$; 4-5w, empty vs. stranger: $p = 0.003$; 5-6w, empty vs. stranger: $p = 0.002$). The four groups spent more time around the

stranger cage than around the empty cage (Fig. 5D; cont, empty vs. stranger: $p = 0.005$; 3-4w, empty vs. stranger: $p < 0.001$; 4-5w, empty vs. stranger: $p < 0.001$; 5-6w, empty vs. stranger: $p < 0.001$). There were no significant differences in sociability index among the four groups (Fig. 5E; $F_{3, 35} = 0.646$, $p = 0.591$, cont vs. 3-4w: $p = 0.198$; cont vs. 4-5w: $p = 0.317$; cont vs. 4-5w: p = 0.559).

Subsequently, a new, unfamiliar mouse was placed in a cage at one end of the experimental apparatus (Fig. 5A). The total distance travelled increased in the social isolation group (5 weeks old) as compared

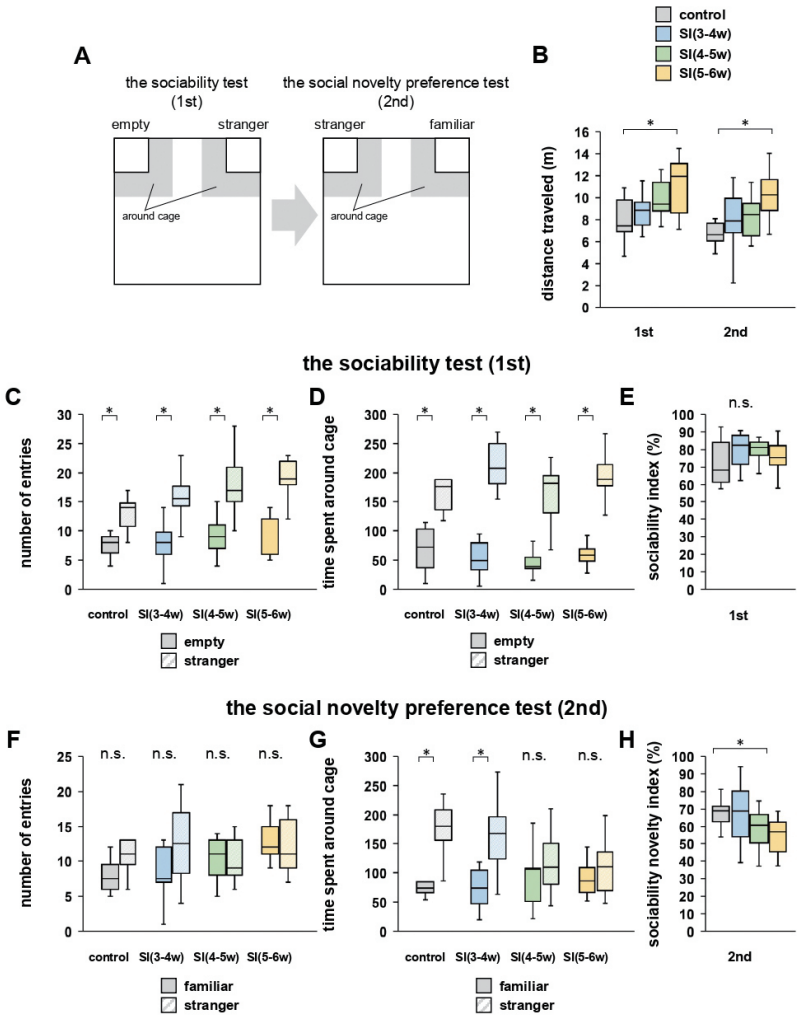

Fig. 5. Effects of one week of social isolation and subsequent group housing on mouse sociability.
(A) Schematic representation of Crawley's sociability and social novelty preference test. In the first session, one cage was empty and the other cage contained an unfamiliar mouse. In the second session, one cage contains a familiar mouse (an unfamiliar mouse in the first session) and the other cage contains a new unfamiliar mouse. (B) Total distance traveled in the sociability test and total distance traveled in the social novelty preference test. (C) Number of times entering around the cage in the sociability test. (D) Time spent around the cage in the sociability test. (E) Sociability index is calculated as the ratio of time spent around strange cages to time spent around all cages. (F) Number of entries into the cage surroundings in the social novelty preference test. (G) Time spent around the cage in the social novelty preference test. (H) Social novelty index is calculated as the ratio of time spent around strange cages to time spent around all cages. Data are presented as box plots (B-H). control: n = 10, social isolated stress (3-4w): n = 10, social isolated stress (4-5w): n = 10, social isolated stress (5-6w): n = 10. * $p < 0.05$. The p-values were calculated using one-way analysis of variance (ANOVA) in (B-H).

