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Short communication

C57BL/6J and C57BL/6N mice show distinct aging-associated behavioral alterations

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ABSTRACT

Aging affects emotional, cognitive, and social functions, increasing susceptibility to neuropsychiatric conditions. C57BL/6 mice are commonly used to study aging mechanisms, yet differences between C57BL/6J and C57BL/6N substrains remain underexplored. This study compared aging-related behavioral changes in these substrains. Aging reduced exploratory activity and heightened anxiety in C57BL/6J, but not C57BL/6N, mice. Conversely, aging reduced social novelty preference in C57BL/6N, but not C57BL/6J, mice. Male mice of both substrains exhibited increased female urine sniffing with age. These findings highlight substrain-specific aging effects, underscoring the importance of substrain selection in behavioral studies of aged mice for drug development.

Aging leads to emotional, cognitive, and social dysfunctions and risks various neuropsychiatric conditions, such as dementia.[1–3] Given the relevance to cognitive impairment in dementia, animal studies of behavioral aging have mainly focused on the cognitive domain so far. However, emotional and social deficits associated with aging have recently gained attention, as these factors often impact the well-being of older adults as significantly as cognitive deficits.[4,5] Therefore, to develop therapeutic drugs that ameliorate various symptoms of dementia, it is crucial to characterize the effects of aging on these behavioral domains and establish a robust behavioral platform for testing the effects of novel compounds on these alterations.

In rodent aging studies, C57BL/6 mice are widely used due to their well-characterized genetic profile and the availability of numerous genetically modified lines within this strain.[6–8] Since multiple genetic factors influence aging and longevity in humans and model animals, it is crucial to select an appropriate genetic background.[9] C57BL/6 strain is composed of two major substrains, C57BL/6J and C57BL/6N. Genomic, metabolic and behavioral differences have been reported between the two substrains.[10–13] In behaviors, C57BL/6J mice show higher exploratory activity, whereas C57BL/6N mice are more susceptible to the pro-depressive effects of chronic corticosterone treatment.[10,14] Between the genomes of C57BL/6J and C57BL/6N, there are 43 structural variants, including 15 overlapping with genes, 34 coding SNPs, and 2 coding indels.[12] Whereas several genes that vary between the substrains have been implicated in emotional and cognitive functions, no specific variant has been determined to account for the behavioral differences between the substrains.

Despite such differences between the substrains, not all behavioral aging studies specified which C57BL/6 substrain was used. When it was specified, C57BL/6J mice have mostly been used to characterize in aging-associated behavioral alterations, including anxiety-like behavior and cognitive deficits,[15–17] whereas only a few studies reported behavioral deficits of aged C57BL/6N mice, such as altered sucrose preference and anxiety.[18,19]

In this study, we aimed to compare emotional and social behaviors of aged C57BL/6J and C57BL/6N mice using multiple tests in different behavioral domains. Refer to the Supplementary Methods for detailed methods for behavioral tests.

We assessed exploratory activity and anxiety-related behaviors in young (8-12-week-old) and aged (75-85-week-old) male and female C57BL/6J mice (Jackson Laboratories Japan, Yokohama, Japan) and C57BL/6N mice (C57BL/6NCrSlc; Japan SLC, Shizuoka, Japan) using the open field and elevated plus maze tests. In the open field test (Fig. 1a), aged C57BL/6J mice showed reduced exploratory activity and

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heightened anxiety compared to young mice, evidenced by shorter travel distances, less time spent in the center zone, and fewer center zone entries normalized to total distance traveled (Fig. 1b–g). Conversely, while aged C57BL/6N mice spent less time in the center zone, they did not differ from young mice in total distance traveled or normalized center zone entries (Fig. 1b–g). Notably, aged C57BL/6J mice spent less time and made fewer entries into the center zone compared to aged C57BL/6N mice, indicating that anxiety increases with age more prominently in C57BL/6J mice (Fig. 1d–g).

