Epitranscriptomic mediators of environmental impacts on mouse behaviours

Momoe Sukegawa

Contents

Abstract

Experiencing the surrounding environment plays an essential role in building our diverse behavioural characteristics based on the dynamic interactions between our genetic factors and environmental factors. However, it is a major challenge for neuroscience to elucidate how this connection is biologically implemented in the nervous system. In this thesis, I aim to test the impacts of neuronal epitranscriptomic regulation on animal behaviours in response to surrounding environments. In Chapter 2, I demonstrate that environmental factors such as enriched environment housing, social isolation housing, and enrichment removal widely impact animal behaviours. In Chapter 3, I investigate the role of epitranscriptomic regulation on gene-environment interaction by using a loss-of-function genetic approach in mice. In Chapter 4, I reveal that m^6A reader YTHDF3 conditional knockout mice showed altered behaviours in their home-cage environment. In Chapter 5, I further investigate the behavioural difference between wild-type mice and m^6A readers YTHDF3 conditional knockout mice and YTHDF1 conditional knockout mice. These studies contribute to our better understanding of how epitranscriptomic regulation may mediate the environmental impact on animal behaviours.

Abbreviations

cKO:	Conditional knock-out
cWT:	Conditional wild-type
EE:	Enriched environment housing
ER:	Enrichment removal
KO:	Knock-out
m^6A:	N6-methyladenosine
mRNA:	messenger RNA
NAc:	Nucleus accumbens
RFID:	Radio frequency identification
SI:	Social isolation housing
ST:	Standard housing

Chapter 1

General introduction

Gene-environment interaction is an essential concept for understanding our diverse behavioural characteristics and capability to adapt to our surrounding environments beyond a simple dichotomy “nature versus nurture”. Our biological systems are built, maintained, and modified by our diverse genetic factors under influence of diverse environmental factors (Briley & Tucker-Drob, 2014; Dick, 2011). In a rapidly changing world with faster and more complicated social demands, elucidating the mechanisms of how environmental factors impact our behaviour and mental health not only contribute to our better basic understanding of behaviour but also to potentially better therapeutic engagement for people showing maladaptive behaviours in specific environments. However, delineating the biological and psychological links between specific environments or specific genetic factors and an individual's behaviour remains challenging (Baumert et al., 2017; Lambert et al., 2019; Yap & Greenberg, 2018).

To address this challenge, here I focus on the epitranscriptomic regulatory mechanisms of gene expression in the brain within the context of gene-environment interaction. The brain is our central organ for processing signals from external and internal world and producing behaviours. In other words, our neural network integrates incoming information of all modalities, and the network itself undergoes corresponding structural and functional alterations (e.g. rewiring) in response to stimuli (Bennett et al., 2018). To induce persistent changes in the neural network, *de novo* transcription and translation (that is, gene expression) in a neural activity-dependent manner (that is, internal responses reflecting environmental inputs) are indispensable (Yap & Greenberg, 2018). Previous research has shown that epigenetic modification on DNA or histone plays an eminent role in regulating environment-dependent gene expression (Feil & Fraga, 2012). Still, epigenetic regulatory mechanisms can neither explain quick responses that occur within minutes upon

a stimulus, nor expression responses that are modulated at the RNA/ translational level. The post-transcriptional mechanisms underlying this process are yet incompletely understood.

Recently, as a previously unknown post-transcriptional (epitranscriptomic) regulatory mechanism that is potentially impactful for dynamic gene expression, a new study field of RNA chemical modifications has been paid strong attention to. One of the most abundant RNA modifications in the mammalian brain is N6-methyladenosine (m^6A); a methylated adenosine of RNA at N6 position. It is known that m^6A regulates gene-expression by affecting RNA properties and metabolism such as RNA structure, alternative splicing, alternative polyadenylation, nuclear export, translation, degradation, stabilisation, and phase-separation (reviewed in He & He, 2021; Murakami & Jaffrey, 2022; Oerum et al., 2021; Shi et al., 2019). m^6A was discovered in the 1970s to exist in messenger RNAs (mRNA) in Novikoff rat hepatoma cells (Desrosiers et al., 1974) and in mouse L cells (Perry & Kelley, 1974) using chromatography. But its transcriptome-wide distribution was only revealed at the beginning of 2010s upon the advancement of next-generation sequencing technology. m^6A entered spotlight again because the m^6A landscape on RNAs revealed high prevalence and conservation suggesting it is functional (Dominissini et al., 2012; Meyer et al., 2012). More than 25% of transcripts in the mammalian transcriptome are estimated to contain m^6A and this number is even higher in the brain up to 50% (Merkurjev et al., 2018). The biotypes RNAs including mRNA, transfer RNA (tRNA), ribosomal RNA (rRNA) microRNA, long non-coding RNA (lncRNA) have m^6A sites on them (reviewed in He & He, 2021; Murakami & Jaffrey, 2022; Oerum et al., 2021; Shi et al., 2019). Many m^6A sites are identified within the consensus motif DRACH (D = G/A/U, R = G/A, H = A/U/C) around the stop codon and 3' UTR (Linder et al., 2015).

With the strong attention to the epitranscriptomic field, regulatory proteins and

their roles on the m^{6}A pathway have been rapidly characterised within the past 10 years. m^{6}A is reversible and readable. For the biogenesis of modified adenosines, a methyl group is installed onto specific sites of RNAs by m^{6}A methyltransferases called m^{6}A "writers". A well-known m^{6}A RNA methyltransferase complex in the nucleus use METTL3/METTL14 as the core subunits. METTL3 (methyltransferase 3) is the catalytic core subunit of this complex (Bokar et al., 1994, 1997). METTL14 (methyltransferase-like protein14) is non-catalytic but facilitates catalytic efficacy by providing structural support to METTL3 (Liu et al., 2014; Ping et al., 2014; Y. Wang et al., 2014). The functional cooperation between METTL3 and METTL14 is well demonstrated by structural dynamics studies (Śledź & Jinek, 2016; P. Wang et al., 2016; X. Wang et al., 2016). Knocking out either METTL3 or METTL14 results in dramatic reduction of m^{6}A modification (Bawankar et al., 2021; Knuckles et al., 2018; Ping et al., 2014; Yue et al., 2018). Important factors responsible for m^{6}A reversibility are m^{6}A demethylases, called m^{6}A "erasers". FTO (fat mass and obesity-associated protein), which is the alkB family of non-heme Fe(II)/α-ketoglutarate (α-KG)-dependent dioxygenases, and ALKBH5 (alkB homolog 5) is a well-known RNA demethylase in the nucleus (Jia et al., 2011; Zheng et al., 2013). These proteins eliminate m^{6}A residues in RNA through oxidation processes (Jia et al., 2011; Zheng et al., 2013). Compared to writers and erasers, many proteins belong to the "readers" category as m^{6}A regulators, suggesting versatile "interpretation" choices of the modified transcriptome. One important class of m^{6}A readers is YTHDF (YT521-B homology domain family) in the cytoplasm. The YTH (YT521-B homology) domain has a m^{6}A -binding pocket of aromatic cage residues (Jones et al., 2022; F. Li et al., 2014; Luo & Tong, 2014; Theler et al., 2014; Xu et al., 2014). YTHDF1, YTHDF2, and YTHDF3 are known members of the family sharing a single highly conserved YTH domain. Previously, it had been considered that YTHDF1, YTHDF2, and YTHDF3 have distinct roles: YTHDF1

enhances RNA translation by recruiting translation initiation factor complex 3 (eIF3) to the modified RNA molecules and promote ribosome loading (Rauch et al., 2018; X. Wang et al., 2015). YTHDF2 recruits carbon catabolite repression 4 (CCR4)- negative on TATA-less (NOT) deadenylase complex and induces RNA degradation (Du et al., 2016; Rauch et al., 2018; X. Wang et al., 2014, 2015), and further mediates RNA endoribonucleolytic cleavage and RNA degradation by recruiting RNase P/MRP (endoribonucleases) (Park et al., 2019). YTHDF3 may affect both RNA translation and RNA degradation possibly by cooperating with YTHDF1 and YTHDF2 (A. Li et al., 2017; Shi et al., 2017), or enhance RNA translation by cooperating with PABP1 and eIF4G2 (Y. Zhang et al., 2019). In contrast, studies by Kontur et al. (2020), Lasman et al. (2020), Zaccara & Jaffrey (2020) have suggested redundant functions of YTHDF1, YTHDF2, and YTHDF3 in triggering RNA degradation. More recently, Flamand et al. (2022) showed that the RNA targets of YTHDF1, YTHDF2, and YTHDF3 are shared, and many RNAs may be bound by more than one YTHDF reader proteins through their lifetime. Furthermore, YTHDF2 may have a more prominent role on RNA degradation than YTHDF1 and YTHDF3.

m^6A epitransctiptomic regulation is known to be related to brain and synaptic functions in neural activity-dependent manners. m^6A -sequencing revealed that synapse-enriched m^6A -modified RNAs are associated with neuronal function (Merkurjev et al., 2018). In post-synapses under NMDA or KCl stimulation, co-localization of m^6A -modified RNA with m^6A eraser ALKBH5, m^6A reader YTHDF1, or YTHDF3 increases (Martinez De La Cruz et al., 2021). In Mettl3 knock-out (KO) neurons, activity-dependent immediate early gene cfos showed blunted expression level and less associated with neuronal activity (Z. Zhang et al., 2018). Flamand & Meyer (2022) shows that m^6A readers YTHDF2 and YTHDF3 mediate RNA localization in neurons. Functionally, in cultured neurons stimulated by KCl,

m^{6}A reader YTHDF1 facilitates translation of m^{6}A -modified RNA, thus enhancing *de novo* protein synthesis (Shi et al., 2018). YTHDF1 or YTHDF3 knockdown led to reduced synaptic transmission, decreased surface expression of AMPA receptors, and altered morphology of dendritic spines in cultured hippocampal neurons (Merkurjev et al., 2018). Furthermore, *in vivo* studies have provided evidence on the role of m^{6}A in brain functions. Knocking-out m^{6}A writer METTL3, eraser FTO, or reader YTHDF1 causes memory deficit in mice (Shi et al., 2018; Walters et al., 2017; Widagdo et al., 2016; Z. Zhang et al., 2018). Stressful stimuli such as restraint stress paradigm changed the global level of m^{6}A in medial prefrontal cortex (mPFC) and amygdala in a region-specific manner in mice (Engel et al., 2018). The stress-related corticoid pathway possibly affects the expression levels of m^{6}A writers, erasers, and readers including Ythdf3 (dos Santos Guilherme et al., 2021; Engel et al., 2018; Yan et al., 2022; Engel et al. reported that increased Ythdf3 mRNA level under glucocorticoid receptor agonist dexamethasone treatment in blood; Yan et al. reported that decreased YTHDF3 protein level under chronic restraint stress in the prefrontal cortex). Enriched environment housing (EE) paradigm, which provides animals with abundant environmental stimuli, increased the global level of m^{6}A in the brain (Qu et al., 2022). Moreover, in human, individuals with mono-allelic loss of Ythdf3 show neurodevelopment disorder (Terkelsen et al., 2022). These findings strongly suggest that the m^{6}A epitranscriptomic regulation serves as a promising molecular system for activity-dependent brain plasticity in response to environmental stimuli. However, although molecular functions of the reader proteins have been well demonstrated, what behavioural consequences caused by missing one of the regulators in specific type of neurons requires further exploration.

In this thesis, I aim to explore the impacts of epitranscriptomic regulation on animal behaviours in response to the dynamic surrounding environment. As the first step, in

Chapter 2 “Environmental factors have critical impacts on animal behaviour”, I set up housing environments of animals and tested behavioural impacts of them. I applied standard housing (ST), enriched environment housing (EE), social isolation housing (SI), and enrichment removal (ER) to wild-type BALB/c mice. The effects of housing environments were explored by behavioural tests covering multiple functional domains to capture the consequences of multimodal enrichment or de-enrichment. Here I demonstrated that housing environments impact a wide range of animal behaviours, possibly via multi-layered biological processes.

Chapter 2

Environmental factors have critical impacts on mice behaviours

2.1 Introduction

In this chapter, I tested the role of environmental factors on animal behaviour. Our surrounding environments critically shape and modify our behaviour. However, it remains an ongoing challenge to elucidate the connections between a set of behaviours and a specific environment.

Rodents allow researchers to use carefully controlled environments and invasive approaches to explore underlying biological processes. In the laboratory standard housing (ST), animals are housed in small cages with a few cage-mates. Social isolation housing (SI) is also often used in laboratory: animals are singly housed in small cages without social contacts. It is known that SI induces hyperactivity and anxiety-like behaviours in rodents (Walker et al., 2019). In contrast, enriched environment housing (EE) is a housing condition which provides rich stimuli over multiple modalities to animals. Although various EE protocols are being used, EE typically consists of a variety of toys such as running wheels in larger cages. It has been shown that EE enhances learning and memory, decreases anxiety-like behaviour, facilitates motor function, and alters communication patterns in animals, for example, increases fighting behaviour (Gubert & Hannan, 2019; Kempermann, 2019; McQuaid et al., 2012; Nithianantharajah & Hannan, 2006). Recently, enrichment removal (ER) housing paradigm to study the effects of negative environmental changes has also attracted attention. When housed in EE and subsequently transferred to ST, thus under ER situation, animals showed stressed-like behaviour and altered corticosterone response (Morano et al., 2019; Smith et al., 2017). Thus, the ER paradigm can be potentially used for studying the effect of negative change of surrounding environments. However, the behavioural effect of ER is underexplored, especially from the aspects of social domains despite their scientific and societal importance.

In this study, I investigated in detail the impact of EE, SI, and ER on animal's behaviours. Previous studies of housing manipulations have paid more attention to emotional and cognitive effects such as learning and memory. In the current study, I also included sensory function, motor function, activity levels, social activities, and stress-coping strategy. This approach allows to consider multiple functional domains involved in the production of behaviour to be considered in a coordinated manner, and to capture the consequences of multimodal enrichment or de-enrichment. Also, here I utilized BALB/c strain mice. BALB/c mice carry a known single-nucleotide polymorphism (SNP) in tryptophan hydroxylase 2 (TPH2) that reduces the enzymatic activity of TPH2 in synthesizing serotonin (X. Zhang et al., 2004). Possibly due to this SNP and reduced serotonin producing capacity, BALB/c mice are considered an overly sensitive strain to the surrounding environment when compared to other mouse strains such as C57BL/6 (Osipova et al., 2009). Thus, they could serve as a research model system representing sensitive human populations.

2.2 Materials and methods

I performed two experiments. In Experiment 1, I explored the effect of long-lasting EE and SI in BALB/c mice. Animals were housed in ST, EE or SI from 3 weeks postnatal until the end of 11 weeks of age. Subsequently, the behavioural test battery was performed on these three groups. In Experiment 2, I explored the effect of acute ER on animals' social interaction patterns. Animals were housed in ST or EE from postnatal 3 weeks until the end of 11 weeks of age. Subsequently, both groups of animals were transferred to ST* (with similar numbers of cage mates and cage size to ST but equipped with video cameras) and kept in ST* for 14 days. Animals' activity level and social behaviour in ST*, especially aggressive behaviour, were measured. Subsequently, I conducted a behavioural test battery. Because of serious animal fighting, I limited the battery to open-field, Crawley's social

interaction and tail suspension tests from ethical perspectives.

2.2.1 Animals

Postnatal days 21–22 (P21–22) weaned male BALB/cCrSlc mice were purchased from Japan SLC (Shizuoka, Japan). Upon receival, animals were randomly assigned to each group for experiments. For stranger animals of the Crawley's social interaction test, postnatal 6- to 7-week-old male C57BL/6NCrSlc mice were purchased from Japan SLC (Shizuoka, Japan). The animal experiments were conducted in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan approved by the Committee on Animal Experimentation of Kyoto University (#42-5).

2.2.2 Housing

Animals were housed in specific-pathogen-free rooms with 12 h of light–dark cycle and fed ad libitum.

ST animals were reared in standard-sized cages (W 234 mm, D 373 mm, H 140 mm, four mice/cage for Experiment 1, three mice/cage for Experiment 2, 220–290 cm^2/mouse) from P21–22 until the end of 11 weeks of age. I reduced the number of animals to three per standard cage in Experiment 2 for better video tracking and behavioural monitoring. No environmental enrichment including hiding spaces was provided. EE animals were reared in open-top arenas (W 900 mm, D 1200 mm, H 450 mm, 20 mice/cage for Experiment 1, 23 mice/cage for Experiment 2, 470–540 cm^2/mouse) from P21–22 until the end of 11 weeks of age (see Figure 1a and Figure 1b). I followed Slater & Cao (2015) to set up objects in EE with additional wooden logs and metal mesh toys. In this protocol, EE has two cages inside to provide water and food to animals. Plastic toys (hollow balls, small arch shelters, small

square shelters, big square shelters, and two-layered shelters) and saucer wheels were obtained from Bio-Serv (New Jersey, USA). Plastic tubes (37–45 mm in diameter) and big metal running wheels were purchased from Sanko (Osaka, Japan). The objects in EE were rearranged and cleaned weekly. SI animals were reared in standard cages (W 234 mm, D 373 mm, H 140 mm, one mouse/cage, 870 cm^2/mouse) from P21–22 until the end of 11 weeks of age. All groups of animals were housed with bedding materials made of white paper.

After the above housing manipulation, during the behavioural test battery in Experiment 1, ST animals and SI animals were housed in standard size cages (W 140 mm, D 265 mm, H 105 mm, four mice/cage for ST animals, one mouse/cage for SI animals). EE animals were housed in semi-EE cages (W 310 mm, D 475 mm, H 295 mm, four mice/cage, 370 cm^2/mouse) to proceed experimental procedure smoothly and avoid stressful situations from being chased by the experimenter in the wide EE arena during behavioural tests. Semi-EE cages contained two saucer wheels, a feeding cage, one or two wooden logs and several plastic toys and tubes also used for EE (see Figure 1c). The schedule to rearrange and clean objects in semi-EE cages was the same to EE.

In Experiment 2, animals housed in ST and animals housed in EE for 9 weeks before they were transferred to ST* (different type standard cage; W 160 mm, D 265 mm, H 300 mm, three mice/cage, 140 cm^2/mouse) with the different type bedding material (pellets of black recycled paper) and ceiling illumination for video recording (O'HARA, Tokyo, Japan). No environmental enrichment including hiding spaces was provided. Animals were housed in ST* during the behavioural test battery.

2.2.3 Procedure

In Experiment 1, the behavioural test battery was conducted on ST, EE and SI animals

(Figure 2a). This test battery included the open-field, Y-maze, light–dark box, elevated-plus maze, rotarod, hot plate, Crawley's social interaction, Porsolt swim, pre-pulse inhibition, Barnes maze, fear-conditioning and tail suspension tests (see Table 1). I performed one behavioural test or trial per day. General health and neurological examination were conducted at the beginning of the test battery, and all experiments were finished before the animals reached 26 weeks of age. The behavioural test battery was performed during the last 7 h of the light cycle. I excluded animals from tests when animals were ailing and not appropriate for conducting behavioural tests from ethical perspectives.

In Experiment 2, animals were transferred to ST* after 9 weeks of ST or EE housing and video recorded for cage activities in ST* 14 days, 24 h a day (Figure 13a). ST* cages were changed in the light phase on day 6 and on day 13. At the beginning and ending of ST*, individual animals were constrained in a transparent cylinder (25 mm in diameter) for a few seconds for their tails to be photographed on both dorsal and ventral sides. These photos were later analysed for numbers of wounds as physical evidence of animal fights. Such evidence was also used for ranking animals in the social structure. Subsequently, I performed the select behavioural test battery (the open-field test, Crawley's social interaction test, and tail suspension test) after handling during the last 7 h of the light cycle (see Table 1). I performed one behavioural test or trial per day.