to the control group (Fig. 5B; $F_{3, 64} = 1.855$, $p = 0.083$, cont vs. 3-4w: $p = 0.522$; cont vs. 4-5w: $p = 0.099$; cont vs. 4-5w: $p = 0.045$). In all groups, there were no significant differences in the number of entries into the familiar mouse cage or around the stranger mouse cage (Fig. 5F; cont, familiar vs. stranger: $p = 0.069$; 3-4w, familiar vs. stranger: $p = 0.213$; 4-5w, familiar vs. stranger: $p = 0.729$; 5-6w, familiar vs. stranger: $p = 0.198$). The control and social isolation groups (3 weeks old) spent more time around the stranger mouse cage than around the familiar mouse cage (Fig. 5G; cont, familiar vs. stranger: $p = 0.001$; 3-4w, familiar vs. stranger: $p = 0.017$; 4-5w, familiar vs. stranger: $p = 0.228$; 5-6w, familiar vs. stranger: $p = 0.843$). There was no significant difference in the time spent around the familiar and stranger mouse cages between the social isolation group (4 weeks old) and the social isolation group (5 weeks old) (Fig. 5G). The sociability novelty index was lower in the social isolation group (5 weeks old) as compared to the control group (Fig. 5H; $F_{3, 35} = 2.431$, $p = 0.083$, cont vs. 3-4w: $p = 0.933$; cont vs. 4-5w: $p = 0.153$; cont vs. 4-5w: $p = 0.036$).

Effects of social isolation and group housing on serum corticosterone

To investigate the mechanism by which social novelty decreases owing to social isolation stress, we evaluated the degree of stress in mice. We investigated the effects of social isolation and group housing on the serum corticosterone levels in mice. Serum corticosterone levels were not significantly different between control and socially isolated mice (Fig. 6; 6w, cont vs. 5-6w: $p = 0.972$; 7w, cont vs. 5-6w: $p = 0.563$). Serum corticosterone levels were not significantly different between control and group-housed mice after social isolation (Fig. 6).

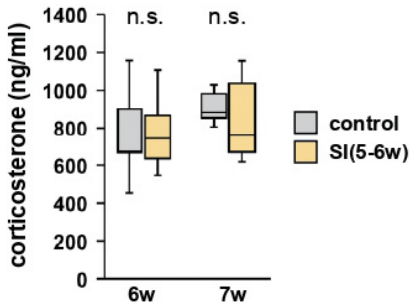

Fig. 6. Effects of 1 week of social isolation and subsequent group housing on serum corticosterone concentrations in mice Comparison of serum corticosterone levels. Data are presented as box plots. control: n = 10, social isolated stress (5-6w): n = 10, social isolated stress and group housed (6-7w): n = 10. * $p < 0.05$. The *p*-values were calculated using one-way analysis of variance (ANOVA).

DISCUSSION

In this study, we revealed that one week of social isolation stress after 4 weeks of age causes a decrease in social novelty in adult mice that cannot be recovered by resocialization. In addition, mice that were subjected to social isolation stress during the third week of life recovered their social novelty to the extent that there was no significant difference from control mice through subsequent resocialization.