In the elevated plus maze test (Fig. 1h), aged C57BL/6J mice exhibited heightened anxiety compared to young C57BL/6J mice, traveling shorter distances, spending less time in open arms, making fewer open-arm entries, and spending more time in closed arms (Fig. 1i–r). In contrast, no significant differences were observed between young and aged C57BL/6N mice in these measures (Fig. 1i–r). Direct comparison of the substrains revealed that aged C57BL/6J mice spent significantly more time in the closed arms than aged C57BL/6N mice (Fig. 1p).

These findings demonstrate that aging preferentially reduces exploratory behavior and increases anxiety in C57BL/6J mice compared to C57BL/6N mice. No significant differences in these behaviors were observed between male and female mice (Supplementary Figs. S1 and S2).

We assessed social behaviors using the sociability and social novelty tests. In the sociability test (Fig. 2a), sniffing time, sniffing bouts, and their ratios for a social versus empty target were indices of sociability. In C57BL/6J mice, aging reduced sniffing time for a social target without affecting that for an empty target (Fig. 2b–e). The ratio of sniffing time

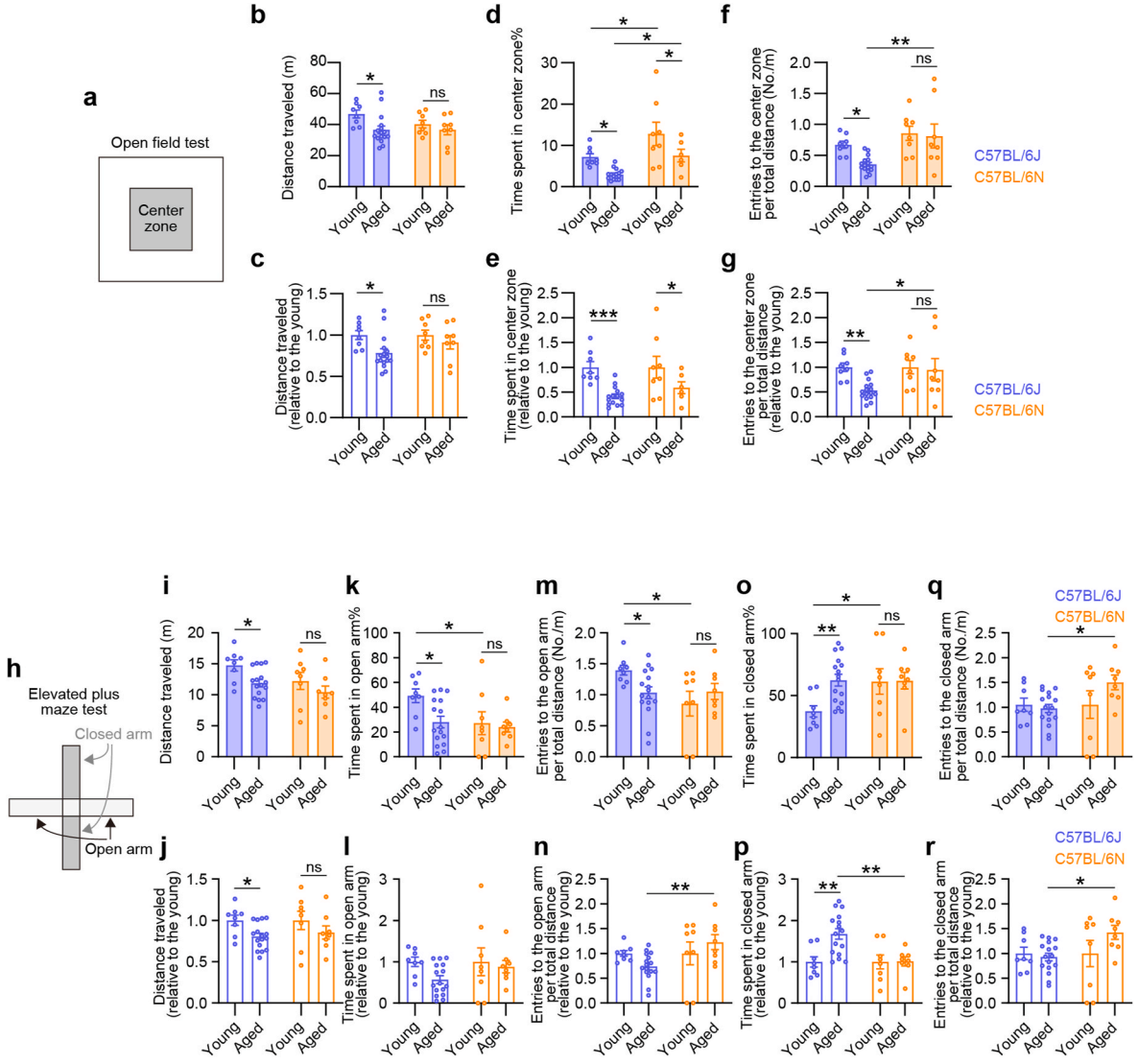

Fig. 1. Aging reduces exploratory behavior and heightens anxiety preferentially in C57BL/6J mice, compared to C57BL/6N mice, in the open field test and in the elevated plus maze test (a) Schematic of the behavioral chamber used in the open field test. (b–g) Total traveled distance (b, c), proportion of time spent in the center zone (d, e), and number of entries to the center zone per total distance traveled (f, g) in the open field test for young and aged C57BL/6J and C57BL/6N mice, without (b, d, f) or with (c, e, g) normalized to the values of young mice of the respective substrains. (h) Schematic of the behavioral chamber used in the elevated plus maze test. (i–r) Total traveled distance (i,j), proportion of time spent in open arms (k,l), number of entries to open arms per total distance traveled (m,n), proportion of time spent in closed arms (o,p), and number of entries to closed arms per total distance traveled (q,r), in the elevated plus maze test for young and aged C57BL/6J and C57BL/6N mice, without (i,k,m,o,q) or with (j,l,n,p,r) normalized to the values of young mice of the respective substrains. Values are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant for Fisher's LSD tests following two-way ANOVA. As a few outliers skewed the statistical results, the outliers were identified separately for the respective groups in each graph using the ROUT method with a 1% false discovery rate and excluded from the corresponding graphs and analyses. Consequently, the outliers were determined independently for different behavioral indices. The following data points were excluded from the analyses: (d,e) 1 out of 16 aged C57BL/6J, 2 out of 8 aged C57BL/6N.