2.2.4 Behavioural experiments

2.2.4.1 General health and neurological examination

General health and neurological examination were performed prior to the test battery. The first day test included rectal temperature, body weight, whisker state, coat state, righting reflex, whisker twitch, ear twitch, reaching and wire hang test. The second day tests included grip strength and epilepsy tests.

In the righting reflex test, animals were lifted up and landed on the floor from their dorsal side. When the righting reflex was normal, animals turned their body and landed from their ventral side. In the whisker twitch and ear twitch test, animals' whiskers or ears were touched gently by a cotton applicator. When twitching was normal, animals' whiskers or ears moved quickly. In the reaching test, animals were lifted up by gripping their tails. When reaching was normal, animals reached for nearby objects which they could see. The wire-hang test was performed with a wire mesh box (O'HARA, Tokyo, Japan). The wire mesh (W 100mm, D100mm, 300mm above the floor) was inverted after animals gripped it, and the latency to fall was recorded (max 60 seconds). Grip strength was measured by a Newton scale with a small wire mesh (W 35 mm, D 43 mm, O'HARA, Tokyo, Japan). Animals gripped the mesh by their forelimbs, and they were gently pulled with their tails until they could not grip it. The best score from three trials was recorded. Epilepsy test was performed with the experimenter juggling a bunch of keys for 2 seconds above animals to test the possibility of seizures (acute convulsion, rigidity, or faint) caused by loud sounds.

2.2.4.2 Open-field test

The open-field test was performed in an arena of W 407 mm, D 407 mm and H 305 mm illuminated at 100 lux (Accuscan Instruments, Ohio, USA), and animals could freely explore the arena for 30 min. Animals were positioned in the front left corner of the arena to start experiments. The total distance travelled, time spent in the centre area (central 30% area) and the number of vertical activities were recorded. For analysis, I calculated the summation of locomotion activities in each 5-min block.

2.2.4.3 Y-maze

The Y-maze was performed in an apparatus (O'HARA, Tokyo, Japan) with three arms arranged at 120° intervals (length: 400 mm, height: 120 mm, lower bottom width: 30 mm,

upper bottom width: 120 mm; illuminated at 100 lux) for 5 min. The total distance travelled and alternation rate (the number to enter all three arms within three entries/ [the total number of entries into arms] -2) were recorded.

2.2.4.4 Light–dark box test

The light–dark box test was performed in an arena of W 405 mm, D 200 mm, H 249 mm (O'HARA, Tokyo, Japan), for 10 min. The arena was equally divided into two chambers separated by a wall: a bright white-coloured chamber with ceiling illumination (550 lux) and a dark black-coloured chamber without illumination. The wall has a hole (W 50 mm, D 30 mm) that allowed animals to move freely between two chambers. Animals were positioned in the dark chamber when the experiment was started and allowed to move freely during the test. The total distance travelled, time spent in each chamber and the latency to enter the light chamber were recorded.

2.2.4.5 Elevated-plus maze

The elevated-plus maze was performed in an arena (O'HARA, Tokyo, Japan) which has four arms (W 50 mm, D 250 mm, 550 mm above the floor; two with 2-mm ledges and the other two with 150-mm transparent walls) and a centre area (W 50 mm, D 50 mm), for 10 min. The experimental room was illuminated at 100 lux. Animals were positioned in the centre area when the experiment was started and allowed to move freely during the test. The total distance travelled, time spent in each arm and the number of entries to arms were recorded.

2.2.4.6 Rotarod test

The rotarod test was performed with a rotarod apparatus (Ugo Basile, Varese, Italy). Six trials (3 trials/day × 2 days) were conducted. The testing room was illuminated at 100 lux. Animals were positioned on a rotating rod (4 rpm, 30 mm in diameter) when the

experiment was started. Speed of rotation was gradually accelerated from 4 to 40 rpm over 5 min. The latency to fall was recorded (max 300 s).

2.2.4.7 Hot plate test

The hot plate test was performed with a hot plate apparatus (W 255 mm, D 255 mm; Columbus Instruments International., Ohio, USA). The experimental room was illuminated at 100 lux. Animals were positioned on a 55°C hot plate. Animals moved freely on the hot plate during the test. The latency to the first foot shake or paw lick was recorded (max 16 s).

2.2.4.8 Crawley's social interaction test

The Crawley's social interaction test was performed in an arena consisted of three chambers (W 200 mm, D 400 mm, H 300 mm each; O'HARA, Tokyo, Japan). The walls of chambers were transparent and had holes (W 50 mm, D 30 mm) which allowed animals to move freely among the three chambers. Two small wire cages (the shape of bottom was 1/4 circle of 100 mm radius, H 100mm) for stranger male C57BL/6N mice (seven - eight weeks of age) were placed in the inner left corner of the left chamber and in the inner corner of the right chamber, respectively. Chambers were illuminated at 6 lux. Subject animals were positioned in the centre chamber when the experiments were started. Subject animals were allowed to move freely during the test. On the first day, all stranger mice were habituated to the small cages in the arena for 10 min. On the second day, subject animals were first placed in the arena with a stranger caged mouse in one side, for 10 min (mouse cage vs. empty cage). Subsequently, subject animals were removed and again placed in the same arena with the previous stranger mouse on the same side with a novel stranger mouse on another side, for 10 min (familiar mouse cage vs. novel mouse cage). The total distance travelled and time spent in the proximity of cages (60 mm around cages) by the tested animal was recorded.

2.2.4.9 Porsolt swim test

The Porsolt swim test was performed in a small round pool (113 mm in diameter, H 216 mm; O'HARA, Tokyo, Japan). The pool was filled with 20°C hypochlorous acid water (pH 6.5 hypochlorous acid) to a height of 75 mm and placed in a white box (100 lux). The mice were tested on two consecutive days, 10 min/day. Animals were allowed to swim freely in the pool during the test. The percent of immobile time, that is, floating status, was recorded.

2.2.4.10 Prepulse inhibition test

The prepulse inhibition test was performed in a startle reflex measurement box (O'HARA, Tokyo, Japan). Acoustic startle responses were measured by stimuli of 90, 100, 110, and 120 dB of white noise (40 ms, 1000–20,000 Hz). Subsequently, prepulse inhibition of acoustic startle responses was measured by pairs of 70 (pre)–120 dB, 75–120 dB, 80–120 dB and 85–120 dB of white noise (40 ms).

Animals were constrained in a transparent cylinder (25 mm in diameter) during the test. The box was illuminated at 3000 lux with under 50 dB background white noise. First, animals were habituated to the test cylinder and box for 300 seconds. Next, acoustic startle responses were measured by acoustic stimuli in the startle stimulus trials. Each stimulus was presented four times (total 16 stimuli) and average startle responses were recorded. Intervals between each stimulus were randomly selected from 5, 10, 15, 20, 25 seconds to prevent animals from predicting the timing of stimuli. Subsequently, prepulse inhibition of acoustic startle responses was measured in the prepulse inhibition trial. Each pair was presented four times (total 16 pairs) and average startle responses were recorded. The prepulse stimuli sound lasted 100 msec before the startle stimulus. Intervals between each paired stimulus were randomly selected from 5, 10, 15, 20, 25 seconds. Amplitudes were

sampled from 400 msec following startle stimuli and averages of amplitudes of the same four stimuli were recorded. The percent of prepulse inhibition was calculated as follows; PPI (%) = (amplitude to 120 dB in the startle stimulus trial amplitude in the prepulse inhibition trial) / amplitude to 120 dB in the startle stimulus trial × 100.

2.2.4.11 Barnes maze

The Barnes maze was performed on a white circular arena (1.0 m in diameter; O'HARA, Tokyo, Japan). Twelve holes (40 mm in diameter) were equally spaced around its circumference. The training session consisted of 16 trials (1 trial/day, 5 min). After 1 day and 8 days of training session, probe tests were conducted.

The arena was 760 mm above the floor and illuminated at 1000 lux. A black escape box (W 160 mm, D 120 mm, H 60 mm) filled with white paper bedding material was placed under one of the holes as the target hole. The position of the target hole was fixed during individual tests and positions of target holes were randomly chosen for different individuals. Four spatial cues (a big blue rectangular, yellow sphere, red quadrangular pyramid, and black coil) were hung from the ceiling in the four corners of the experimental room. The arena was rotated every day to avoid animals using olfactory or proximal cues in the arena. The training session consisted of 16 trials (1 trial/day, 5 minutes). Animals were placed in the centre area inside of white opaque cylinder (110mm in diameter, H 168 mm), and when the experiments started, the cylinder was taken out. The starting point was randomised within the centre area. Animals moved freely in the arena during the test. The total distance travelled and the latency to enter the targeted hole was recorded. When animals did not enter the target hole within 5 minutes, they were guided to the target hole and left there for 30 seconds. The latency was recorded as 300 seconds in this case. One day after the training session, the first probe test for 5 minutes was conducted in the absence of the

target hole, to confirm that the animals were only guided by the distal spatial cues. After the first probe test, one training trial was conducted as a retraining. A second probe test was conducted one week after the first probe test with the same procedure. Time spent around holes was recorded. Missing values in data were complemented by means of the value of the previous trial and next trial. When missing values were in the data of trial 16, they were complemented by the values of trial 15. If missing values were in sequential trials, the data was excluded from analysis. Here data had some missing values due to administrative failures in the training session (14 missing points/ total 445 data collecting points), and processed as mentioned above.

2.2.4.12 Fear-conditioning test

The fear-conditioning test was performed over 3 days (apparatus were from O'HARA, Tokyo, Japan). On the first day, conditioning was conducted. On the second day, a contextual test was conducted. On the third day, a cue test was conducted.

On the first day, conditioning was conducted in a test chamber illuminated at 100 lux (W 327 mm, D 250 mm, H 284 mm). Animals could move freely in the chamber for 8 minutes. First, animals were habituated to the chamber for 120 seconds. Second, Three CS [conditioned stimulus, 30 seconds of white noise (55 dB, 500 20000 Hz)) – US (unconditioned stimulus, last 2 seconds of the tone, 0.30 mA electrical foot shock) pairs were presented (120-150 second, 240-270 second, 360-390 second). On the second day contextual test was conducted in the same chamber. Animals could move freely in the chamber during the test for 5 minutes without white noise or foot shock. On the third day, cue test was conducted in a new triangular chamber (333 mm × 333 mm × 333 mm, H 400 mm). The chamber was illuminated at 10 lux. Animals could move freely in the chamber during the test for 6 minutes. First, animals were habituated to the chamber for 180

seconds (pre-cue phase). Second, 180 seconds of 55 dB white noise was presented (180-360 second) without foot shock (cue phase). The percent of immobile time (freezing) was recorded.

2.2.4.13 Tail suspension test

The tail suspension test was performed for 10 min. The base of the tail of the mouse was taped onto a metal board, and animals were suspended 270 mm above the floor in a white box (100 lux; O'HARA, Tokyo, Japan). The percent of immobile time was recorded.

2.2.4.14 Tail-wound counting and ranking

The number of tail wounds (red or dark red scab, scratch and internal bleeding) from both ventral and dorsal sides was counted manually in the pre-ST* and post-ST* photos and added for each animal. The ER (EE ->ST*) animals with the fewest wounds in each post-ST* cage were regarded as 'ER_α' animals (one α animal/cage) and the other two 'ER_others'.

2.2.4.15 Aggressive behaviour evaluation in ST* (video analysis)

The number of aggressive interactions in ST* was counted manually in recorded videos. Aggressive interactions include chasing, wrestling, boxing and mounting. When multiple aggressive interactions occurred within 3 s, they were regarded as a single continuous aggressive interaction thus counted as one (Sano et al., 2016). If multiple mounting behaviours occurred within 3 s, they were counted as one. A 60-min video records starting 1 h after lights-off were utilised for this analysis, based on the increased activity level and aggressive behaviour during the early dark phase (Todd et al., 2018).

2.2.4.16 Activity level and social behaviour evaluation in ST* (video analysis)

Activity level data (the summation of the number of different pixels between consecutive two flames [8 flames/s] in each 1 min) and social behaviour data (the average of the number of particles; how many clusters of animals were in a cage in each 1 min) were

recorded by the software and apparatus included in this system (O'HARA, Tokyo, Japan). If all three animals took distance from other animals, the number of particles was three. If all three animals stick together, the number of particles was one. For analysis, the average activity level data and social behaviour data in every 12 h were calculated by averaging each data of 1 min including each 12 h.

2.2.5 Statistical analysis

Statistical analysis and graphs were conducted using R version 4.0.0 (R Core Team, 2022). Analysis of variance (ANOVA) and Holm's sequentially rejective Bonferroni procedure (Holm's method) were carried out by R function “anovakun” version 4.8.5 (Iseki, 2020). For single-factor experiments, I presented the outputs of Holm's method (e.g., bodyweight measurement). For two-factor experiments, I presented the outputs of ANOVA and subsequent analysis by Holm's method (e.g., the prepulse inhibition test). I excluded animals from analysis when I noticed administrative failures of experimental procedures on them.

2.3 Results

The housing environments, both of long-term housing manipulation and acute change of housing environments, critically affected animal’s behaviour.

In Experiment 1, I observed significant effects of long-term housing environments over behavioural tests to assess animal’s physiological states, sensory function, motor function, activity level and anxiety, stress-coping strategy, and learning and memory. Animals housed in EE and SI showed increased body weight and rectal temperature (Figure 2a-b), enhanced sensory function (Figure 3), and altered motor function (Figure 2d-g). Especially EE animals showed clearly enhanced performance in both of sensory tasks and motor tasks. In activity level and anxiety tasks, EE animals showed less locomotion level, meanwhile SI animals showed increased locomotion, decreased anxiety-like behaviour, and

increased vertical activities (Figure 4-7). Their stress-coping strategy was also changed; EE animals showed increased immobility, meanwhile SI animals showed decreased immobility (Figure 9). Furthermore, EE showed improved performance in the fear-conditioning test, and SI animals showed less performance in the fear-conditioning test and the Barnes maze (Figure 10-12). Note that EE animals did not escape to the holes during the training session of Barnes maze, suggesting less anxiety for blight light and the spacious open arena. Thus they were excluded from this test. There was no significant difference between EE animals and SI animals in the time spent with mouse or novel mouse of Crawley's social interaction test (Figure 8).

In Experiment 2, I observed impacts of enrichment removal. ER animals showed indirect (the number of tail wounds) and direct (chasing, wrestling, boxing, and mounting) evidence of increased fighting behaviour under ER (Figure 13). The tendency of number of chasing, wrestling, and boxing behaviours were dynamically changed over two weeks on ER animals, whereas the number of mounting behaviour of them was gradually increased. In addition, ER animals were less active during the dark phase, and more active during the light phase (Figure 14a). Their physical distance toward cage mates were kept longer over the dark and light phases (Figure 14b). In the subsequent behavioural tests, ER animals showed lower locomotion level, less anxiety-like behaviour, and increased immobility (Figure 15-17). ER_α animals moved vertically more in the open-field test, and showed most increased immobility in the tail-suspension test. In contrast, ER_other animals showed relatively less locomotion level and less anxiety-like behaviour. There was no significant difference among ST animals, ER_α animals, and ER_other animals in the time spent with mouse or novel mouse of the Crawley's social interaction test (Figure 16).

2.4 Discussion

I found that long-term housing manipulations such as EE and SI affected physiological states and various behavioural performances including sensory, motor, activity level, and stress-coping domain in BALB/c male mice. Also I revealed that an unexpected and forced transfer to a less enriched environment, ER, induced stress/frustration-like behaviour in BALB/c male mice. These results suggest that our behavioural screening approach might successfully capture the consequences of multimodal enrichment or de-enrichment, and that long-term environmental factors affected underlying information processing and the physiological functions for behaviours. For a detailed discussion and the possibility of multi-layered effects of environments from basic physiological functions to behavioural strategy, see 2.4.1. Furthermore, it demonstrated here that ER is a significant stressor that induces immediate and intense behavioural consequences. It is suggested that the characteristics of the animals reared in EE were in strong conflict with the de-enriched environment, thus inducing 'maladaptive' behaviours that may serve as a model of direct relevance to human health. See detailed discussion for 2.4.2.

Together, this chapter contributes to our understanding of the roles of surrounding environments on animal's behaviours and development of ER paradigm as a relevant stressor (in addition to other stressors such as movement restriction, social isolation, social defeat, etc). Biological correlates have not yet been established for ER given that ER as a new model of life stressor has only emerged recently. In contrast, environmental manipulations using EE had started in the 1960's and ample biological evidence has been associated with EE. Specifically, in rats, EE increases dendritic spines in all cortical layers (Johansson & Belichenko, 2002), while social isolation has the opposite effect (Bryan & Riesen, 1989). EE also enhances neurogenesis concurrent with enhanced spatial learning and memory, and activity-dependent upregulation of a variety of growth-promoting factors

such as BDNF and GAP43 (reviewed in McDonald et al., 2018). Whether such molecular and structural changes in the cortex and hippocampus requires epitranscriptomic regulation is unknow. In the next chapter, I apply long-lasting housing manipulations to genetically engineered animals to investigate the epitranscriptomic regulatory roles in mediating environmental impact on animal behaviour.

2.4.1 Experiment 1

2.4.1.1 General health and motor function

EE and SI housing affected the physiological states of animals: increased body weight and increased rectal temperature. Furthermore, EE animals showed enhanced motor function, as expected from their housed environment. In contrast, SI animals showed inconsistent performances in the grip-strength test, wire-hang test, and rotarod test. It is possibly because of excessive reaction to novel situations of SI animals. See also 2.4.1.3 Activity level and anxiety-like behaviour and 2.4.1.5 Stress-coping strategy.

2.4.1.2 Sensory function

Sensitivities to various auditory and tactile stimuli as well as prepulse inhibition were enhanced in EE animals. Regarding the effect of EE on sensory function, a limited number of previous studies reported inconsistent results. In the hot-plate test, EE animals showed a faster response in NMRI (Rabadán et al., 2020), slower response in 129S6/SvEv/Tac (Abramov et al., 2008), and no significant difference in C57BL/6 (Abramov et al., 2008), compared with ST animals. In the acoustic startle response test and the prepulse-inhibition test, results of this study were consistent with some previous research (Chen et al., 2010; Varty et al., 1995). The present study provides further insights into the impact of environmental factors on animal's sensory function.

SI animals also showed enhanced prepulse inhibition. Again, previous studies are

limited. Geyer et al. (1993), Varty et al. (1995), Wilkinson et al. (1994) , and Cilia et al. (2005) reported that SI induced disrupted prepulse inhibition. Chen et al. (2010) reported that stressful stimuli such as chronic restraint induced enhanced prepulse inhibition. The present study adds a new research example to the response of prepulse inhibition under stress.

2.4.1.3 Activity level and anxiety-like behaviour

Overall, EE animals showed decreased locomotion and anxiety-like behaviour in relevant tasks. In contrast, SI animals showed increased locomotion, decreased anxiety-like behaviour, and increased vertical activities.

Here, possibly, EE animals were wary of, or had less motivation to move in novel but narrow experimental chambers during the testing. EE animals showed less anxiety-like behaviours, possibly because they were already habituated to an open space. The result of the training session in Barnes maze, using relatively wider experimental arena, supports this interpretation – EE animals' activity levels gradually increased over days in a wide arena without obvious escaping behaviour to the holes. On the other hand, SI animals moved excessively in novel situations during the tests, possibly because of chronic social deprivation including sensory stimuli from other animals. Here, SI animal's hyperactivity could interfere the explanation of their anxiety-like behaviour. See also discussion in 2.4.1.5 Stress-coping strategy and 2.4.1.6 Learning and memory.