Social isolation stress during the 3rd, 4th, and 5th weeks of life did not cause significant changes in body weight or muscle strength in adulthood. In previous studies, various groups have measured the metabolic outcomes of mice raised in social isolation, with mixed results. Some groups have reported that social isolation and housing stress do not affect weight gain and/or obesity in C57BL/6 mice[41, 42]. Meanwhile, other studies have reported obesity and increased fat cell size[43, 44]. Mice are typically maintained at room temperature (20-24℃), which is a comfortable temperature range for experimenters (humans). This housing environment is below the comfort temperature (29-33℃), defined as the ambient temperature at which energy is expended solely to maintain the mouse's basal metabolic rate[45–47]. Therefore, in the breeding rooms, mice typically counteract stress by

building nests and congregating for warmth. Social thermoregulation is not possible in mice subjected to social isolation stress. Therefore, mice expend additional energy to maintain their core body temperature and remain in a state of chronic cold stress[47, 48]. Exacerbating cold stress causes mice to expend additional energy to maintain their body temperature[49]. Although social isolation stress was expected to lead to weight gain, this study did not find such a result. The lack of significant differences in body weight between the groups may be due to stress relief through resocialization. Further studies on stress and weight gain are required to clarify these findings.

In the sociality test, although each group showed no abnormalities in social behaviour, the social isolation rearing stress groups showed a decrease in social novelty at 4 weeks of age only, at 5 weeks of age only, at 3 and 4 weeks of age, and at 5 and 6 weeks of age. In the social novelty recognition test, the difference in the time spent in the surroundings of strangers and familiar mice reflected the short-term memory and social exploration temperament of familiar mice. Preference for social novelty is defined as the tendency to spend more time with unfamiliar mice than with familiar mice[39]. Low interest in social novelty and excessive avoidance often occur in parallel[50]. Although the expression of preference for social novelty is thought to be intuitive, its magnitude and sensitivity can be influenced by environmental factors and the internal balance between social approach and avoidance[51]. The drive to approach and explore new conspecifics is inherent in social animals and may promote optimal social functioning. Since the exploration of novelty often involves risky behavior, approaching new stimuli may require not only the motivation to approach and explore but also the inhibition of risk aversion[52]. Therefore, there are two possible frameworks for how the balance between approach and avoidance controls selection toward social novelty. One possible explanation for this is the hedonic approach to social novelty. When a mouse encounters an unfamiliar mouse, the hedonic and/or motivational value of social novelty is strengthened to overcome risk aversion, leading to a preference for social novelty[53]. The second assumption is that individuals are inherently averse to risk. Under conditions of anxiety and social fear, inherent risk aversion is enhanced, leading to avoidance of social novelty[54]. A lack of social novelty preference is commonly associated with neuropsychiatric disorders[55]. Abnormalities in the discrimination of social cues, impaired social skills, and difficulty maintaining social relationships are characteristic features of several psychiatric disorders, neurodevelopmental disorders, and neurodegenerative diseases[56, 57]. In particular, impaired social novelty preference in adolescents has been identified as an important early marker of autism[39]. In the social isolation rearing stress group (4 weeks, 5 weeks, 3-4 weeks, and 5-6 weeks after birth), which showed a decrease in social novelty, but subsequent resocialization did not have any effect on reducing stress. The results of this study indicate that the stress of social isolation and rearing after four weeks of age can cause behavioral abnormalities that affect mice throughout their lives. Social isolation causes behavioral changes in adult rodents. Similarly, social isolation causes similar symptoms in patients suffering from neuropsychiatric disorders such as attention-deficit hyperactivity disorder, obsessive-compulsive disorder, autism, schizophrenia, and depression[58]. There is a wealth of data showing that exposure to stressful events early in life can increase the risk of mental illnesses such as mood and anxiety disorders[59–61].

The basic maturational task of adolescence is the acquisition of social competence, and the main focus of social motivation is peer acceptance and integration[62, 63]. Stable positive social contact with