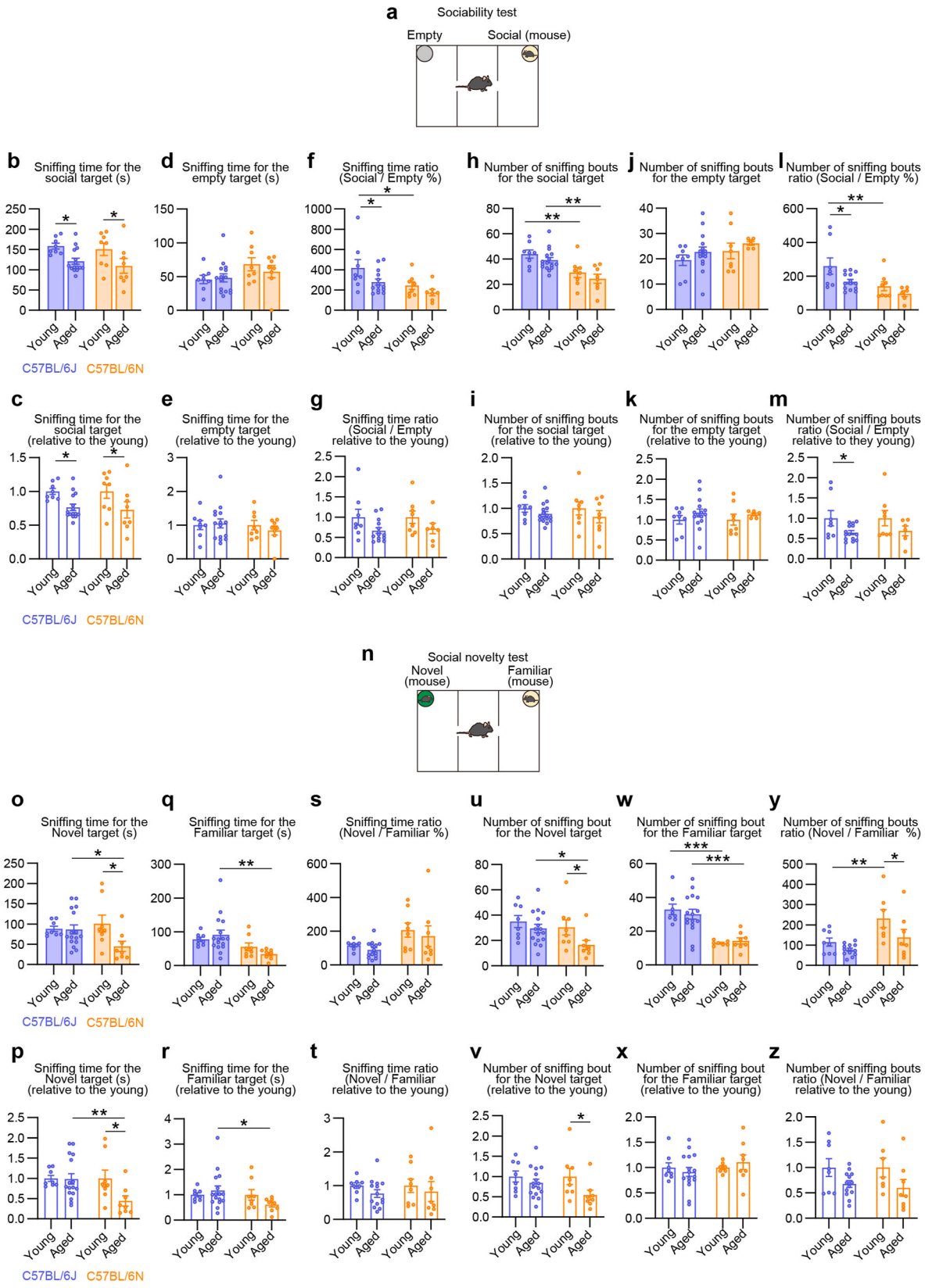

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Fig. 2. Aging reduces sociability and social novelty preference preferentially in C57BL/6N mice, compared to C57BL/6J mice. (a) Schematic of the behavioral chamber used in the sociability test. (b–g) Time spent sniffing a social target (b, c) or an empty target (d, e) and the ratio of sniffing time for a social target relative to an empty target (f, g) for young and aged C57BL/6J and C57BL/6N mice without (b, d, f) or with (c, e, g) normalized to the values of young mice of the respective substrains. (h–m) The number of sniffing bouts for a social target (h, i) or an empty cage (j, k) and the ratio of the number of sniffing bouts of social target relative to an empty target (l, m) for young and aged C57BL/6J and C57BL/6N mice without (h, j, l) or with (i, k, m) normalized to the values of young mice of the respective substrains. (n) Schematic of the behavioral chamber used in the social novelty test. (o–z) Time spent sniffing a novel social target (o,p) or a familiar one (q,r) and the ratio of sniffing time for a novel social target relative to a familiar one (s,t) for young and aged C57BL/6J and C57BL/6N mice without (o,q,s) or with (p,r,t) normalized to the values of young mice of the respective substrains. (u–z) The number of sniffing bouts for a novel social target (u,v) or a familiar one (w,x) and the ratio of the number of sniffing bouts for a novel social target relative to a familiar one (y,z) for young and aged C57BL/6J and C57BL/6N mice without (u,w,y) or with (v,x,z) normalized to the values of young mice of the respective substrains. Values are shown as means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns, not significant for Fisher's LSD tests following two-way ANOVA. As a few outliers skewed the statistical results, the outliers were identified separately for the respective groups in each graph using the ROUT method with a 1% false discovery rate and excluded from the corresponding graphs and analyses. Consequently, the outliers were determined independently for different behavioral indices. The following data points were excluded from the analyses: (b–e) 1 out of 16 aged C57BL/6J; (f,g) 2 out of 16 aged C57BL/6J, 1 out of 8 aged C57BL/6N; (j,k) 1 out of 16 aged C57BL/6J, 2 out of 8 aged C57BL/6N; (l,m) 2 out of 16 aged C57BL/6J, 2 out of 8 aged C57BL/6N; (s,t) 2 out of 16 aged C57BL/6J; (w,x) 1 out of 8 young C57BL/6N; and (y,z) 3 out of 16 aged C57BL/6J, 1 out of young C57BL/6N.