2.4.1.4 Sociality

In Crawley's social interaction test, there was no significant difference between ST animals and EE or SI animals. However, when comparing ST with EE or SI data of the percent of time staying around the mouse cage, the effect size *r* is .42 (ST vs. EE) or .36 (ST vs. SI), not a very small value, suggesting that EE and SI animals have altered sociality but the sample size in this experiment was small.

2.4.1.5 Stress-coping strategy

In the Porsolt swim and the tail suspension tests, EE animals showed higher immobility. In contrast, SI animals showed less immobility. According to Commons et al. (2017), high immobility can be interpreted as a coping strategy to conserve energy against unavoidable stressors, whereas low immobility can be interpreted as a coping strategy to consume more energy. Note that EE animals showed especially higher immobility on day 2 in the Porsolt swim test, which can be interpreted as cognitive enhancement against the repeated unavoidable stressor.

Previous reports have also shown that EE has 'anti-depressant like' effect or 'no effect' and SI had 'depressogenic' or 'no effect' measured using immobility time in the Porsolt swim test (reviewed by Bogdanova et al., 2013). This difference could be originated from experimental conditions such as strain difference and/or animal ages during housing manipulation (Huang et al., 2021; Mesa-Gresa et al., 2021). Alternatively, this result could simply be attributed to the lower/higher locomotor activity of EE/SI animals during behavioural tests. Since the context of the experiments is extremely important for setting up the behavioural outcomes, although I could not exclude the possible effects of semi-EE housing and animal fights and the influence of experimental schedule during the behavioural test battery, overall results from my test battery were consistent with each other.

2.4.1.6 Learning and memory

Here, EE animals showed "better" performance in the fear-conditioning test, and SI animals showed "not as good" performance in the fear-conditioning test and the Barnes maze, compared to ST animals. However, interpretations of these results may not be straightforward, given the physiological changes and different sensitivities to the novel

environment during the tests. Although previous research reported that EE induces enhanced learning and memory, and SI disrupts them (Bianchi et al., 2006; Nithianantharajah & Hannan, 2006), my study shows potential alternative interpretations as followings.

EE animals showed increased freezing in the fear-conditioning test that uses foot shocks as unconditioned stimuli (US). The increased sensitivities to tactile stimuli and hypoactivity in overall behavioral test battery in EE animals provides alternative explanation to the "enhanced memory" observation measured as increased freezing time although they do not exclude the possibility of enhanced learning and memory function. Also, EE animals showed altered behavioural patterns in the Y-maze chamber and Barnes maze tests. Thus, it is likely that their performance in these mazes does not appropriately reflect memory function as how the measurements from these tests are interpreted.

SI animals showed less freezing in the fear-conditioning test. Also, in the Barnes maze, SI animals showed less staying time around the target in probe tests compared to ST animals, despite their similar learning curve in the training session. Possibly, SI animals' performance in these tests is associated with both increased locomotion activities in novel environments and altered learning and memory function.

2.4.2 Experiment 2

2.4.2.1 Fighting behaviour, activity level, and social behaviour under ER

ER animals showed stress/frustration-like behaviours. First, ER animals became more aggressive toward cage mates after being moved to standard-sized cages. The aggressive behaviours such as chasing, wrestling, boxing, and mounting changed dynamically over two weeks of monitor period. The dynamics could be interpreted as following sequential reactions by the animals: on day 0 and day 1 in ST*, the animals

reacted to the abrupt environmental change with increased aggressive behaviour. On day 8, the animals were acclimated to ST* and showed less aggressive behaviour in a more stable hierarchical structure. On day 13, the combination of the establishment of new social relationships and the extended burden of enrichment deprivation led to aggressive behaviour to emerge again. Second, ER animals showed unorthodox activity levels in the dark and light phases, which could cause stress-induced insufficient resting. Third, they kept more physical distances from cage mates.

What may be triggering these stress/frustration-like behaviours in the ER animals? Firstly a “bored” problem that the animals can not exercise or interact with previous cage mates. Secondly, a “shrank space” problem that the animal can not keep their personal space or escape from other aggressive behaviours causing collapse of group dynamics in the narrower spaces such (e.g. overcrowding effect). Such behaviours may also be related to the serotonin synthesis-related genetic polymorphism of BALB/c. As previously reviewed by Gubert & Hannan (2019), and observed by wound counting at the timing of pre-ST* in Experiment 2, EE itself could induce aggressive behaviour. And, the homozygous 1473G SNP in the Tph2 gene in BALB/c mice could have augmented aggressive behaviour because of their lower activity in serotonin synthesis compared to other mice strains (Giles et al., 2018; Osipova et al., 2009).

2.4.2.2 Behavioural test battery on ER animals

Overall, ER animals showed similar performances with EE animals in Experiment 1, such as lower locomotion, less anxiety-like behaviour, and energy-conserving coping strategy against an unavoidable stressful condition. Thus, losing environmental enrichment did not deprive ER animals of all previously obtained behavioural characteristics in EE. Note that animals in Experiment 2 showed more travelled distance in test chambers compared to

in Experiment 1. It is possible that the ceiling lights for video-recording in the ST* cages had acquainted the animals to them so the animals were less intimidated by the light during the behavioural test battery.

When behaviours were analysed based on their social ranking in the cage, ER_α animals showed increased vertical movement in the open-field test, which could be interpreted as enhanced novelty-seeking behaviour related to escape (Lever et al., 2006). Furthermore, ER_α animals showed most energy-conserving coping strategy in the tail-suspension test. In contrast, ER_other animals showed relatively less activity level and less anxiety-like behaviour. More evidence from future ER studies is necessary to interpret these results appropriately and establishing biological correlates to ER in the future will be essential for understanding the mechanisms underlying such behaviours.

Chapter 3

RNA modification is a novel regulator of gene-environment interaction

Chapter 4

Epitranscriptomic regulation via m^6A reader YTHDF3 affects mice behaviours

Chapter 5

Analysis of m^6A reader YTHDF1 conditional-knockout mice behaviours

Chapter 6

General discussion

References

Abramov, U., Puussaar, T., Raud, S., Kurrikoff, K., & Vasar, E. (2008). Behavioural differences between C57BL/6 and 129S6/SvEv strains are reinforced by environmental enrichment. *Neuroscience Letters*, *443*(3), 223–227. https://doi.org/10.1016/j.neulet.2008.07.075

Baumert, A., Schmitt, M., Perugini, M., Johnson, W., Blum, G., Borkenau, P., Costantini, G., Denissen, J. J. A., Fleeson, W., Grafton, B., Jayawickreme, E., Kurzius, E., MacLeod, C., Miller, L. C., Read, S. J., Roberts, B., Robinson, M. D., Wood, D., & Wrzus, C. (2017). Integrating Personality Structure, Personality Process, and Personality Development. *European Journal of Personality*, *31*(5), 503–528. https://doi.org/10.1002/per.2115

Bawankar, P., Lence, T., Paolantoni, C., Haussmann, I. U., Kazlauskiene, M., Jacob, D., Heidelberger, J. B., Richter, F. M., Nallasivan, M. P., Morin, V., Kreim, N., Beli, P., Helm, M., Jinek, M., Soller, M., & Roignant, J.-Y. (2021). Hakai is required for stabilization of core components of the m6A mRNA methylation machinery. *Nature Communications*, *12*(1), Article 1. https://doi.org/10.1038/s41467-021-23892-5

Bennett, S. H., Kirby, A. J., & Finnerty, G. T. (2018). Rewiring the connectome: Evidence and effects. *Neuroscience and Biobehavioral Reviews*, *88*, 51–62. https://doi.org/10.1016/j.neubiorev.2018.03.001

Bianchi, M., Fone, K. F. C., Azmi, N., Heidbreder, C. A., Hagan, J. J., & Marsden, C. A. (2006). Isolation rearing induces recognition memory deficits accompanied by cytoskeletal alterations in rat hippocampus. *European Journal of Neuroscience*, *24*(10), 2894–2902. https://doi.org/10.1111/j.1460-9568.2006.05170.x

Bogdanova, O. V., Kanekar, S., D'Anci, K. E., & Renshaw, P. F. (2013). Factors influencing behavior in the forced swim test. *Physiology & Behavior*, *118*, 227–239. https://doi.org/10.1016/j.physbeh.2013.05.012

Bokar, J. A., Rath-Shambaugh, M. E., Ludwiczak, R., Narayan, P., & Rottman, F. (1994). Characterization and partial purification of mRNA N6-adenosine methyltransferase from HeLa cell nuclei. Internal mRNA methylation requires a multisubunit complex. *Journal of Biological Chemistry*, *269*(26), 17697–17704. https://doi.org/10.1016/S0021-9258(17)32497-3

Bokar, J. A., Shambaugh, M. E., Polayes, D., Matera, A. G., & Rottman, F. M. (1997). Purification and cDNA cloning of the AdoMet-binding subunit of the human mRNA (N6-adenosine)-methyltransferase. *RNA*, *3*(11), 1233–1247.

Briley, D. A., & Tucker-Drob, E. M. (2014). Genetic and Environmental Continuity in Personality Development: A Meta-Analysis. *Psychological Bulletin*, *140*(5), 1303–1331. https://doi.org/10.1037/a0037091

Bryan, G. K., & Riesen, A. H. (1989). Deprived somatosensory-motor experience in stumptailed monkey neocortex: Dendritic spine density and dendritic branching of layer IIIB pyramidal cells. *Journal of Comparative Neurology*, *286*(2), 208–217. https://doi.org/10.1002/cne.902860206

Chen, Y., Mao, Y., Zhou, D., Hu, X., Wang, J., & Ma, Y. (2010). Environmental enrichment and chronic restraint stress in ICR mice: Effects on prepulse inhibition of startle and Y-maze spatial recognition memory. *Behavioural Brain Research*, *212*(1), 49–55. https://doi.org/10.1016/j.bbr.2010.03.033

Cilia, J., Hatcher, P. D., Reavill, C., & Jones, D. N. C. (2005). Long-term evaluation of isolation-rearing induced prepulse inhibition deficits in rats: An update. *Psychopharmacology*, *180*(1), 57–62. https://doi.org/10.1007/s00213-004-2139-5

Desrosiers, R., Friderici, K., & Rottman, F. (1974). Identification of Methylated Nucleosides in Messenger RNA from Novikoff Hepatoma Cells. *Proceedings of the National Academy*

of Sciences, *71*(10), 3971–3975. https://doi.org/10.1073/pnas.71.10.3971

Dick, D. M. (2011). Gene-Environment Interaction in Psychological Traits and Disorders. *Annual Review of Clinical Psychology*, *7*(1), 383–409. https://doi.org/10.1146/annurev-clinpsy-032210-104518

Dominissini, D., Moshitch-Moshkovitz, S., Schwartz, S., Salmon-Divon, M., Ungar, L., Osenberg, S., Cesarkas, K., Jacob-Hirsch, J., Amariglio, N., Kupiec, M., Sorek, R., & Rechavi, G. (2012). Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. *Nature*, *485*(7397), Article 7397. https://doi.org/10.1038/nature11112

dos Santos Guilherme, M., Tsoutsouli, T., Todorov, H., Teifel, S., Nguyen, V. T. T., Gerber, S., & Endres, K. (2021). N6 -Methyladenosine Modification in Chronic Stress Response Due to Social Hierarchy Positioning of Mice. *Frontiers in Cell and Developmental Biology*, *9*. https://www.frontiersin.org/articles/10.3389/fcell.2021.705986

Du, H., Zhao, Y., He, J., Zhang, Y., Xi, H., Liu, M., Ma, J., & Wu, L. (2016). YTHDF2 destabilizes m6A-containing RNA through direct recruitment of the CCR4–NOT deadenylase complex. *Nature Communications*, *7*(1), Article 1. https://doi.org/10.1038/ncomms12626

Engel, M., Eggert, C., Kaplick, P. M., Eder, M., Röh, S., Tietze, L., Namendorf, C., Arloth, J., Weber, P., Rex-Haffner, M., Geula, S., Jakovcevski, M., Hanna, J. H., Leshkowitz, D., Uhr, M., Wotjak, C. T., Schmidt, M. V., Deussing, J. M., Binder, E. B., & Chen, A. (2018). The Role of m6A/m-RNA Methylation in Stress Response Regulation. *Neuron*, *99*(2), 389-403.e9. https://doi.org/10.1016/j.neuron.2018.07.009

Feil, R., & Fraga, M. F. (2012). Epigenetics and the environment: Emerging patterns and implications. *Nature Reviews Genetics*, *13*(2), Article 2.

https://doi.org/10.1038/nrg3142

Flamand, M. N., Ke, K., Tamming, R., & Meyer, K. D. (2022). Single-molecule identification of the target RNAs of different RNA binding proteins simultaneously in cells. *Genes & Development*. https://doi.org/10.1101/gad.349983.122

Flamand, M. N., & Meyer, K. D. (2022). M6A and YTHDF proteins contribute to the localization of select neuronal mRNAs. *Nucleic Acids Research*, *50*(8), 4464–4483. https://doi.org/10.1093/nar/gkac251

Geyer, M. A., Wilkinson, L. S., Humby, T., & Robbins, T. W. (1993). Isolation rearing of rats produces a deficit in prepulse inhibition of acoustic startle similar to that in schizophrenia. *Biological Psychiatry*, *34*(6), 361–372. https://doi.org/10.1016/0006-3223(93)90180-L

Giles, J. M., Whitaker, J. W., Moy, S. S., & Fletcher, C. A. (2018). Effect of Environmental Enrichment on Aggression in BALB/cJ and BALB/cByJ Mice Monitored by Using an Automated System. *Journal of the American Association for Laboratory Animal Science*, *57*(3), 236–243. https://doi.org/10.30802/AALAS-JAALAS-17-000122

Gubert, C., & Hannan, A. J. (2019). Environmental enrichment as an experience-dependent modulator of social plasticity and cognition. *Brain Research*, *1717*, 1–14. https://doi.org/10.1016/j.brainres.2019.03.033

He, P. C., & He, C. (2021). m6A RNA methylation: From mechanisms to therapeutic potential. *The EMBO Journal*, *40*(3), e105977. https://doi.org/10.15252/embj.2020105977

Huang, H., Wang, Q., Guan, X., Zhang, X., Zhang, Y., Cao, J., & Li, X. (2021). Effects of enriched environment on depression and anxiety-like behavior induced by early life stress: A comparison between different periods. *Behavioural Brain Research*, *411*,

113389. https://doi.org/10.1016/j.bbr.2021.113389

Iseki, R. (2020). Anovakun. http://riseki.php.xdomain.jp/

Jia, G., Fu, Y., Zhao, X., Dai, Q., Zheng, G., Yang, Y., Yi, C., Lindahl, T., Pan, T., Yang, Y.-G., & He, C. (2011). N6-Methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nature Chemical Biology*, *7*(12), Article 12. https://doi.org/10.1038/nchembio.687

Johansson, B. B., & Belichenko, P. V. (2002). Neuronal Plasticity and Dendritic Spines: Effect of Environmental Enrichment on Intact and Postischemic Rat Brain. *Journal of Cerebral Blood Flow & Metabolism*, *22*(1), 89–96. https://doi.org/10.1097/00004647-200201000-00011

Jones, A. N., Tikhaia, E., Mourão, A., & Sattler, M. (2022). Structural effects of m6A modification of the Xist A-repeat AUCG tetraloop and its recognition by YTHDC1. *Nucleic Acids Research*, *50*(4), 2350–2362. https://doi.org/10.1093/nar/gkac080

Kempermann, G. (2019). Environmental enrichment, new neurons and the neurobiology of individuality. *Nature Reviews Neuroscience*, *20*(4), Article 4. https://doi.org/10.1038/s41583-019-0120-x

Knuckles, P., Lence, T., Haussmann, I. U., Jacob, D., Kreim, N., Carl, S. H., Masiello, I., Hares, T., Villaseñor, R., Hess, D., Andrade-Navarro, M. A., Biggiogera, M., Helm, M., Soller, M., Bühler, M., & Roignant, J.-Y. (2018). Zc3h13/Flacc is required for adenosine methylation by bridging the mRNA-binding factor Rbm15/Spenito to the m6A machinery component Wtap/Fl(2)d. *Genes & Development*, *32*(5–6), 415–429. https://doi.org/10.1101/gad.309146.117

Kontur, C., Jeong, M., Cifuentes, D., & Giraldez, A. J. (2020). Ythdf m6A Readers Function Redundantly during Zebrafish Development. *Cell Reports*, *33*(13), 108598.

https://doi.org/10.1016/j.celrep.2020.108598

Lambert, K., Eisch, A. J., Galea, L. A. M., Kempermann, G., & Merzenich, M. (2019). Optimizing brain performance: Identifying mechanisms of adaptive neurobiological plasticity. *Neuroscience & Biobehavioral Reviews*, *105*, 60–71. https://doi.org/10.1016/j.neubiorev.2019.06.033

Lasman, L., Krupalnik, V., Viukov, S., Mor, N., Aguilera-Castrejon, A., Schneir, D., Bayerl, J., Mizrahi, O., Peles, S., Tawil, S., Sathe, S., Nachshon, A., Shani, T., Zerbib, M., Kilimnik, I., Aigner, S., Shankar, A., Mueller, J. R., Schwartz, S., … Hanna, J. H. (2020). Context-dependent functional compensation between Ythdf m6A reader proteins. *Genes & Development*, *34*(19–20), 1373–1391. https://doi.org/10.1101/gad.340695.120

Li, A., Chen, Y.-S., Ping, X.-L., Yang, X., Xiao, W., Yang, Y., Sun, H.-Y., Zhu, Q., Baidya, P., Wang, X., Bhattarai, D. P., Zhao, Y.-L., Sun, B.-F., & Yang, Y.-G. (2017). Cytoplasmic m6A reader YTHDF3 promotes mRNA translation. *Cell Research*, *27*(3), Article 3. https://doi.org/10.1038/cr.2017.10

Li, F., Zhao, D., Wu, J., & Shi, Y. (2014). Structure of the YTH domain of human YTHDF2 in complex with an m6A mononucleotide reveals an aromatic cage for m6A recognition. *Cell Research*, *24*(12), Article 12. https://doi.org/10.1038/cr.2014.153

Linder, B., Grozhik, A. V., Olarerin-George, A. O., Meydan, C., Mason, C. E., & Jaffrey, S. R. (2015). Single-nucleotide-resolution mapping of m6A and m6Am throughout the transcriptome. *Nature Methods*, *12*(8), Article 8. https://doi.org/10.1038/nmeth.3453

Liu, J., Yue, Y., Han, D., Wang, X., Fu, Y., Zhang, L., Jia, G., Yu, M., Lu, Z., Deng, X., Dai, Q., Chen, W., & He, C. (2014). A METTL3–METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. *Nature Chemical Biology*, *10*(2), Article 2.

https://doi.org/10.1038/nchembio.1432

Luo, S., & Tong, L. (2014). Molecular basis for the recognition of methylated adenines in RNA by the eukaryotic YTH domain. *Proceedings of the National Academy of Sciences*, *111*(38), 13834–13839. https://doi.org/10.1073/pnas.1412742111

Martinez De La Cruz, B., Markus, R., Malla, S., Haig, M. I., Gell, C., Sang, F., Bellows, E., Sherif, M. A., McLean, D., Lourdusamy, A., Self, T., Bodi, Z., Smith, S., Fay, M., Macdonald, I. A., Fray, R., & Knight, H. M. (2021). Modifying the m6A brain methylome by ALKBH5-mediated demethylation: A new contender for synaptic tagging. *Molecular Psychiatry*, *26*(12), Article 12. https://doi.org/10.1038/s41380-021-01282-z