peers is a prerequisite for satisfying the need to belong, a core social motive thought to underlie a variety of social behaviors[64, 65]. Colonial species have complex social structures and stable bonds play important roles in maintaining their health, strength, and survival[8, 66]. The neural basis of the effects of stress during development is poorly understood, and stressful events are thought to cause structural and functional impairments in brain regions responsible for human emotional behavior[67–69]. Many brain regions are thought to be involved in social affiliative behaviors, including the amygdala, hippocampus, prefrontal cortex, and anterior cingulate cortex[70–72]. Many studies have shown that social isolation in young mice causes abnormalities in social behavior and structural changes in the brain after adulthood[73]. Social buffering plays an important role in psychological and physiological well-being[74–76]. Resocialization in humans and mouse models is evidenced by restored myelination, normalized behavior, and improved cognitive performance[31, 34]. In this study, we showed that behavioral abnormalities did not improve after social isolation and rearing stress experienced after 4 weeks of age, even after subsequent resocialization. Individual brain regions are highly malleable in response to environmental stimuli during the early stages of development, known as critical periods. These critical periods are temporally staggered across the brain, with the primary sensory areas of the neocortex maturing earlier than areas of higher-order integration such as language[77]. After the critical period, plasticity decreases significantly and learning becomes more difficult[78, 79]. Although the existence of a critical period of sociality in mice has not yet been clarified, this study suggests that this period of sociality in mice may end by the fourth week of life.

Murine and human developmental timelines are not linearly correlated[80]. Postnatally, during the first month, mice mature approximately 150 times faster than humans. Subsequently, between one and six months of age, this ratio decreases to approximately 45 times faster. Murine adulthood, typically reached between three and six months, is considered developmentally analogous to human adulthood between 20 and 30 years of age. While not empirically validated, it is posited that murine development between one and two months corresponds to human adolescence, roughly equivalent to 12 to 17 years of age[81]. Specifically, the third and fourth postnatal weeks in mice are often considered comparable to human ages of 8-10 and 10-12 years, respectively, the latter aligning with early adolescence[82, 83]. Extrapolating these findings to humans, this research suggests a potential critical period for social development around 10-12 years of age.

Animals typically exhibit several physiological responses to chronic stress[84]. Corticosterone is an important stress marker in nonhuman mammals. Serum corticosterone levels are commonly measured to detect stress responses in mice. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis[85]. In this study, there was no significant difference in serum corticosterone concentration between the control and experimental groups, either during social isolation stress or during subsequent group housing. Previous studies have also reported that social isolation in adult mice causes a decrease in corticosterone levels[86]. Reduced adrenal activity in response to chronic stress has also been reported in animal models of social defeat[87]. Interestingly, it has been reported that rats with decreased serum corticosterone levels exhibit increased anxiety-like behavior[88]. Results from previous studies and the present study suggest that social isolation stress may be a suitable model for studying behavioral and molecular changes associated with anxiety-like behaviors[89]. However, further research is required to clarify how social isolation and group housing influence the decline in social novelty.

The use of only male mice in our study is a limitation of our study. Further studies are required to determine whether similar results can be obtained in female mice. In this study, behavioral experiments were conducted after 9 weeks of age to clarify behavioral abnormalities in mice after maturation. Therefore, the period of resocialization in each group was different. Further research is needed to extend the period of resocialization for groups with behavioral abnormalities that may improve behavioral abnormalities.

CONCLUSION

This study showed that the stress of social isolation rearing during the third week of life improves behavioral abnormalities through resocialization. The stress of social isolation and rearing after 4 weeks of age showed that behavioral abnormalities did not improve, even with resocialization. This result suggests that a critical period for sociality in mice may exist at approximately 3 weeks after birth.

LIST OF ABBREVIATIONS

ANOVA, analyses of variance

DECLARATIONS

Authors' contributions

All authors had full access to all study data and take full responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: S.Mori., H.Ueno., M.Okamoto., and T.Ishihara. Acquisition of data: S.Mori, H.Ueno, Y.Tkahashi, S.Murakami and E.Kitano. Analysis and interpretation of data: S.Mori, H.Ueno and Y.Takahashi. Drafting of the manuscript: S.Mori, H.Ueno and M.Okamoto. Critical revision of the manuscript for important intellectual content: S.Murakami, K.Wani, M.Tetsuji, Y.Matsumoto, and T.Ishihara. Statistical analysis: S.Mori, H.Ueno and Y.Takahashi. Study supervision: M.Okamoto and T.Ishihara.

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Competing interests

The authors declare no competing interests.

Availability of data and materials

The dataset is available on reasonable request from the corresponding author.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All animal experiments were performed in accordance with the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised in 1996) and approved by the Committee for Animal Experiments at the Kawasaki Medical School Advanced Research Centre.

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