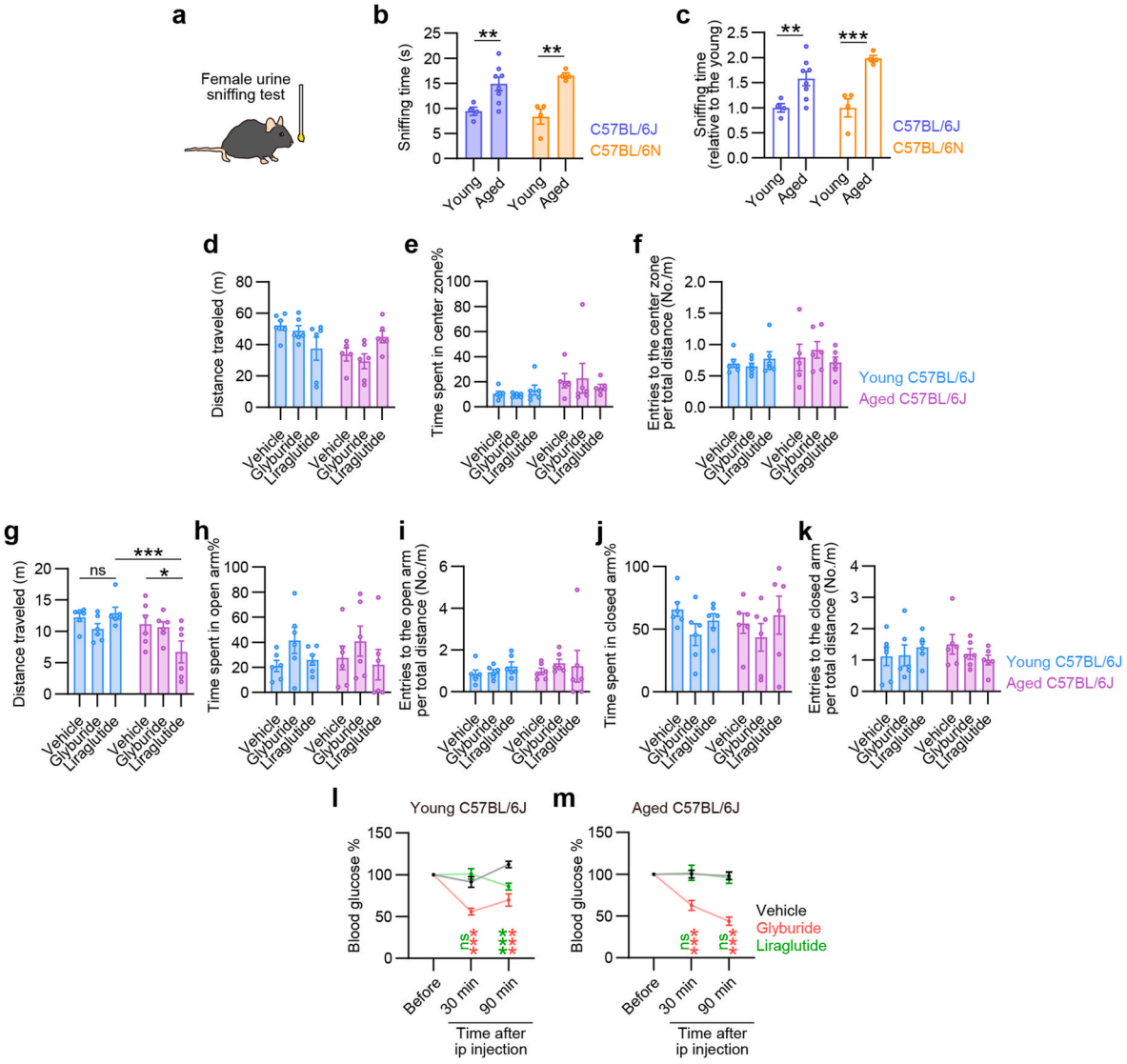

Fig. 3. Aging increases female urine sniffing in both C57BL/6J and C57BL/6N mice, and glyburide treatment appeared to ameliorate anxiety in both young and aged C57BL/6J mice. (a) Schematic of the female urine sniffing test. (b,c) Time spent sniffing female urine for young and aged C57BL/6J and C57BL/6N mice without (b) or with (c) normalized to the values of young mice of the respective substrains. (*d*–f) Total traveled distance (d), proportion of time spent in the center zone (e), and number of entries to the center zone per total distance traveled (f) in the open field test for young and aged C57BL/6J mice, following intraperitoneal injection of either vehicle control, glyburide, or liraglutide. (g–k) Total traveled distance (g), proportion of time spent in open arms (h), number of entries to open arms per total distance traveled (i), proportion of time spent in closed arms (j), and number of entries to closed arms per total distance traveled (k), in the elevated plus maze test for young and aged C57BL/6J mice, following intraperitoneal injection of either vehicle control, glyburide, or liraglutide. (l,m) Blood glucose levels of young (l) or aged (m) C57BL/6J mice before and after intraperitoneal injections of vehicle control, glyburide, or liraglutide. Values are shown as means ± SEM. $\#P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant for Fisher's LSD tests following two-way ANOVA (b–k) or one-way ANOVA (l), or Dunnett's multiple comparisons test between vehicle and drugs following two-way repeated measures ANOVA (l,m).