McDonald, M. W., Hayward, K. S., Rosbergen, I. C. M., Jeffers, M. S., & Corbett, D. (2018). Is Environmental Enrichment Ready for Clinical Application in Human Post-stroke Rehabilitation? *Frontiers in Behavioral Neuroscience*, *12*, 135. https://doi.org/10.3389/fnbeh.2018.00135

McQuaid, R. J., Audet, M.-C., & Anisman, H. (2012). Environmental enrichment in male CD-1 mice promotes aggressive behaviors and elevated corticosterone and brain norepinephrine activity in response to a mild stressor. *Stress*, *15*(3), 354–360. https://doi.org/10.3109/10253890.2011.623249

Merkurjev, D., Hong, W.-T., Iida, K., Oomoto, I., Goldie, B. J., Yamaguti, H., Ohara, T., Kawaguchi, S., Hirano, T., Martin, K. C., Pellegrini, M., & Wang, D. O. (2018). Synaptic N6-methyladenosine (m6A) epitranscriptome reveals functional partitioning of localized transcripts. *Nature Neuroscience*, *21*(7), 1004–1014. https://doi.org/10.1038/s41593-018-0173-6

Mesa-Gresa, P., Ramos-Campos, M., & Redolat, R. (2021). Behavioral impact of experience based on environmental enrichment: Influence of age and duration of exposure in

male NMRI mice. *Developmental Psychobiology*, *63*(5), 1071–1081. https://doi.org/10.1002/dev.22093

Meyer, K. D., Saletore, Y., Zumbo, P., Elemento, O., Mason, C. E., & Jaffrey, S. R. (2012). Comprehensive Analysis of mRNA Methylation Reveals Enrichment in 3' UTRs and near Stop Codons. *Cell*, *149*(7), 1635–1646. https://doi.org/10.1016/j.cell.2012.05.003

Morano, R., Hoskins, O., Smith, B. L., & Herman, J. P. (2019). Loss of Environmental Enrichment Elicits Behavioral and Physiological Dysregulation in Female Rats. *Frontiers in Behavioral Neuroscience*, *12*. https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00287

Murakami, S., & Jaffrey, S. R. (2022). Hidden codes in mRNA: Control of gene expression by m6A. *Molecular Cell*, *82*(12), 2236–2251. https://doi.org/10.1016/j.molcel.2022.05.029

Nithianantharajah, J., & Hannan, A. J. (2006). Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews Neuroscience*, *7*(9), Article 9. https://doi.org/10.1038/nrn1970

Oerum, S., Meynier, V., Catala, M., & Tisné, C. (2021). A comprehensive review of m6A/m6Am RNA methyltransferase structures. *Nucleic Acids Research*, *49*(13), 7239–7255. https://doi.org/10.1093/nar/gkab378

Osipova, D. V., Kulikov, A. V., & Popova, N. K. (2009). C1473G polymorphism in mouse tph2 gene is linked to tryptophan hydroxylase-2 activity in the brain, intermale aggression, and depressive-like behavior in the forced swim test. *Journal of Neuroscience Research*, *87*(5), 1168–1174. https://doi.org/10.1002/jnr.21928

Park, O. H., Ha, H., Lee, Y., Boo, S. H., Kwon, D. H., Song, H. K., & Kim, Y. K. (2019).

Endoribonucleolytic Cleavage of m6A-Containing RNAs by RNase P/MRP Complex. *Molecular Cell*, *74*(3), 494-507.e8. https://doi.org/10.1016/j.molcel.2019.02.034

Perry, R. P., & Kelley, D. E. (1974). Existence of methylated messenger RNA in mouse L cells. *Cell*, *1*(1), 37–42. https://doi.org/10.1016/0092-8674(74)90153-6

Ping, X.-L., Sun, B.-F., Wang, L., Xiao, W., Yang, X., Wang, W.-J., Adhikari, S., Shi, Y., Lv, Y., Chen, Y.-S., Zhao, X., Li, A., Yang, Y., Dahal, U., Lou, X.-M., Liu, X., Huang, J., Yuan, W.-P., Zhu, X.-F., … Yang, Y.-G. (2014). Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase. *Cell Research*, *24*(2), Article 2. https://doi.org/10.1038/cr.2014.3

Qu, W., Li, Q., Wang, M., Zhao, X., Wu, J., Liu, D., Hong, S., Yang, Y., Shu, Q., & Li, X. (2022). M6A Modification Involves in Enriched Environment-Induced Neurogenesis and Cognition Enhancement. *Frontiers in Cell and Developmental Biology*, *10*. https://www.frontiersin.org/articles/10.3389/fcell.2022.903179

R Core Team. (2022). R: The R Project for Statistical Computing. *R Foundation for Statistical Computing, Vienna, Austria*. https://www.R-project.org/

Rabadán, R., Ramos-Campos, M., Redolat, R., & Mesa-Gresa, P. (2020). Physical activity and environmental enrichment: Behavioural effects of exposure to different housing conditions in mice. *Acta Neurobiologiae Experimentalis*, *79*(4), 374–385. https://doi.org/10.21307/ane-2019-035

Rauch, S., He, C., & Dickinson, B. C. (2018). Targeted m6A Reader Proteins To Study Epitranscriptomic Regulation of Single RNAs. *Journal of the American Chemical Society*, *140*(38), 11974–11981. https://doi.org/10.1021/jacs.8b05012

Shi, H., Wang, X., Lu, Z., Zhao, B. S., Ma, H., Hsu, P. J., Liu, C., & He, C. (2017). YTHDF3 facilitates translation and decay of N6-methyladenosine-modified RNA. *Cell*

Research, *27*(3), 315–328. https://doi.org/10.1038/cr.2017.15

Shi, H., Wei, J., & He, C. (2019). Where, When, and How: Context-Dependent Functions of RNA Methylation Writers, Readers, and Erasers. *Molecular Cell*, *74*(4), 640–650. https://doi.org/10.1016/j.molcel.2019.04.025

Shi, H., Zhang, X., Weng, Y.-L., Lu, Z., Liu, Y., Lu, Z., Li, J., Hao, P., Zhang, Y., Zhang, F., Wu, Y., Delgado, J. Y., Su, Y., Patel, M. J., Cao, X., Shen, B., Huang, X., Ming, G., Zhuang, X., … Zhou, T. (2018). M6A facilitates hippocampus-dependent learning and memory through YTHDF1. *Nature*, *563*(7730), Article 7730. https://doi.org/10.1038/s41586-018-0666-1

Slater, A. M., & Cao, L. (2015). A Protocol for Housing Mice in an Enriched Environment. *JoVE (Journal of Visualized Experiments)*, *100*, e52874. https://doi.org/10.3791/52874

Śledź, P., & Jinek, M. (2016). Structural insights into the molecular mechanism of the m6A writer complex. *ELife*, *5*, e18434. https://doi.org/10.7554/eLife.18434

Smith, B. L., Lyons, C. E., Correa, F. G., Benoit, S. C., Myers, B., Solomon, M. B., & Herman, J. P. (2017). Behavioral and physiological consequences of enrichment loss in rats. *Psychoneuroendocrinology*, *77*, 37–46. https://doi.org/10.1016/j.psyneuen.2016.11.040

Terkelsen, T., Brasch-Andersen, C., Illum, N., Busa, T., Missirian, C., Chandler, K., Holden, S. T., Jensen, U. B., & Fagerberg, C. R. (2022). Mono-allelic loss of YTHDF3 and neurodevelopmental disorder: Clinical features of four individuals with 8q12.3 deletions. *Clinical Genetics*, *101*(2), 208–213. https://doi.org/10.1111/cge.14083

Theler, D., Dominguez, C., Blatter, M., Boudet, J., & Allain, F. H.-T. (2014). Solution structure of the YTH domain in complex with N6-methyladenosine RNA: A reader of

methylated RNA. *Nucleic Acids Research*, *42*(22), 13911–13919. https://doi.org/10.1093/nar/gku1116

Varty, G. B., Higgins, G. A., & Higgins, G. A. (1995). Examination of drug-induced and isolation-induced disruptions of prepulse inhibition as models to screen antipsychotic drugs. *Psychopharmacology*, *122*(1), 15–26. https://doi.org/10.1007/BF02246437

Walters, B. J., Mercaldo, V., Gillon, C. J., Yip, M., Neve, R. L., Boyce, F. M., Frankland, P. W., & Josselyn, S. A. (2017). The Role of The RNA Demethylase FTO (Fat Mass and Obesity-Associated) and mRNA Methylation in Hippocampal Memory Formation. *Neuropsychopharmacology*, *42*(7), Article 7. https://doi.org/10.1038/npp.2017.31

Wang, P., Doxtader, K. A., & Nam, Y. (2016). Structural Basis for Cooperative Function of Mettl3 and Mettl14 Methyltransferases. *Molecular Cell*, *63*(2), 306–317. https://doi.org/10.1016/j.molcel.2016.05.041

Wang, X., Feng, J., Xue, Y., Guan, Z., Zhang, D., Liu, Z., Gong, Z., Wang, Q., Huang, J., Tang, C., Zou, T., & Yin, P. (2016). Structural basis of N6-adenosine methylation by the METTL3–METTL14 complex. *Nature*, *534*(7608), Article 7608. https://doi.org/10.1038/nature18298

Wang, X., Lu, Z., Gomez, A., Hon, G. C., Yue, Y., Han, D., Fu, Y., Parisien, M., Dai, Q., Jia, G., Ren, B., Pan, T., & He, C. (2014). N6-methyladenosine-dependent regulation of messenger RNA stability. *Nature*, *505*(7481), Article 7481. https://doi.org/10.1038/nature12730

Wang, X., Zhao, B. S., Roundtree, I. A., Lu, Z., Han, D., Ma, H., Weng, X., Chen, K., Shi, H., & He, C. (2015). N6-methyladenosine Modulates Messenger RNA Translation Efficiency. *Cell*, *161*(6), 1388–1399. https://doi.org/10.1016/j.cell.2015.05.014

Wang, Y., Li, Y., Toth, J. I., Petroski, M. D., Zhang, Z., & Zhao, J. C. (2014). N6-methyladenosine modification destabilizes developmental regulators in embryonic stem cells. *Nature Cell Biology*, *16*(2), Article 2. https://doi.org/10.1038/ncb2902

Widagdo, J., Zhao, Q.-Y., Kempen, M.-J., Tan, M. C., Ratnu, V. S., Wei, W., Leighton, L., Spadaro, P. A., Edson, J., Anggono, V., & Bredy, T. W. (2016). Experience-Dependent Accumulation of N6-Methyladenosine in the Prefrontal Cortex Is Associated with Memory Processes in Mice. *Journal of Neuroscience*, *36*(25), 6771–6777. https://doi.org/10.1523/JNEUROSCI.4053-15.2016

Wilkinson, L. S., Killcross, S. S., Humby, T., Hall, F. S., Geyer, M. A., & Robbins, T. W. (1994). Social Isolation in the Rat Produces Developmentally Specific Deficits in Prepulse Inhibition of the Acoustic Startle Response Without Disrupting Latent Inhibition. *Neuropsychopharmacology*, *10*(1), Article 1. https://doi.org/10.1038/npp.1994.8

Xu, C., Wang, X., Liu, K., Roundtree, I. A., Tempel, W., Li, Y., Lu, Z., He, C., & Min, J. (2014). Structural basis for selective binding of m6A RNA by the YTHDC1 YTH domain. *Nature Chemical Biology*, *10*(11), Article 11. https://doi.org/10.1038/nchembio.1654

Yan, L., Wei, J., Yang, F., Wang, M., Wang, S., Cheng, T., Liu, X., Jia, Y., So, K.-F., & Zhang, L. (2022). Physical Exercise Prevented Stress-Induced Anxiety via Improving Brain RNA Methylation. *Advanced Science*, *9*(24), 2105731. https://doi.org/10.1002/advs.202105731

Yap, E.-L., & Greenberg, M. E. (2018). Activity-Regulated Transcription: Bridging the Gap between Neural Activity and Behavior. *Neuron*, *100*(2), 330–348. https://doi.org/10.1016/j.neuron.2018.10.013

Yue, Y., Liu, J., Cui, X., Cao, J., Luo, G., Zhang, Z., Cheng, T., Gao, M., Shu, X., Ma, H., Wang, F., Wang, X., Shen, B., Wang, Y., Feng, X., He, C., & Liu, J. (2018). VIRMA mediates

preferential m6A mRNA methylation in 3'UTR and near stop codon and associates with alternative polyadenylation. *Cell Discovery*, *4*(1), Article 1. https://doi.org/10.1038/s41421-018-0019-0

Zaccara, S., & Jaffrey, S. R. (2020). A Unified Model for the Function of YTHDF Proteins in Regulating m6A-Modified mRNA. *Cell*, *181*(7), 1582-1595.e18. https://doi.org/10.1016/j.cell.2020.05.012

Zhang, X., Beaulieu, J.-M., Sotnikova, T. D., Gainetdinov, R. R., & Caron, M. G. (2004). Tryptophan Hydroxylase-2 Controls Brain Serotonin Synthesis. *Science*, *305*(5681), 217–217. https://doi.org/10.1126/science.1097540

Zhang, Y., Wang, X., Zhang, X., Wang, J., Ma, Y., Zhang, L., & Cao, X. (2019). RNA-binding protein YTHDF3 suppresses interferon-dependent antiviral responses by promoting FOXO3 translation. *Proceedings of the National Academy of Sciences*, *116*(3), 976–981. https://doi.org/10.1073/pnas.1812536116

Zhang, Z., Wang, M., Xie, D., Huang, Z., Zhang, L., Yang, Y., Ma, D., Li, W., Zhou, Q., Yang, Y.-G., & Wang, X.-J. (2018). METTL3-mediated N6-methyladenosine mRNA modification enhances long-term memory consolidation. *Cell Research*, *28*(11), Article 11. https://doi.org/10.1038/s41422-018-0092-9

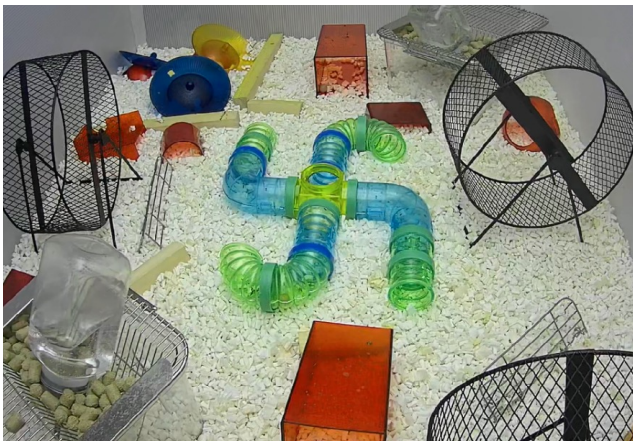
Zheng, G., Dahl, J. A., Niu, Y., Fedorcsak, P., Huang, C.-M., Li, C. J., Vågbø, C. B., Shi, Y., Wang, W.-L., Song, S.-H., Lu, Z., Bosmans, R. P. G., Dai, Q., Hao, Y.-J., Yang, X., Zhao, W.-M., Tong, W.-M., Wang, X.-J., Bogdan, F., … He, C. (2013). ALKBH5 Is a Mammalian RNA Demethylase that Impacts RNA Metabolism and Mouse Fertility. *Molecular Cell*, *49*(1), 18–29. https://doi.org/10.1016/j.molcel.2012.10.015

Figures and tables

Figure 1

EE with an open-top arena and semi-EE (enriched environment housing).

a



b

c

(a) Light phase of EE. (b) Dark phase of EE. (c) Semi-EE where EE animals were kept during the behavioural test battery.

Table 1

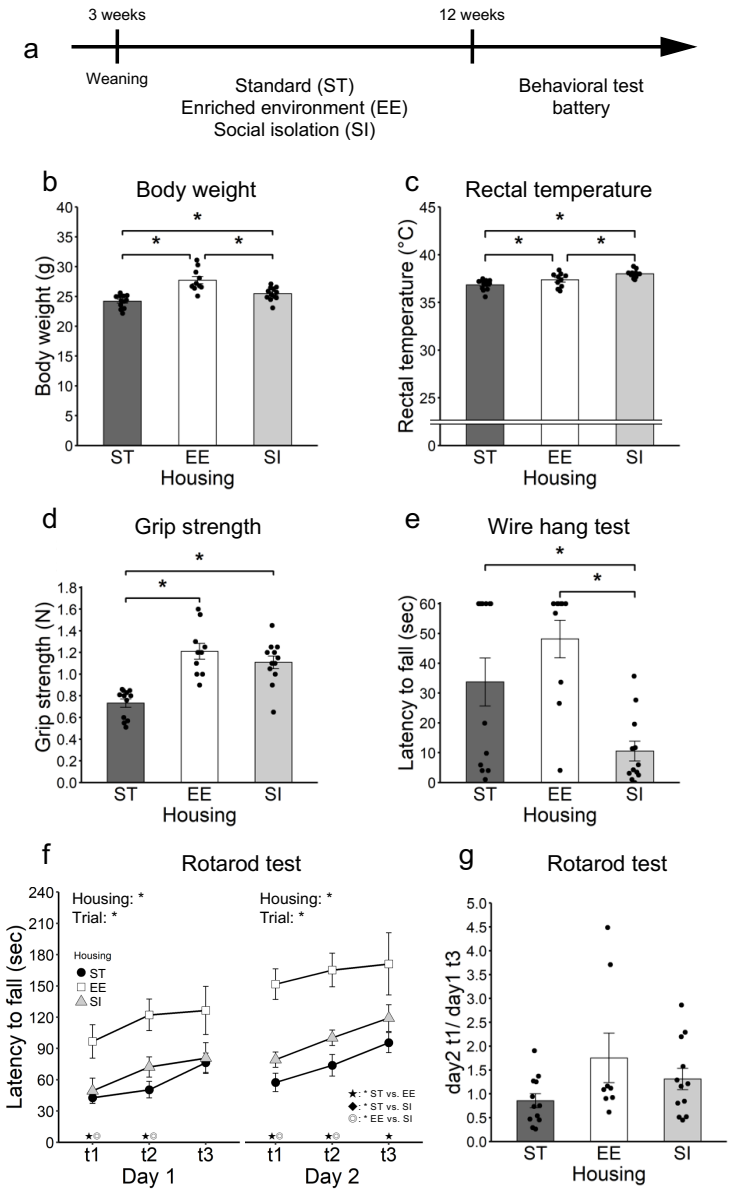
Schedule of behavioural test batteries in Chapter 2.

	Experiment 1	Experiment 2
Health check and/or handling	0-5	0
Open-field	2-6	1
Y-maze	3-8	
Elevated-plus maze	6-9	
Rotarod	7-29	
Hot plate	10-29	
Social interaction	11-20	4-8
Porsolt swim	14-35	
Prepulse inhibition	19-31	
Light-dark box	20-93	
Barnes maze	32-76	
Fear-conditioning	53-79	
Tail suspension	50-90	11

(day)

Figure 2

General health and motor function.



(a) The schema of Experiment 1. (b) Body weight. (c) Rectal temperature. (d) Grip strength. (e) Latency to fall in the wire hang test. (f) Latency to fall in the rotarod test. (g) The ratio of performances of day 2 trial 1/day 1 trial 3 in the rotarod test. Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. ST: standard housing. EE: enriched environment housing. SI: social isolation housing. See below for detailed results.

General health and neurological examinations on whisker state, coat state, righting reflex, whisker twitch, ear twitch, reaching and epilepsy detected no abnormalities (data not shown).

In bodyweight (g) measurement (Figure 2b; 12 ST animals [*Mean* = 24.21, *SEM* = 0.31] vs. 10 EE animals [*Mean* = 27.74, *SEM* = 0.60] vs. 12 SI animals [*Mean* = 25.48, *SEM* = 0.32]), there were significant differences between ST and EE groups ($p < .001$, adjusted $p < .001$, $r = .73$), between ST and SI groups ($p = .031$, adjusted $p = .031$, $r = .38$) and between EE and SI groups ($p = .001$, adjusted $p = .001$, $r = .57$). EE animals had the heaviest body weight, and ST animals had the lightest weight.