for social versus empty targets decreased with aging, though young and aged mice distributions overlapped considerably (Fig. 2f and g). Similarly, the ratio of sniffing bouts for social versus empty targets declined with age, though changes in individual sniffing bouts were not statistically significant (Fig. 2h–m). In C57BL/6N mice, aging also reduced sniffing time for a social target without affecting the empty target, but the ratio remained unchanged (Fig. 2b–g). Aging did not affect sniffing bouts or their ratio in these mice (Fig. 2h–m). These results suggest that aging modestly reduces sociability in both C57BL/6J and C57BL/6N mice, with overlapping distributions between young and aged mice.

In the social novelty test (Fig. 2n), sniffing time, sniffing bouts, and their ratios for novel versus familiar social targets indexed novelty preference. In C57BL/6J mice, aging did not alter sniffing time or bouts for novel or familiar targets, nor their ratios (Fig. 2o–z). In C57BL/6N mice, aging reduced sniffing time and bouts for novel social targets without affecting familiar targets. The ratio of bouts for novel versus familiar targets decreased with aging, though changes in time ratios were not statistically significant (Fig. 2o–z). These findings suggest that aging preferentially reduces social novelty preference in C57BL/6N mice compared to C57BL/6J mice. No sex differences were observed (Supplementary Figs. S3 and S4).

Lastly, we evaluated female urine sniffing behavior in male mice (Fig. 3a). Aged C57BL/6J and C57BL/6N males spent more time sniffing female urine than young males (Fig. 3b and c). This indicates that aging increases female urine sniffing in both mouse strains.