In rectal temperature (°C) measurement (Figure 2c; 12 ST animals [*Mean* = 36.85, *SEM* = 0.16] vs. 10 EE animals [*Mean* = 37.36, *SEM* = 0.23] vs. 12 SI animals [*Mean* = 38.00, *SEM* = 0.12]), there were significant differences between ST and EE groups ($p = .044$, adjusted $p = .044$, $r = .35$), between ST and SI groups ($p < .001$, adjusted $p < .001$, $r = .67$) and between EE and SI groups ($p = .012$, adjusted $p = .024$, $r = .43$). SI animals had the highest, and ST animals had the lowest rectal temperature.

In grip strength measurement (Figure 2d; 12 ST animals vs. 10 EE animals vs. 12 SI animals), there were significant differences between ST and EE groups ($p < .001$, adjusted $p < .001$, $r = .73$) and between ST and SI groups ($p < .001$, adjusted $p < .001$, $r = .66$). EE and SI animals showed enhanced grip strength compared with ST animals.

The latency to fall in the wire hang test is shown in Figure 2e (12 ST animals vs. 10 EE animals vs. 12 SI animals). There were significant differences between ST and SI groups ($p = .011$, adjusted $p = .021$, $r = .44$) and between EE and SI groups ($p < .001$, adjusted $p = .001$, $r = .60$). SI animals dropped from the wire mesh earlier than ST and EE animals.

The latency to fall in day 1 is shown in Figure 2f left (12 ST animals vs. 8 EE animals

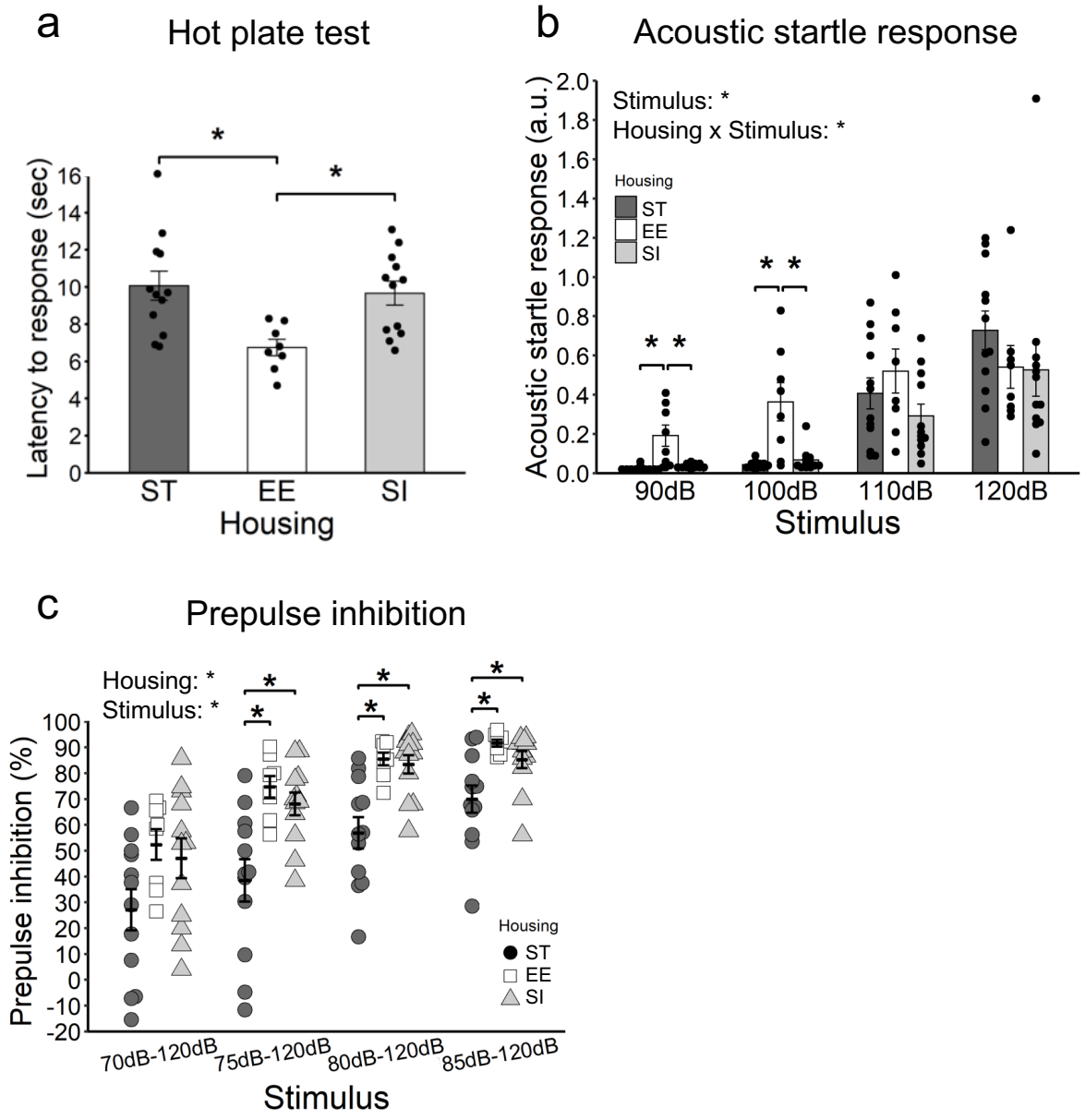
vs. 12 SI animals). A 3 (housing; ST, EE and SI; between-animal) × 3 (trial; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 29) = 9.78$, $p = .001$, $\eta_p^2 = .403$]. The subsequent analysis revealed that there were significant differences between ST and EE groups ($p < .001$, adjusted $p = .001$, $r = .62$) and between EE and SI groups ($p = .002$, adjusted $p = .003$, $r = .54$). EE animals rode on rotarods longer than ST and SI animals in day 1. The main effect of trial was significant [$F(2, 58) = 7.23$, $p = .002$, $\eta_p^2 = .200$]. The interaction between housing and trial was not significant [$F(4, 58) = 0.39$, $p = .818$, $\eta_p^2 = .026$].

The latency to fall in day 2 is shown in Figure 2f right (12 ST animals vs. 8 EE animals vs. 12 SI animals). A 3 (housing; ST, EE, and SI; between-animal) × 3 (trial; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 29) = 18.57$, $p < .001$, $\eta_p^2 = .562$]. The subsequent analysis revealed that there were significant differences between ST and EE groups ($p < .001$, adjusted $p < .001$, $r = .75$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .63$). EE animals stayed on the rotarod longer than ST and SI animals on day 2. The main effect of trial was significant [$F(2, 58) = 7.64$, $p = .001$, $\eta_p^2 = .209$]. The interaction between housing and trial was not significant [$F(4, 58) = 0.31$, $p = .872$, $\eta_p^2 = .021$].

The ratio of performances of day 2 trial 1/day 1 trial 3 is shown in Figure 2g (12 ST animals vs. 8 EE animals vs. 12 SI animals). There was no significant difference between ST and EE groups ($p = .042$, adjusted $p = .126$, $r = .37$), between ST and SI groups ($p = .236$, adjusted $p = .472$, $r = .22$) or between EE and SI groups ($p = .304$, adjusted $p = .472$, $r = .19$).

Figure 3

Sensory function.



(a) Latency to response in the hot plate test. (b) Acoustic startle response. a.u.: arbitrary unit. (c) Prepulse inhibition. Bars in the centre represent the means. Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

The latency to response is shown in Figure 3a (12 ST animals vs. 8 EE animals vs. 12 SI animals). There were significant differences between ST and EE groups (p = .003, adjusted p = .009, r = .52) and between EE and SI groups (p = .008, adjusted p = .016, r = .47). EE animals responded earlier to foot heat than ST and SI animals.

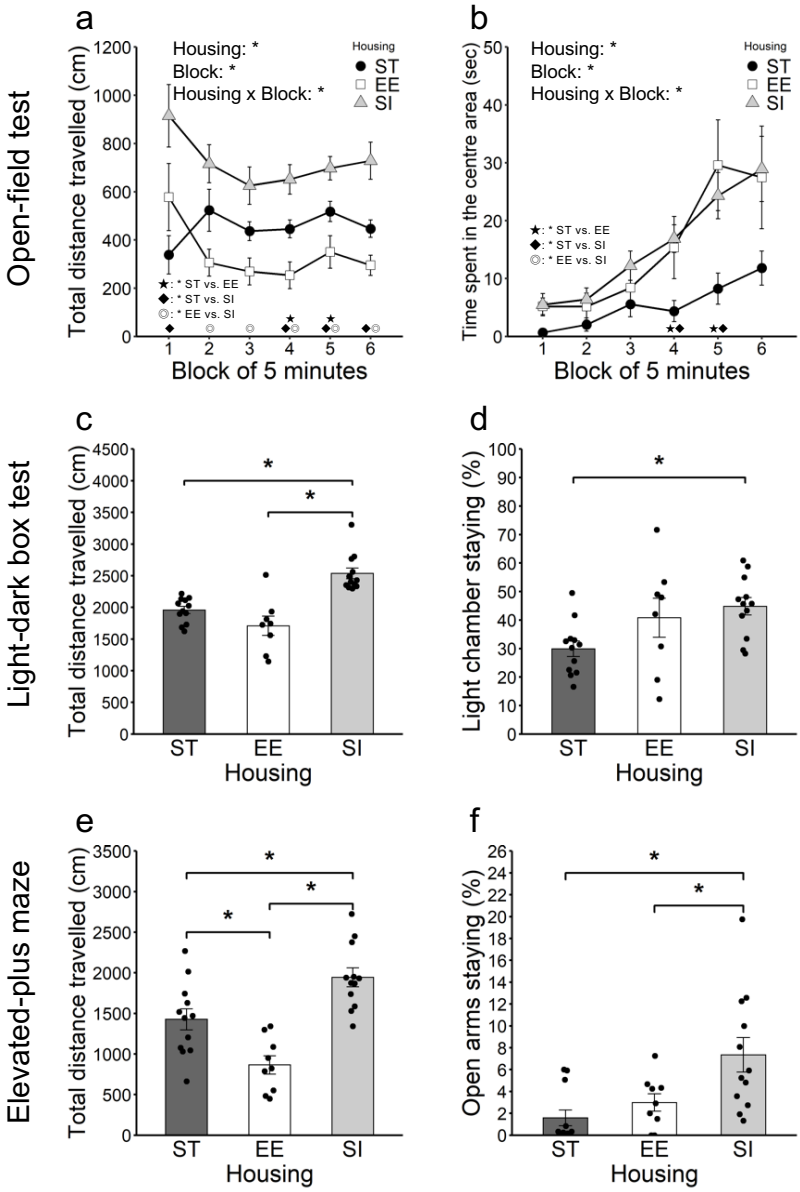
Acoustic startle response is shown in Figure 3b (12 ST animals vs. 8 EE animals vs. 12 SI animals). A 3 (housing; ST, EE, and SI; between-animal) × 4 (stimulus; within-animal) ANOVA was conducted. The main effect of housing was not significant [$F(2, 29) = 2.24$, $p = .125$, $\eta_p^2 = .134$]. The main effect of stimulus was significant [$F(3, 87) = 44.43$, $p < .001$, $\eta_p^2 = .605$]. The interaction between housing and stimulus was significant [$F(6, 87) = 3.00$, $p = .010$, $\eta_p^2 = .171$]. The subsequent analysis revealed that there were significant differences at 90 dB between ST and EE groups ($p < .001$, adjusted $p < .001$, $r = .66$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .64$), and there were significant differences at 100 dB between ST and EE groups ($p < .001$, adjusted $p < .001$, $r = .68$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .65$). EE animals showed enhanced startle response compared with ST and SI animals to 90- and 100-dB acoustic stimuli, indicating enhanced response to relatively weak acoustic stimuli in EE animals.

Prepulse inhibition rate is shown in Figure 3c (12 ST animals vs. 8 EE animals vs. 12 SI animals). A 3 (housing; ST, EE, and SI; between-animal) × 4 (stimulus; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 29) = 8.98$, $p = .001$, $\eta_p^2 = .383$]. The subsequent analysis revealed that there were significant differences between ST and EE groups ($p = .001$, adjusted $p = .002$, $r = .57$) and between ST and SI groups ($p = .002$, adjusted $p = .004$, $r = .54$). EE and SI animals showed enhanced prepulse inhibition compared with ST animals. The main effect of stimulus was significant [$F(3, 87) = 72.05$, $p < .001$, $\eta_p^2 = .713$]. The interaction between housing and stimulus was not significant [$F(6,$

87) = 1.16, p = .334, η_p^2 = .074].

Figure 4

Activity level and anxiety-like behaviour.



(a) Total distance travelled in the open-field test. (b) Time spent in the centre area in the open-field test. (c) Total distance travelled in the light–dark box test. (d) Percent of time spent in the light chamber in the light–dark box test. (e) Total distance travelled in the elevated-plus maze. (f) Percent of time staying in open arms in the elevated-plus maze. Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See

below for detailed results.

The total distance travelled is shown in Figure 4a (12 ST animals vs. 9 EE animals vs. 12 SI animals). A 3 (housing; ST, EE, and SI; between-animal) × 6 (block; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 30) = 12.57$, $p < .001$, $\eta_p^2 = .456$]. The subsequent analysis revealed that there were significant differences between ST and SI groups ($p = .001$, adjusted $p = .002$, $r = .55$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .65$). SI animals showed longer total distance travelled than ST and EE animals. The main effect of block was significant [$F(5, 150) = 3.93$, $p = .002$, $\eta_p^2 = .116$]. The interaction between housing and block was significant [$F(10, 150) = 3.39$, $p = .001$, $\eta_p^2 = .184$]. The subsequent analysis revealed that EE animals travelled less than ST or SI animals at all but the first block, and SI animals travelled more than ST or EE animals at all blocks.

Time spent in the centre area is shown in Figure 4b (12 ST animals vs. 9 EE animals vs. 12 SI animals). A 3 (housing; ST, EE, and SI; between-animal) × 6 (block; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 30) = 5.02$, $p = .013$, $\eta_p^2 = .251$]. The subsequent analysis revealed that there were significant differences between ST and EE groups ($p = .017$, adjusted $p = .034$, $r = .42$) and between ST and SI groups ($p = .008$, adjusted $p = .023$, $r = .46$). EE and SI animals spent more time in the centre area than ST animal. The main effect of block was significant [$F(5, 150) = 26.23$, $p < .001$, $\eta_p^2 = .467$]. The interaction between housing and block was significant [$F(10, 150) = 2.26$, $p = .017$, $\eta_p^2 = .131$]. The subsequent analysis revealed that EE and SI animals spent more time in the centre area than ST animals at the fourth and fifth blocks.

The total distance travelled is shown in Figure 4c (12 ST animals vs. 8 EE animals vs. 12 SI animals). There were significant differences between ST and SI groups ($p < .001$,

adjusted $p < .001$, $r = .66$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .74$). SI animals travelled more than ST and EE animals.

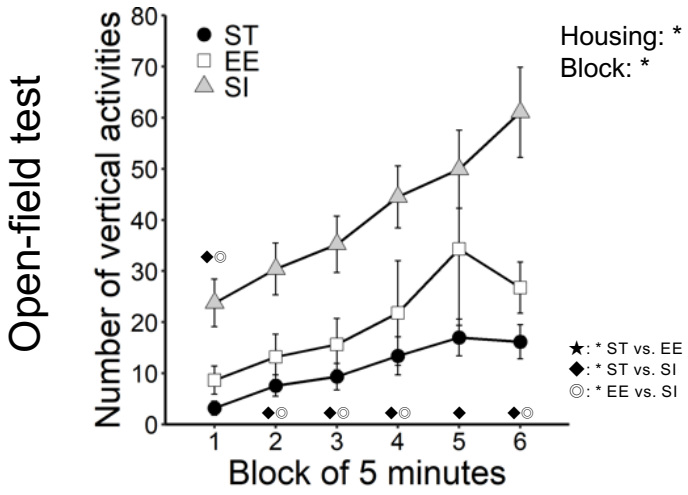
The percent of time animals spent in the light chamber is shown in Figure 4d (12 ST animals vs. 8 EE animals vs. 12 SI animals). There were significant differences between ST and SI groups ($p = .008$, adjusted $p = .025$, $r = .47$). SI animals spent more time in the light chamber than ST animals.

The total distance travelled is shown in Figure 4e (12 ST animals vs. 9 EE animals vs. 12 SI animals). There were significant differences between ST and EE groups ($p = .004$, adjusted $p = .008$, $r = .50$), between ST and SI groups ($p = .004$, adjusted $p = .008$, $r = .50$) and EE and SI group ($p < .001$, adjusted $p < .001$, $r = .74$). SI animals showed the longest and EE animals showed the shortest total distance travelled.

The percent of time staying in open arms is shown in Figure 4f (12 ST animals vs. 9 EE animals vs. 12 SI animals). There were significant differences between ST and SI groups ($p = .001$, adjusted $p = .003$, $r = .56$) and between EE and SI groups ($p = .015$, adjusted $p = .030$, $r = .43$). SI animals spent more time in open arms than ST and EE animals.

Figure 5

The number of vertical activities in the open-field test.

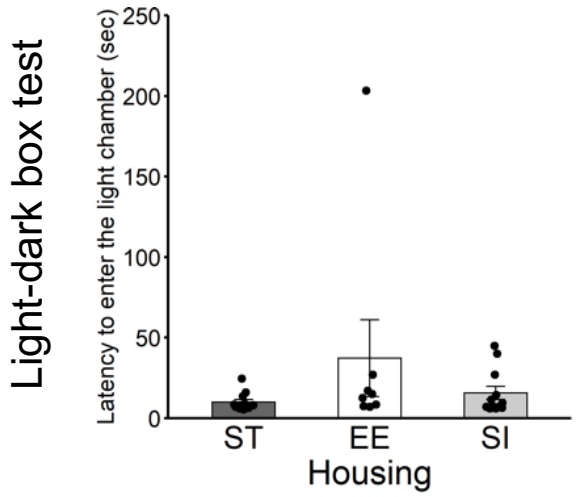


Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

The number of vertical activities is shown in Figure 5 (12 ST animals vs. 9 EE animals vs. 12 SI animals). A 3 (housing; ST, EE, and SI; between-animal) × 6 (block; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 30) = 11.83$, $p < .001$, $\eta_p^2 = .441$]. The subsequent analysis revealed that there were significant differences between ST and SI groups ($p < .001$, adjusted $p < .001$, $r = .66$) and between EE and SI groups ($p = .004$, adjusted $p = .009$, $r = .49$). SI animals showed more vertical activities than ST and EE animals. The main effect of block was significant [$F(5, 150) = 15.32$, $p < .001$, $\eta_p^2 = .338$]. The interaction between housing and block was not significant [$F(10, 150) = 1.60$, $p = .112$, $\eta_p^2 = .096$].

Figure 6

The latency to enter the light chamber in the light-dark box test.