To exploit our behavioral findings to pharmacological research, we examined whether metabolic dysregulation contributes to heightened anxiety in aged C57BL/6J mice with antidiabetic drugs glyburide or liraglutide, which have distinct pharmacological actions, in the open field test and the elevated plus maze test. Although aging-related differences in anxiety levels became unclear, perhaps due to increased anxiety associated with intraperitoneal injections of even the vehicle solution, neither drug significantly affected anxiety levels in either test (Fig. 3d–f,h-k). Nonetheless, liraglutide, but not glyburide, selectively reduced locomotor activity in aged mice during the elevated plus maze test (Fig. 3g). Notably, liraglutide did not alter blood glucose levels 30 min post-injection (Fig. 3l and m), when the behavioral experiments were conducted. This suggests that its effect on locomotor activity in aged mice is independent of its glucose-lowering action and instead may be attributed to other GLP-1-related mechanisms.

In the present study examined aging-associated behavioral changes in C57BL/6J and C57BL/6N mice, revealing genetic and behavioral domain-specific effects. Aging reduced exploratory activity and heightened anxiety in C57BL/6J mice, while reducing social novelty preference in C57BL/6N mice. Both substrains exhibited increased female urine sniffing, indicating a common behavioral change. Differences in breeding conditions, including microbiomes, due to vendor sources may have influenced the findings, but the results highlight the importance of substrain selection for studying behavioral aging.

Our findings suggest that aging affects multiple behavioral domains differently, depending on genetic background. Consequently, aging does not uniformly impact all behaviors; instead, distinct patterns of behavioral deficits may emerge among individuals with varying genetic backgrounds. It should be noted that the interpretation of the results of each behavioral test needs great care. The level of anxiety is influenced by the motivation to explore novel environments. Similarly, social novelty preference relies on general learning and memory abilities. To address this complexity, a behavioral battery incorporating a comprehensive set of experiments may be required.

Among these genetic differences, the deletion of the Nnt gene that selectively occurs in C57BL/6J mice has been shown to cause impaired insulin secretion and glucose intolerance.[20] Since glucose intolerance reportedly leads to heightened anxiety,[21] aging may have different effects on anxiety in C57BL/6J and C57BL/6N mice, possibly due to their distinct metabolic profiles. Importantly, rodent studies have indicated that not all individuals exhibit aging-associated cognitive deficits, even with identical genetic backgrounds, pointing to non-genetic factors underlying the variability in behavioral aging.[22,23] To clarify the genetic and non-genetic mechanisms of behavioral aging, it is crucial to categorize aged individuals based on their specific patterns of behavioral deficits.

We found that the GLP-1 agonist liraglutide suppressed locomotor activity in aged, but not young, mice. While preclinical research has emphasized the potential therapeutic benefits of GLP-1 agonists for psychiatric and neurological disorders,[24,25] this finding raises a potential concern: GLP-1 agonists may have age-dependent effects that could impair certain behavioral functions in older individuals. As many animal models for neurological disorders are designed to exhibit behavioral dysfunctions at relatively early stages of life, they may fail to detect therapeutic effects of novel compounds that become evident only after aging. This study underscores the importance of establishing a behavioral testing platform tailored to drug development for aged population.

By leveraging behavioral differences between C57BL/6J and C57BL/6N substrains, this study highlights variability in behavioral aging influenced by genetics and behavioral domains. These findings pave the way for identifying diverse aging patterns, potentially enabling targeted anti-aging strategies based on individual biological mechanisms.

CRediT authorship contribution statement

Rui Yamada: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Hirotaka Nagai:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization. **Io Horikawa:** Investigation. **Wenran Qiu:** Investigation. **Yunhui Zhu:** Investigation. **Kohei Ota:** Investigation. **Tomoyuki Furuyashiki:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

Data availability

The datasets generated during and/or analyzed during the current study are available in Supplementary Tables 1 and 2 or from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2025.01.002.

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