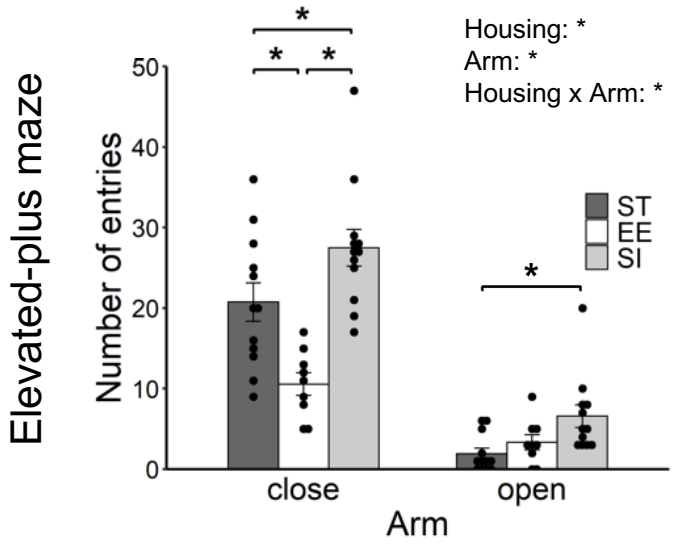


Error bars represent standard errors of the mean. See below for detailed results.

The latency to enter the light chamber is shown in Figure 6 (12 ST animals vs. 8 EE animals vs. 12 SI animals). There was no significant difference between ST and EE groups (p = .093, adjusted p = .280, r = .31), between ST and SI groups (p = .692, adjusted p = .692, r = .07) or between EE and SI groups (p = .179, adjusted p = .358, r = .25).

Figure 7

The number of entries into arms in the elevated-plus maze.



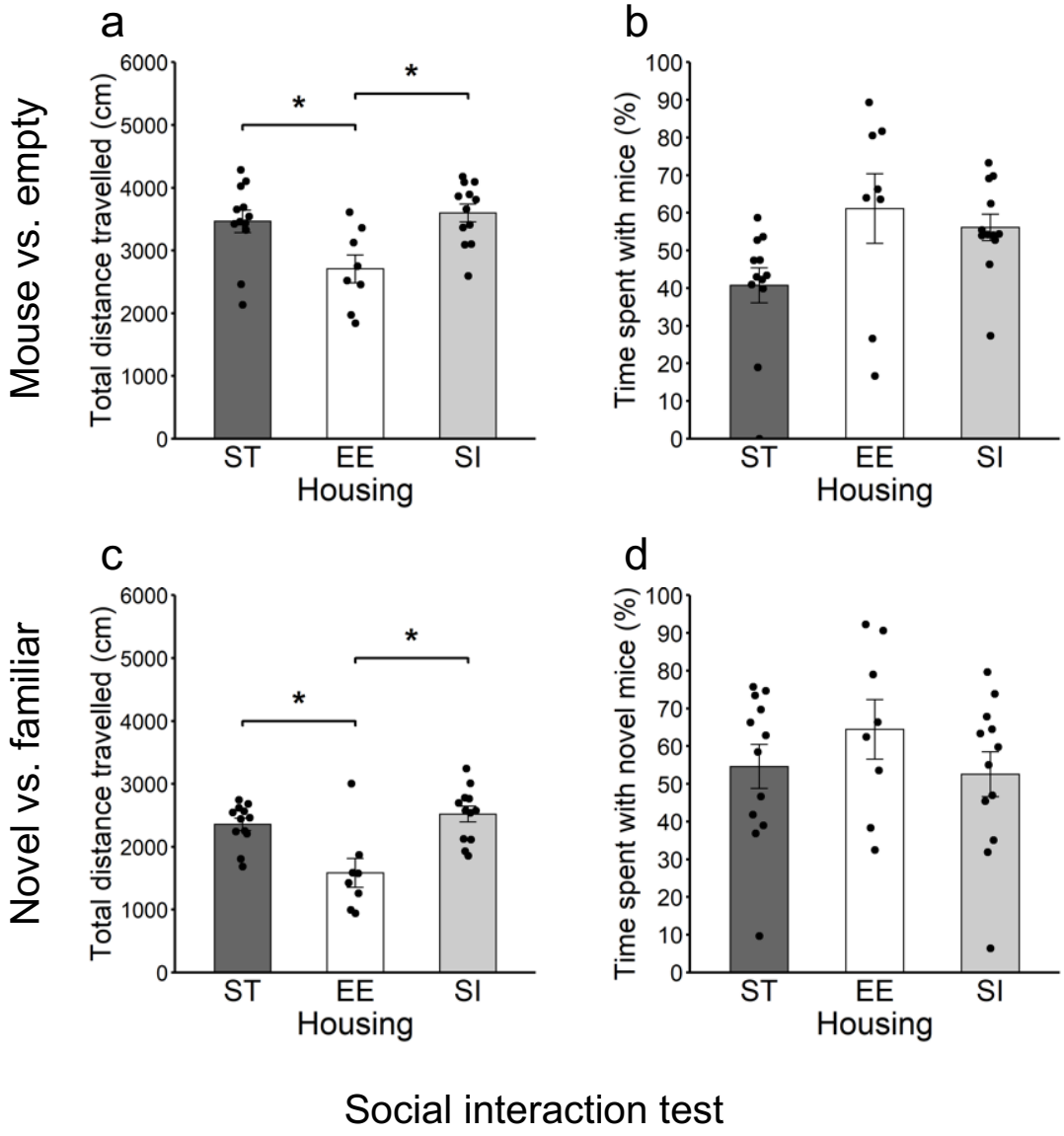
Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

The number of entries into arms is shown in Figure 7 (12 ST animals vs. 9 EE animals vs. 12 SI animals). A 3 (housing; ST, EE and SI; between-animal) × 2 (arm; close and open; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 30) = 13.62$, $p < .001$, $\eta_p^2 = .476$]. The subsequent analysis revealed that there were significant differences at closed arms between ST and EE groups ($p = .033$, adjusted $p = .033$, $r = .38$), between ST and SI groups ($p = .004$, adjusted $p = .008$, $r = .50$) and EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .68$). SI animals entered to arms most frequently, and EE animals entered to arms least frequently. The main effect of arm was significant [$F(1, 30) = 153.13$, $p < .001$, $\eta_p^2 = .836$]. The interaction between housing and arm was significant [$F(2, 30) = 10.35$, $p < .001$, $\eta_p^2 = .408$]. The subsequent analysis revealed that SI animals entered to closed arms most frequently and EE animals entered to closed arms least

frequently, and SI animals entered to open arms more than ST animals.

Figure 8

Sociality (Crawley's social interaction test).



(a) Total distance travelled in the trial of mouse cage versus empty cage. (b) Percent of time staying around the mouse cage in the trial of mouse cage versus empty cage. (c) Total distance travelled in the trial of novel mouse cage versus familiar mouse cage. (d) Percent of time staying around the novel mouse cage in the trial of novel mouse cage versus familiar

mouse cage. Error bars represent standard errors of the mean. See below for detailed results.

The total distance travelled in the mouse cage versus empty cage trial is shown in Figure 8a (12 ST animals vs. 8 EE animals vs. 12 SI animals). There were significant differences between ST and EE groups (p = .008, adjusted p = .015, r = .47) and between EE and SI groups (p = .002, adjusted p = .006, r = .53). EE animals showed shorter total distance travelled than ST and SI animals.

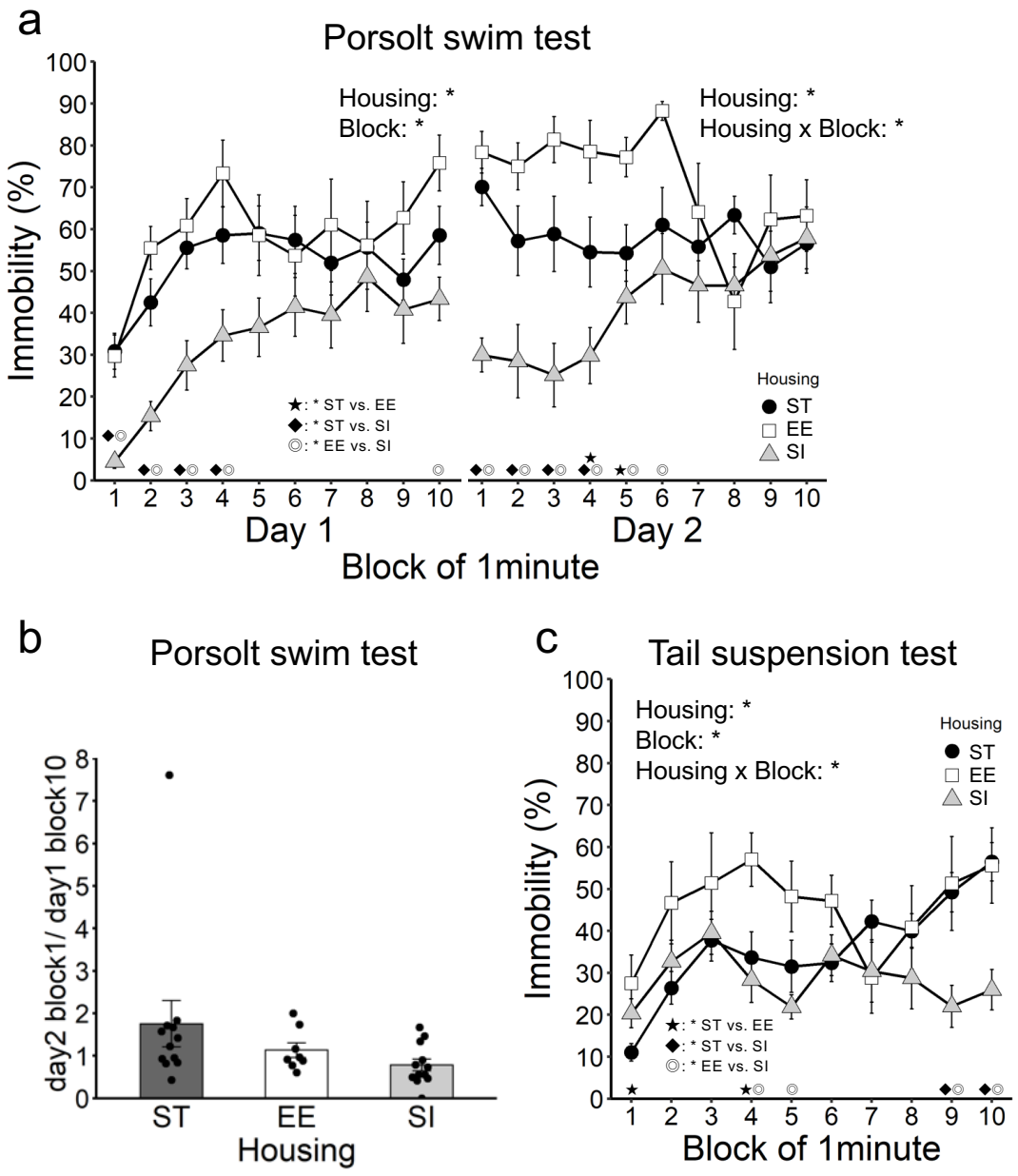
The percent of time staying around the novel mouse cage is shown in Figure 8b (12 ST animals vs. 8 EE animals vs. 12 SI animals). There was no significant difference between ST and EE groups (p = .019, adjusted p = .056, r = .42), between ST and SI groups (p = .044, adjusted p = .088, r = .36) or between EE and SI groups (p = .545, adjusted p = .545, r = .11).

The total distance travelled in the familiar mouse cage versus novel mouse cage is shown in Figure 8c (12 ST animals vs. 8 EE animals vs. 12 SI animals). There were significant differences between ST and EE groups (p = .001, adjusted p = .002, r = .56) and between EE and SI groups (p < .001, adjusted p < .001, r = .63). EE animals showed shorter total distance travelled than ST and SI animals.

The percent of time staying around the novel mouse cage is shown in Figure 8d (12 ST animals vs. 8 EE animals vs. 12 SI animals). There was no significant difference between ST and EE groups (p = .312, adjusted p = .665, r = .19), between ST and SI groups (p = .807, adjusted p = .807, r = .05) or between EE and SI groups (p = .222, adjusted p = .665, r = .23).

Figure 9

Stress-coping strategy.



(a) Percent of immobile time in the Porsolt swim test. (b) Ratio of immobile percent of day2 block 1/day1 block 10 in the Porsolt swim test. (c) Percent of immobile time in the tail suspension test. Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

The percent of immobile time in day 1 is shown in Figure 9a left (12 ST animals vs. 8 EE animals vs. 12 SI animals). A 3 (housing; ST, EE, and SI; between-animal) × 10 (block; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 29) = 13.15$, $p < .001$, $\eta_p^2 = .476$]. The subsequent analysis revealed that there were significant differences between ST and SI groups ($p = .001$, adjusted $p = .001$, $r = .58$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .66$). SI animals showed lower immobility than ST and EE animals on day 1. The main effect of block was significant [$F(9, 261) = 8.96$, $p < .001$, $\eta_p^2 = .236$]. The interaction between housing and block was not significant [$F(18, 261) = 1.03$, $p = .425$, $\eta_p^2 = .066$].

The percent of immobile time on day 2 is shown in Figure 9a right (12 ST animals vs. 8 EE animals vs. 12 SI animals). A 3 (housing; ST, EE, and SI; between-animal) × 10 (block; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 29) = 10.57$, $p < .001$, $\eta_p^2 = .422$]. The subsequent analysis revealed that there were significant differences between ST and SI groups ($p = .008$, adjusted $p = .015$, $r = .47$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .64$). Again, SI animals showed lower immobility than ST and EE animals on day 2. The main effect of block was not significant [$F(9, 261) = 1.26$, $p = .258$, $\eta_p^2 = .042$]. The interaction between housing and block was significant [$F(18, 261) = 3.14$, $p < .001$, $\eta_p^2 = .178$]. The subsequent analysis revealed that EE animals showed higher immobility than ST or SI animals in blocks 1–6, and SI animals showed lower immobility than ST or EE animals in blocks 1–6.

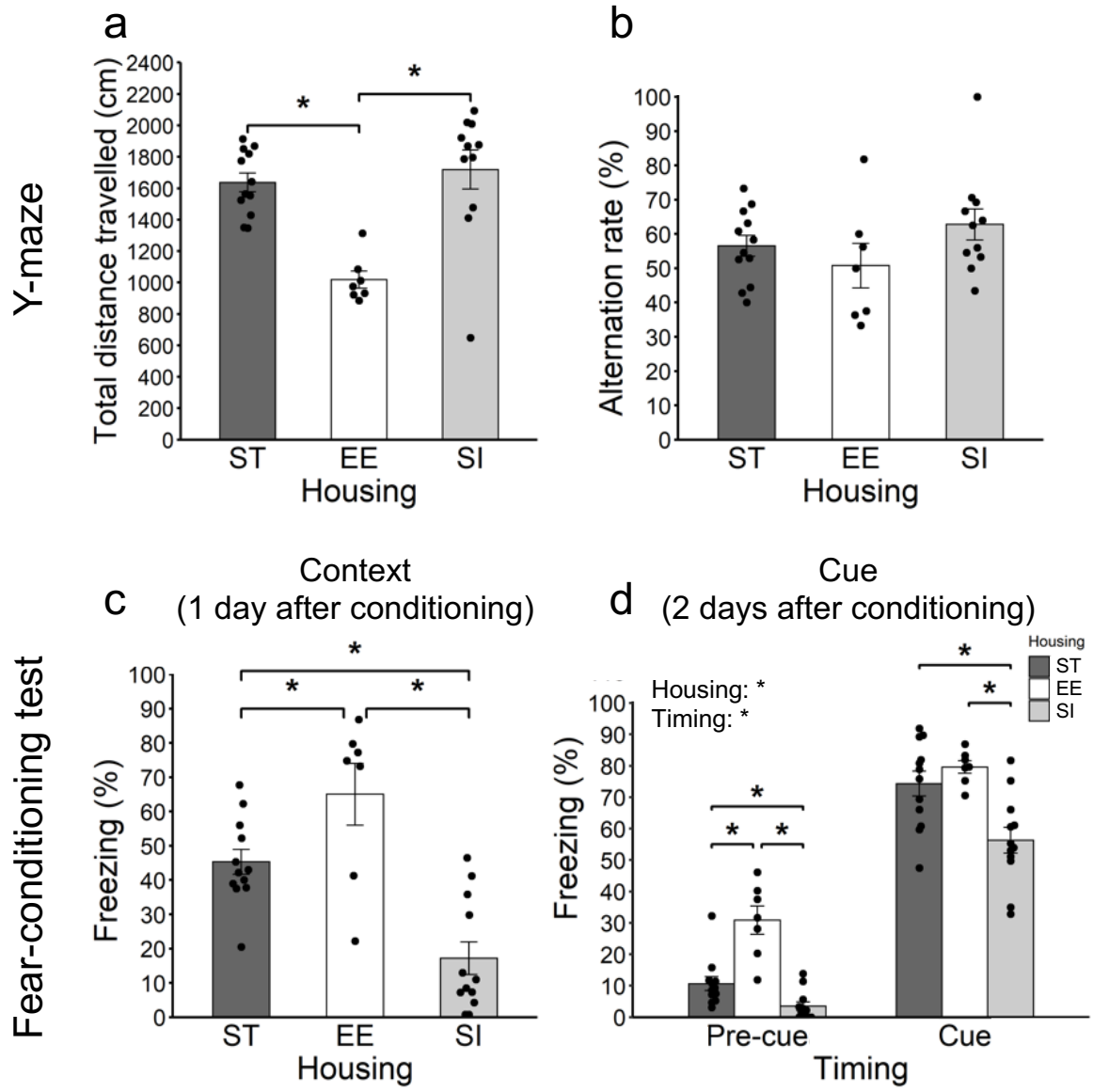
The ratio of the immobile percent of day 2 block 1/day 1 block 1 is shown in Figure 9b (12 ST animals vs. 8 EE animals vs. 12 SI animals). There was no significant difference between ST and EE groups ($p = .278$, adjusted $p = .556$, $r = .20$), between ST and SI groups ($p = .063$, adjusted $p = .189$, $r = .34$) or between EE and SI groups ($p = .538$, adjusted

p = .556, r = .11).

The percent of immobile time is shown in Figure 9c (12 ST animals vs. 7 EE animals vs. 12 SI animals). A 3 (housing; ST, EE, and SI; between-animal) × 10 (block; within-animal) ANOVA was conducted. The main effect of housing was significant [F(2, 28) = 5.23, p = .012, η_p^2 = .272]. The subsequent analysis revealed that there was a significant difference between EE and SI groups (p = .003, adjusted p = .010, r = .52). SI animals showed lower immobility than EE animals. The main effect of block was significant [F(9, 252) = 5.67, p < .001, η_p^2 = .168]. The interaction between housing and block was significant [F(18, 252) = 2.87, p < .001, η_p^2 = .170]. The subsequent analysis revealed that EE animals showed higher immobility than ST or SI animals in blocks 1, 4, 5, 9 and 10, and SI animals showed lower immobility than ST or EE animals in blocks 4, 5, 9 and 10.

Figure 10

Spatial working memory and associative fear memory.



(a) Total distance travelled in the Y-maze. (b) Alternation rate in the Y-maze. (c) Percent of freezing time in contextual memory test. (d) Percent of freezing time in cued memory test. Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

The total distance travelled is shown in Figure 10a (12 ST animals vs. 7 EE animals vs. 11 SI animals). There were significant differences between ST and EE groups ($p < .001$, adjusted $p < .001$, $r = .65$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .69$). EE animals showed shorter total distance travelled than ST and SI animals.

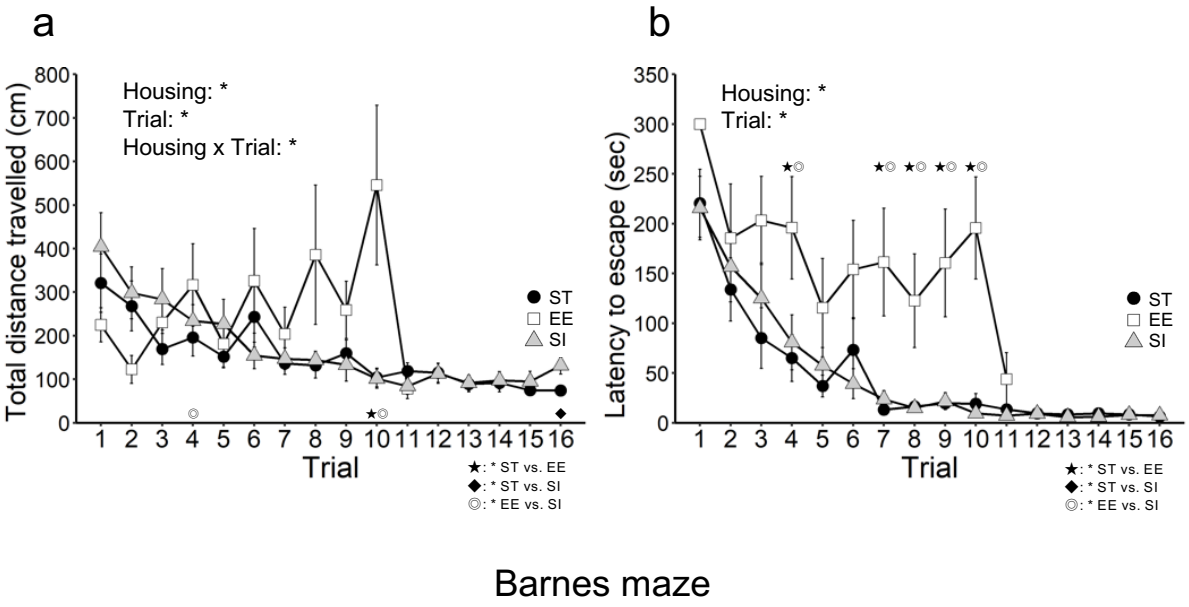
Alternation rate is shown in Figure 10b (12 ST animals vs. 7 EE animals vs. 11 SI animals). There was no significant difference between ST and EE groups ($p = .390$, adjusted $p = .590$, $r = .17$), between ST and SI groups ($p = .295$, adjusted $p = .590$, $r = .20$) or between EE and SI groups ($p = .086$, adjusted $p = .259$, $r = .32$).

Percent of freezing time in context test is shown in Figure 10c (12 ST animals vs. 7 EE animals vs. 12 SI animals). There were significant differences between ST and EE groups ($p = .022$, adjusted $p = .022$, $r = .42$), between ST and SI groups ($p < .001$, adjusted $p = .001$, $r = .61$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .74$). EE animals showed most, and SI animals showed least, freezing.

Percent of freezing time in the cue test is shown in Figure 10d (12 ST animals vs. 7 EE animals vs. 12 SI animals). A 3 (housing; ST, EE and SI; between-animal) × 2 (timing; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 28) = 22.97$, $p < .001$, $\eta_p^2 = .621$]. The subsequent analysis revealed that there were significant differences between ST and EE groups ($p = .002$, adjusted $p = .002$, $r = .54$), between ST and SI groups ($p = .001$, adjusted $p = .001$, $r = .59$) and between EE and SI groups ($p < .001$, adjusted $p < .001$, $r = .78$). EE animals showed most, and SI animals showed least, freezing. The main effect of timing was significant [$F(1, 28) = 460.46$, $p < .001$, $\eta_p^2 = .943$]. The interaction between housing and timing was not significant [$F(2, 28) = 3.12$, $p = .060$, $\eta_p^2 = .182$].

Figure 11

The training session in the Barnes maze.



(a) Total distance travelled. (b) Latency to escape to the target hole. Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

In the training sessions, I found that EE mice did not escape to the hiding holes as expected to be the default behaviour in this test, suggesting less motivation in EE mice to avoid bright light and spacious open place. I excluded EE animals from this further testing at training trial 12, because it is likely the result could not be used to appropriately evaluate the learning and memory functions of EE animals.

The total distance travelled in the training session is shown in Figure 11a. In trial 1 - 11 (12 ST animals vs. 7 EE animals vs. 11 SI animals), a 3 (housing; ST, EE, and SI; between-animal) × 11 (trial; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(2, 27) = 4.01$, $p = .030$, $\eta_p^2 = .229$]. The subsequent analysis revealed that there

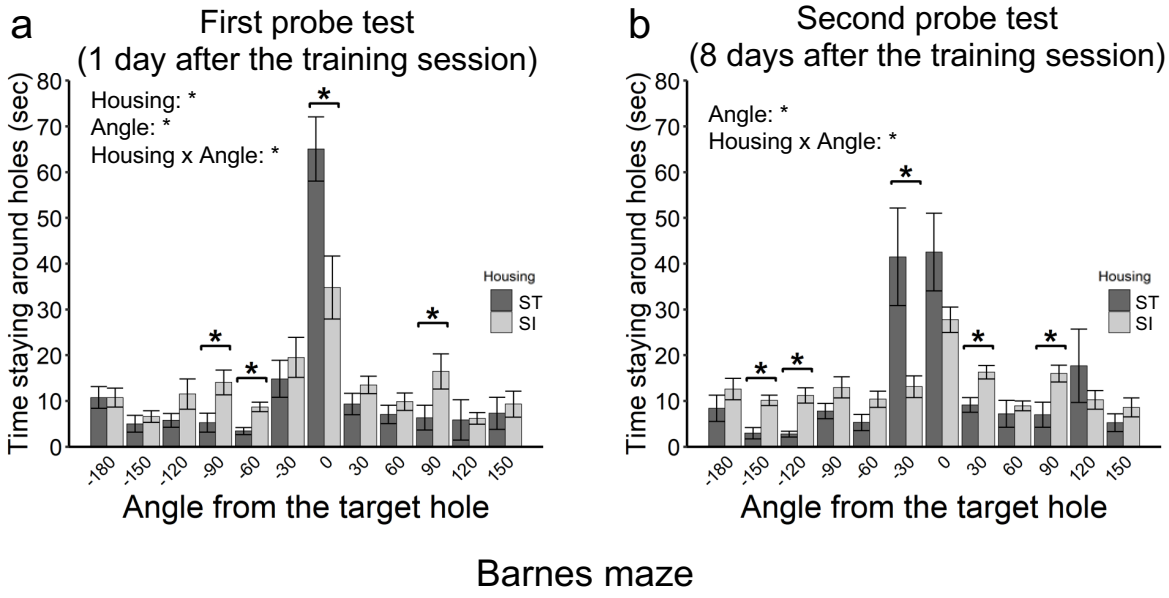
were significant differences between ST and EE group (p = .013, adjusted p = .040, r = .45) and between EE and SI group (p = .022, adjusted p = .044, r = .42). EE animals showed longer total distance travelled than ST and SI animals. The main effect of trial was significant [F(10, 270) = 3.03, p = .001, η_p^2 = .101]. The interaction between housing and trial was significant [F(20, 270) = 3.17, p < .001, η_p^2 = .190]. The subsequent analysis revealed that EE animals showed longer total distance travelled than ST or SI animals in trial 4 and 10. In trial 12 - 16 (12 ST animals vs. 11 SI animals), a 2 (housing; ST and SI; between-animal) × 5 (trial; within-animal) ANOVA was conducted. The main effect of housing was not significant [F(1, 21) = 1.03, p =.321, η_p^2 = .047]. The main effect of trial was not significant [F(4, 84) = 0.91, p = .464, η_p^2 = .041]. The interaction between housing and trial was not significant [F(4, 84) = 1.01, p = .407, η_p^2 = .046].

The latency to escape to the target hole in the training session is shown in Figure 11b. In trial 1 - 11 (12 ST animals vs. 7 EE animals vs. 11 SI animals), a 3 (housing; ST, EE, and SI; between-animal) × 11 (trial; within-animal) ANOVA was conducted. The main effect of housing was significant [F(2, 27) = 14.63, p < .001, η_p^2 = .520]. The subsequent analysis revealed that there were significant differences between ST and EE group (p < .001, adjusted p < .001, r = .69) and between EE and SI group (p < .001, adjusted p < .001, r = .67). EE animals showed longest latency than ST and SI animals. The main effect of trial was significant [F(10, 270) = 17.98, p < .001, η_p^2 = .400]. The interaction between housing and trial was not significant [F(20, 270) = 1.06, p = .390, η_p^2 = .073]. In trial 12 - 16 (12 ST animals vs. 11 SI animals), a 2 (housing; ST and SI; between-animal) × 5 (trial; within-animal) ANOVA was conducted. The main effect of housing was not significant [F(1, 21) = 0.96, p =.339, η_p^2 = .044]. The main effect of trial was not significant [F(4, 84) = 0.84, p = .506, η_p^2 = .038]. The interaction between housing and trial was not significant [F(4, 84) = .0.83, p = .512, η_p^2

= .038].

Figure 12

Spatial reference memory (the Barnes maze).



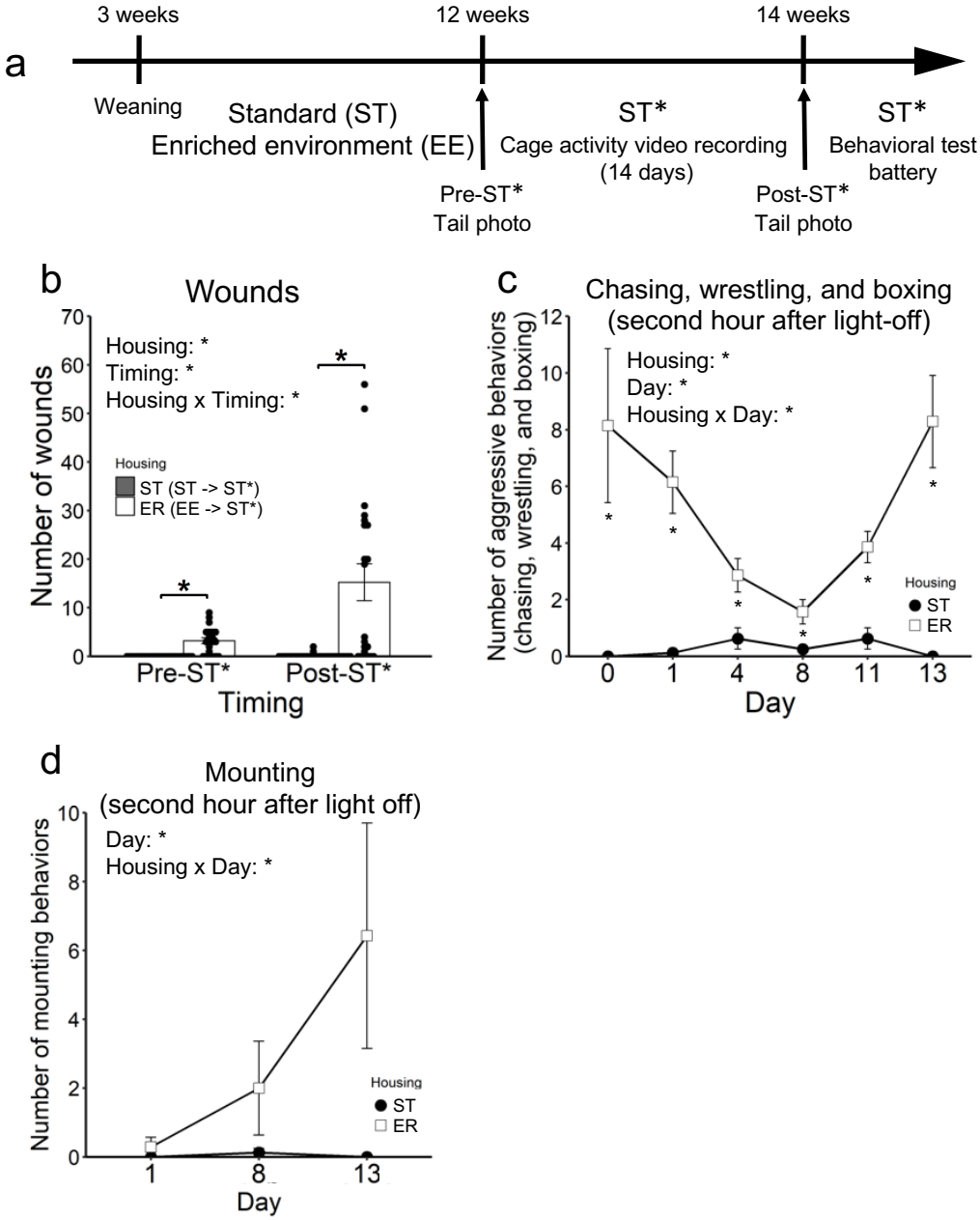
(a) Time staying around holes in the first probe test. (b) Time staying around holes in the second probe test. Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

Time staying around holes in the first probe test (1-day post-training) is shown in Figure 12a (12 ST animals vs. 12 SI animals). A 2 (housing; ST and SI; between-animal) × 12 (angle; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(1, 22) = 7.86$, $p = .010$, $\eta_p^2 = .263$], indicating that SI animals stayed around the area of holes longer than ST animals. The main effect of angle was significant [$F(11, 242) = 25.67$, $p < .001$, $\eta_p^2 = .539$]. The interaction between housing and angle was significant [$F(11, 242) = 4.74$, $p < .001$, $\eta_p^2 = .177$]. The subsequent analysis revealed that ST animals stayed around the target hole (0 angles) longer than SI animals, whereas SI animals stayed around holes of −90, −60 and 90 angles longer than ST animals.

The time staying around holes in the second probe test (eight days post-training) is shown in Figure 12b (12 ST animals vs. 12 SI animals). A 2 (housing; ST and SI; between-animal) × 12 (angle; within-animal) ANOVA was conducted. The main effect of housing was not significant [$F(1, 22) = 0.02$, $p = .897$, $\eta_p^2 < .001$]. The main effect of angle was significant [$F(11, 242) = 10.55$, $p < .001$, $\eta_p^2 = .324$]. The interaction between housing and angle was significant [$F(11, 242) = 4.26$, $p < .001$, $\eta_p^2 = .162$]. The subsequent analysis revealed that ST animals stayed around the hole of 30 angles longer than SI animals, whereas SI animals stayed around holes of −150, −120, 30 and 90 angles longer than ST animals, suggesting less memory acuity in the SI mice.

Figure 13

Fighting behaviour under enrichment removal.



(a) The schema of Experiment 2. (b) the number of tail wounds pre-ST* and post-ST*. (c) The number of aggressive interactions (chasing, wrestling, and boxing) within the second hour after light-off in ST*. (d) The number of mounting behaviour within the second hour after light-off in ST*. Asterisks represent adjusted $p < .05$. See below for detailed results.

The numbers of tail wounds before and after ST* are shown in Figure 13b (24 ST [ST - > ST*] animals vs. 21 ER [EE - > ST*] animals). A 2 (housing; ST and ER; between-animal) × 2 (timing; pre-ST* and post- ST*; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(1, 43) = 22.98$, $p < .001$, $\eta_p^2 = .348$], indicating that ER animals had more wounds than ST animals. The main effect of timing was significant [$F(1, 43) = 12.63$, $p = .001$, $\eta_p^2 = .227$]. The interaction between housing and timing was significant [$F(1, 43) = 12.81$, $p = .001$, $\eta_p^2 = .230$]. The subsequent analysis revealed that ER animals had more wounds than ST animals in both pre-ST* and post-ST*; furthermore, the number of wounds of ER animals increased in post-ST* compared with pre-ST*.

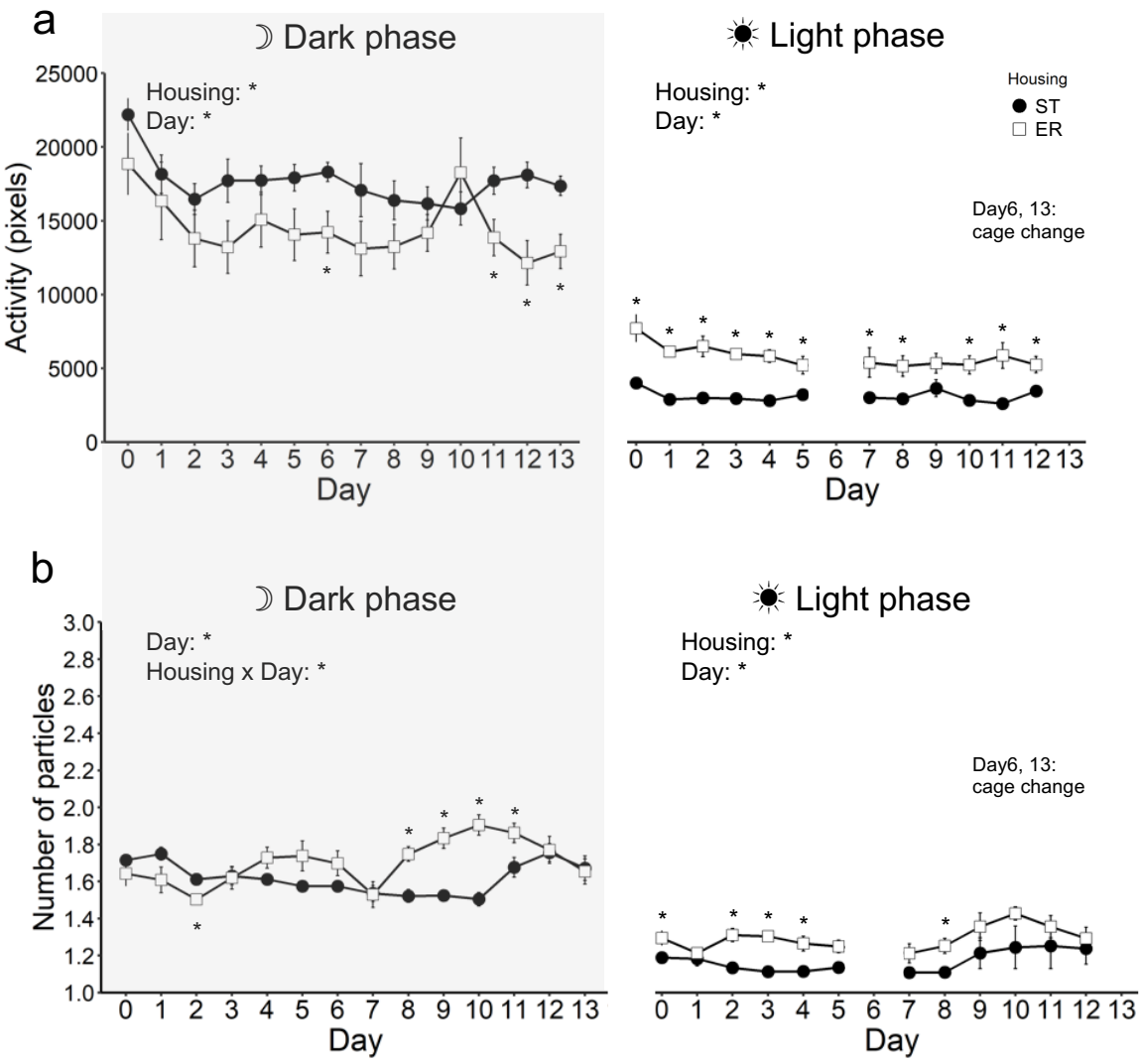
The numbers of aggressive behaviour (chasing, wrestling, and boxing) under ST* are shown in Figure 13c (eight ST cages vs. seven ER cages). A 2 (housing; ST and ER; between-animal) × 6 (day; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(1, 13) = 88.10$, $p < .001$, $\eta_p^2 = .871$], indicating that ER animals showed chasing, wrestling and boxing more often than ST animals. The main effect of day was significant [$F(5, 65) = 3.67$, $p = .006$, $\eta_p^2 = .220$]. The interaction between housing and day was significant [$F(5, 65) = 4.97$, $p = .001$, $\eta_p^2 = .277$], indicating different temporal dynamics of aggressive behaviour between ST and ER animals. The subsequent analysis did not show any significant differences between any days on the data of ER animals.

The number of mounting behaviour under ST* is shown in Figure 13d (eight ST cages vs. seven ER cages). A 2 (housing; ST and ER; between-animal) × 3 (day; within-animal) ANOVA was conducted. The main effect of housing was not significant [$F(1, 13) = 3.81$, $p = .073$, $\eta_p^2 = .223$]. The main effect of day was significant [$F(2, 26) = 4.41$, $p = .022$, $\eta_p^2 = .254$]. The interaction between housing and day was significant [$F(2, 26) = 4.51$, $p = .021$, $\eta_p^2 = .258$], indicating different temporal dynamics of mounting behaviour between

ST and ER animals. The subsequent analysis did not show any significant differences between any days on the data of ER animals.

Figure 14

Activity level and social behaviour under enrichment removal.



(a) Average of the activity level in the dark phase (left) and the light phase (right). (b) The average of number of particles of animals in the dark phase (left) and the light phase (right). Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

The average of activity level in the dark phase is shown in Figure 14a left (eight ST cages vs. seven ER cages). A 2 (housing; ST and ER; between-animal) × 14 (day; within-

animal) ANOVA was conducted. The main effect of housing was significant [$F(1, 13) = 5.73$, $p = .033$, $\eta_p^2 = .306$], indicating that ER animals showed lower activity level than ST animals in the dark phase. The main effect of day was significant [$F(13, 169) = 3.27$, $p < .001$, $\eta_p^2 = .201$]. The interaction between housing and day was not significant [$F(13, 169) = 1.39$, $p = .170$, $\eta_p^2 = .096$].

The average of activity level in the light phase is shown in Figure 14a right (eight ST cages vs. seven ER cages). A 2 (housing; ST and ER; between-animal) × 12 (day; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(1, 13) = 28.75$, $p < .001$, $\eta_p^2 = .689$], indicating that ER animals showed higher activity level than ST animals in the light phase. The main effect of day was significant [$F(11, 143) = 3.23$, $p = .001$, $\eta_p^2 = .199$]. The interaction between housing and day was not significant [$F(11, 143) = 1.62$, $p = .099$, $\eta_p^2 = .111$].

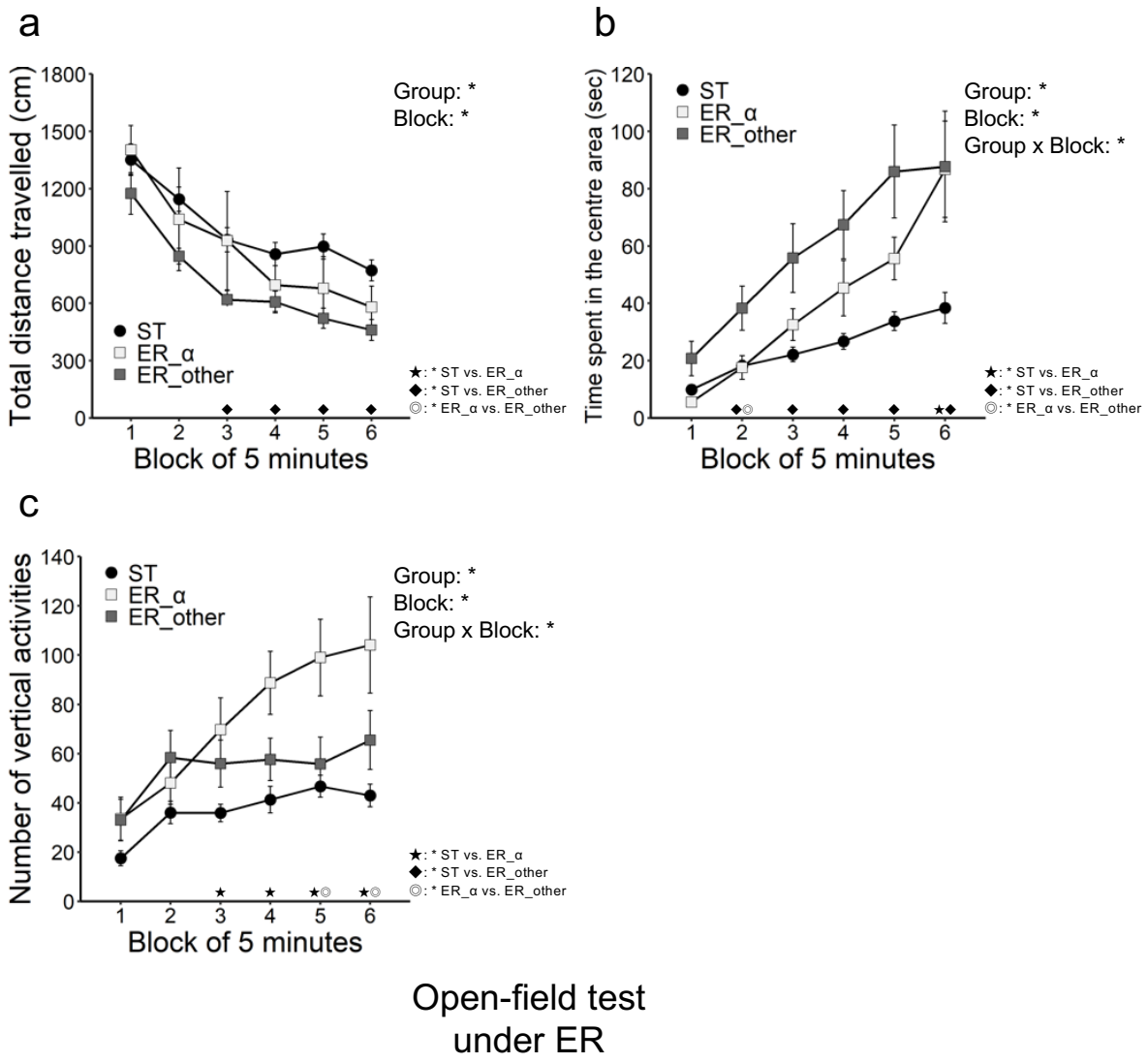
The average of number of particles in the dark phase is shown in Figure 14b left (eight ST cages vs. seven ER cages). A 2 (housing; ST and ER; between-animal) × 14 (day; within-animal) ANOVA was conducted. The main effect of housing was not significant [$F(1, 13) = 3.87$, $p = .071$, $\eta_p^2 = .230$]. The main effect of day was significant [$F(13, 169) = 4.93$, $p < .001$, $\eta_p^2 = .275$]. The interaction between housing and day was significant [$F(13, 169) = 7.51$, $p < .001$, $\eta_p^2 = .366$]. The subsequent analysis revealed that ER animals kept longer distances from other cage mates than ST animals in the dark phase on days 8, 9, 10 and 11, whereas ST animals kept longer distances than ER animals on day 2.

The average of number of particles in the light phase is shown in Figure 14b right (eight ST cages vs. seven ER cages). A 2 (housing; ST and ER; between-animal) × 12 (day; within-animal) ANOVA was conducted. The main effect of housing was significant [$F(1, 13) = 5.14$, $p = .041$, $\eta_p^2 = .283$], indicating that ER animals are more distanced from other

cage mates than ST animals in the light phase. The main effect of day was significant [F(11, 143) = 1.97, p = .035, η_p^2 = .132]. The interaction between housing and day was not significant [F(11, 143) = 0.63, p = .804, η_p^2 = .046].

Figure 15

Activity level and anxiety-like behaviour of ER animals.



(a) Total distance travelled in the open-field test. (b) Time spent in the centre area in the open-field test. (c) The number of vertical activities in the open-field test. Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

The average number of wounds in ER_α animals after ST* was 0.14 (seven animals,

SEM = 0.14) and in ER_other animals was 22.79 (14 animals, *SEM* = 4.51).

The total distance travelled is shown in Figure 15a (24 ST animals vs. 7 ER_α animals vs. 14 ER_other animals). A 3 (group; ST, ER_α, and ER_other; between-animal) × 6 (block; within-animal) ANOVA was conducted. The main effect of group was significant [$F(2, 42) = 4.74$, $p = .014$, $\eta_p^2 = .184$]. The subsequent analysis revealed that there was significant difference between ST and ER_other groups ($p = .004$, adjusted $p = .011$, $r = .43$). ER_other animals showed less total distance travelled compared with ST animals. The main effect of block was significant [$F(5, 210) = 60.02$, $p < .001$, $\eta_p^2 = .588$]. The interaction between group and block was not significant [$F(10, 210) = 1.17$, $p = .315$, $\eta_p^2 = .053$].

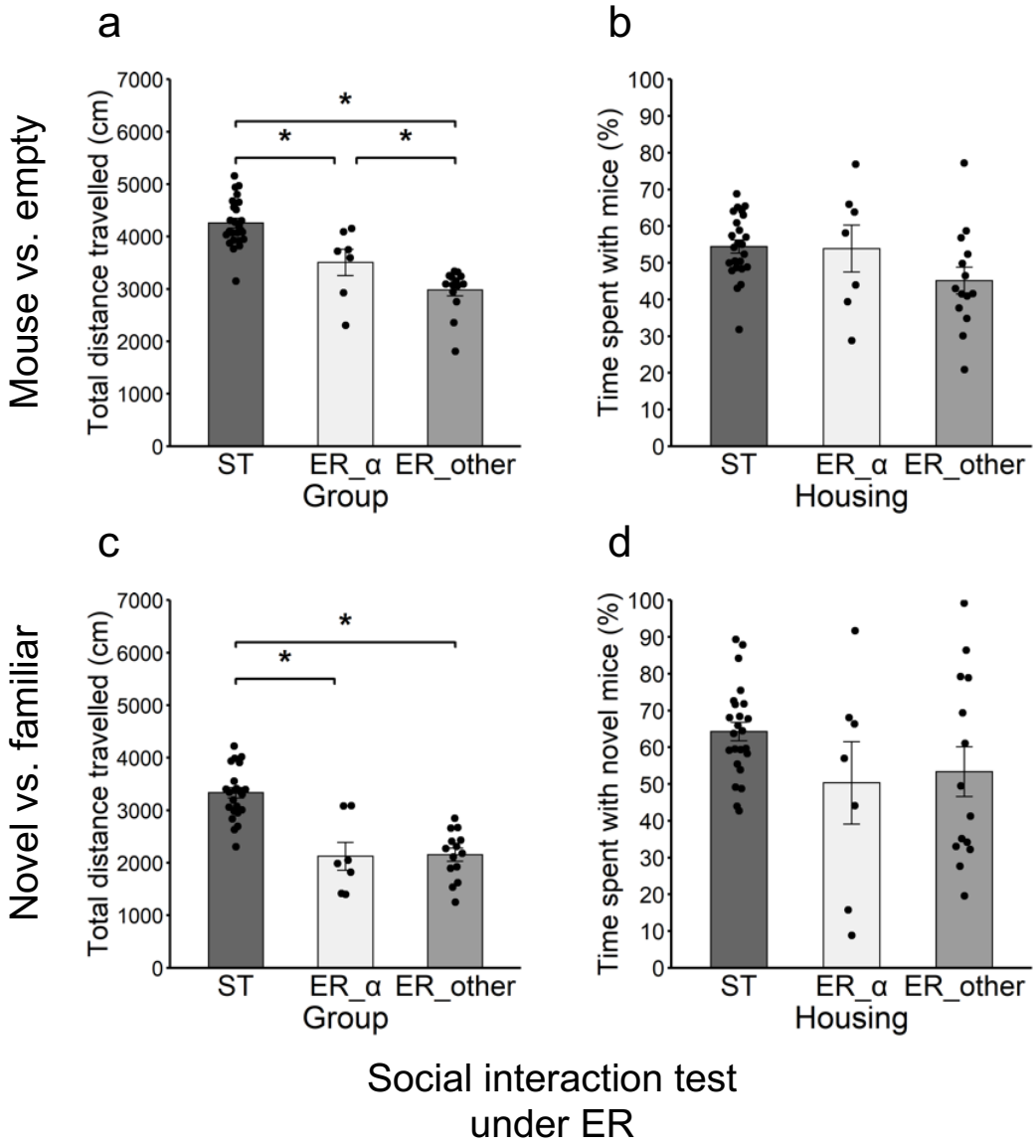
Time spent in the centre area is shown in Figure 15b (24 ST animals vs. 7 ER_α animals vs. 14 ER_other animals). A 3 (group; ST, ER_α, and ER_other; between-animal) × 6 (block; within-animal) ANOVA was conducted. The main effect of group was significant [$F(2, 42) = 9.22$, $p = .001$, $\eta_p^2 = .305$]. The subsequent analysis revealed that there was a significant difference between ST and ER_other groups ($p < .001$, adjusted $p < .001$, $r = .55$). ER_other animals spent more time in the centre area than ST animals. The main effect of block was significant [$F(5, 210) = 37.39$, $p < .001$, $\eta_p^2 = .471$]. The interaction between group and block was significant [$F(10, 210) = 4.04$, $p < .001$, $\eta_p^2 = .161$]. The subsequent analysis revealed that ER_α animals spent more time in the centre area than ST animals at the sixth block, and ER_other animals spent more time in the centre area than ST animals or ER_α animals at all but the first block.

The numbers of vertical activities are shown in Figure 15c (24 ST animals vs. 7 ER_α animals vs. 14 ER_other animals). A 3 (group; ST, ER_α, and ER_other; between-animal) × 6 (block; within-animal) ANOVA was conducted. The main effect of group was significant [$F(2, 42) = 7.00$, $p = .002$, $\eta_p^2 = .250$]. The subsequent analysis revealed that there was a

significant difference between ST and ER_α groups (p = .001, adjusted p = .003, r = .48). ER_α animals showed more vertical activities than ST animals. The main effect of block was significant [F(5, 210) = 25.80, p < .001, η_p^2 = 381]. The interaction between group and block was significant [F(10, 210) = 4.01, p < .001, η_p^2 = .160]. The subsequent analysis revealed that ER_α animals showed more vertical activities than ST animals or ER_other animals at all but the first two blocks.

Figure 16

The sociality of ER animals (Crawley's social interaction test).



(a) Total distance travelled in the trial of mouse cage vs. empty cage. (b) Percent of time staying around the mouse cage in the trial of mouse cage vs. empty cage. (c) Total distance travelled in the trial of novel mouse cage vs. familiar mouse cage. (d) Percent of time staying around the novel mouse cage in the trial of novel mouse cage vs. familiar mouse cage. Error bars represent standard errors of the mean. See below for detailed results.

The total distance travelled in the mouse cage versus empty cage trial is shown in Figure 16a (24 ST animals vs. 7 ER_α animals vs. 14 ER_other animals). There were significant differences between ST and ER_α groups (p = .001, adjusted p = .002 r = .49), between ST and ER_other groups (p < .001, adjusted p < .001, r = .77) and between ER_α and ER_other groups (p = .024, adjusted p = .024, r = .34). ST animals showed longest, and ER_other animals showed shortest total distance travelled.

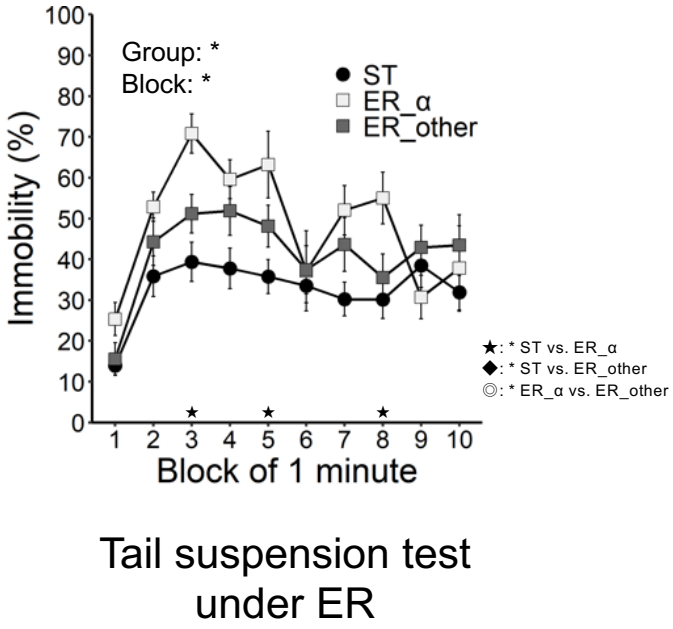
The percent of time staying around the novel mouse cage is shown in Figure 16b (24 ST animals vs. 7 ER_α animals vs. 14 ER_other animals). There was no significant difference between ST and ER_α groups (p = .915, adjusted p = .915, r = .02), between ST and ER_other groups (p = .025, adjusted p = .076, r = .34) or between ER_α and ER_other groups (p = .120, adjusted p = .240, r = .24).

The total distance travelled in the familiar mouse cage versus novel mouse cage trial is shown in Figure 16c (24 ST animals vs. 7 ER_α animals vs. 14 ER_other animals). There were significant differences between ST and ER_α groups (p < .001, adjusted p < .001, r = .64) and between ST and ER_other groups (p < .001, adjusted p < .001, r = .72). ST animals showed longer total distance travelled than ER_α and ER_other animals.

The percent of time staying around the novel mouse cage is shown in Figure 16d (24 ST animals vs. 7 ER_α animals vs. 14 ER_other animals). There was no significant difference between ST and ER_α groups (p = .116, adjusted p = .348, r = .24), between ST and ER_other groups (p = .118, adjusted p = .348, r = .24) or between ER_α and ER_other groups (p = .745, adjusted p = .745, r = .05).

Figure 17

Stress-coping strategy of ER animals.



Percent of immobile time in the tail suspension test. Error bars represent standard errors of the mean. Asterisks represent adjusted $p < .05$. See below for detailed results.

The percent of immobile time is shown in Figure 17 (23 ST animals vs. 7 EE animals vs. 13 SI animals). A 3 (group; ST, ER_α, and ER_other; between-animal) × 10 (block; within-animal) ANOVA was conducted. The main effect of group was significant [$F(2, 40) = 7.37$, $p = .002$, $\eta_p^2 = .269$]. The subsequent analysis revealed that there was a significant difference between ST and ER_α groups ($p = .001$, adjusted $p = .003$, $r = .49$) and between ST and ER_other groups ($p = .020$, adjusted $p = .040$, $r = .36$). ST animals showed lower immobility than ER_α and ER_other animals. The main effect of block was significant [$F(9, 360) = 9.23$, $p < .001$, $\eta_p^2 = .187$]. The interaction between group and block was not significant [$F(18, 360) = 1.29$, $p = .190$, $\eta_p^2 = .061$].

Acknowledgements

I would like to express my sincere thanks to Prof. Dan Ohtan Wang and members of Ohtan laboratory. They have always taught me what good science is and how to perform it. Experiences in this lab are one of my best treasures in my life. I greatly appreciate helpful advice and encouragement from Prof. Jun Suzuki, Prof. James-Alan Hejna, and Prof. Peter Carlton. I wish to thank Dr. Toru Yoshihara, Ms. Mariko Hayashi, and Ms. Yasuko Fukuda, Division for Mouse Behavior Analysis, Medical Research Support Center, Graduate School of Medicine, Kyoto University, Japan, for their numerous supports of behavioural experiments. I am also grateful to Dr. Toshihiro Endo, Phenovance LLC, for his great guide of IntelliCage experiments.

This thesis is based on the following peer-reviewed research paper.

Momoe Sukegawa, Toru Yoshihara, Shengqun Hou, Masahide Asano, Anthony J. Hannan, and Dan Ohtan Wang

Long-lasting housing environment manipulation and acute loss of environmental enrichment impact BALB/c mice behaviour in multiple functional domains

European Journal of Neuroscience, Volume 55, Issue 5, Pages 1118-1140, 2022.