Semaglutide Administration in Healthy Mice Alters Behaviour Related to Stress and Motivation

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SUMMARY

Semaglutide, a GLP-1 receptor agonist used primarily to regulate blood sugar and appetite, has been studied mostly in models of obesity and diabetes. Yet, little is known about its effects on healthy animals. In this study, daily dosing of semaglutide (0.1 mg/kg) in healthy C57BL/6JRj male wildtype mice led to noticeable changes in behavioural tests commonly used to evaluate stress and motivation. The mice showed increased jumping in the open field, reduced shredding of nestlets, less interest in sniffing female urine, and heightened activity in the forced swim test. These behavioural shifts may indicate potential side effects of semaglutide in healthy animals, providing a crucial baseline for understanding how the drug could affect conditions like obesity, addiction, or cognitive decline.

Semaglutide[1,2], a long-acting glucagon-like peptide-1 (GLP-1) analogue, traditionally used for the treatment of diabetes, has recently emerged as an efficient therapeutic agent for obesity[3]. Preclinical research in rodent models has therefore focused on obese and/or diabetic subjects, as it was originally developed for metabolic control. Therefore, its effects in healthy, non-obese rodents remain largely underexplored. This study aims to investigate the behavioural impacts of semaglutide in healthy C57BL/6JRj mice, providing insights into its potential broader effects and establishing a baseline for future research.

In this study, semaglutide was administered to adult healthy male C57BL/6JRj wildtype mice, a commonly used strain in behavioural research. We tested different administration protocols. The first protocol consisted of subcutaneous injections of semaglutide (Ozempic®, 0.1 mg/kg) on a weekly basis for eight weeks. This regimen mirrors human clinical practice for chronic conditions[3]. The second protocol consisted of an injection of the same dose on a daily basis for eight days, accounting for the higher metabolic rate in mice and aiming to explore short-term effects[4]. All mice received the same amount of drug overall (8 injections). After completing the injection regimen, the mice underwent a series of behavioural tests designed to evaluate different aspects of behaviour and emotional state (Figure 1A). Throughout the study period, we monitored food and water consumption and body weight to assess any physiological changes. These metrics were crucial for understanding the overall impact of semaglutide on health and behaviour. As previously reported in rodents[5–9], we observed a substantial weight loss after only a single injection that continued throughout the treatment length (Figure 1B). Notably, food and water consumption were also reduced (Figure 1B), which may have contributed to the observed weight loss. In the following

paragraphs, we report the behavioural changes associated with the daily injection treatment.

The open field test, a standard measure of locomotion and anxiety, revealed no changes in any of the measured variables (faecal boli, time spent in the field's centre, distance travelled, path efficiency, maximum speed, mean speed, time spent immobile, number of rotations, or number of clockwise rotations; Figure 1C). However, we observed a significant increase in jumping behaviour (Figure 1C), with the semaglutide group jumping significantly more than the control group (Median = 9 vs. 1; U = 31, p = 0.0151; Mann-Whitney U test). This could indicate more attempts to escape the testing box, as the jumping was most frequently observed towards the walls.

In order to evaluate anxiety and compulsive behaviour, we applied two complementary tests: the marble burying test and the nestlet shredding test[10,11]. The nestlet shredding test (Figure 2B) demonstrated a significant reduction in shredding among the semaglutide-treated mice (Mean = 1.615) compared to controls (Mean = 6.187), with a difference of 4.572 ± 1.117 ($t(22) = 4.095$, $p = 0.0005$, $R^2 = 0.4325$). This finding suggests an increase in anxiety or a shift in exploratory behaviour, potentially related to semaglutide's impact on the central nervous system. However, no significant changes were observed in the marble burying test (Figure 2A) between the groups (U = 44.50, p = 0.1157).

The Female Urine Sniffing Test (FUST) (Figure 2C) is used to evaluate reward-seeking behaviour in rodents[12] by measuring the time spent sniffing cotton swabs soaked in different substances. First, the rodent is exposed to a swab soaked in water, followed by one soaked in urine from females in the estrous cycle. Healthy male rodents typically spend significantly more time sniffing the female urine, indicating natural reward-seeking behaviour. A decrease in this behaviour is linked to anhedonia. A two-way repeated measures ANOVA revealed a significant interaction between smell type and treatment ($F(1, 22) = 4.310$, $p = 0.0498$), indicating that semaglutide treatment affected sniffing behaviour differently depending on the stimulus. Specifically, the number of sniffs directed at urine was significantly reduced in the semaglutide-treated group compared to controls, representing a 27.21% reduction (Semaglutide: 13.83 sniffs, Control: 19 sniffs; p = 0.0051). Total sniffing time also showed a significant smell x treatment interaction ($F(1, 22) = 4.881$, $p = 0.0379$), with the semaglutide-treated group spending significantly less time sniffing urine, representing a 33.9% reduction (Semaglutide: 22.18 seconds, Control: 33.6 seconds; p = 0.0108). Despite these reductions, both groups still showed a significant overall difference between sniffing water and urine ($F(1, 22) = 81.94$, $p < 0.0001$ for number of sniffs; $F(1, 22) = 76.16$, $p < 0.0001$ for sniffing time).

The forced swimming test (Figure 2D), commonly used to evaluate depressive-like behaviour and resilience[13], revealed a significant decrease in immobility time in the semaglutide-treated group (Median = 17.40 vs. 69.05, p = 0.0121, Mann-Whitney U = 29), which accounted for a 74.8% reduction. Additionally, when examining immobility per minute, a two-way repeated measures ANOVA showed a significant effect of treatment over time ($F(1, 22) = 8.643$, $p = 0.0076$), with semaglutide-treated mice spending significantly less time immobile in minutes 3, 4, and 5 compared to controls (p values of 0.0309, 0.0001, and 0.0131, respectively). The overall reduction in immobility could suggest a potential improvement in coping behaviour or a reduction in depressive-like responses.

In contrast to the daily-injected group, the weekly-injection group did not exhibit significant behavioural changes. This lack of effect highlights the potential influence of dosing frequency on the behavioural outcomes associated with semaglutide. It suggests that the

daily dosing regimen may be more likely to elicit behavioural changes, which could be due to differences in drug metabolism or pharmacodynamics. Despite the absence of significant behavioural changes, the weekly injection regimen was still effective in promoting weight loss. This indicates that while dosing frequency may influence behavioural outcomes, the metabolic effects of semaglutide on weight reduction remain robust even with less frequent administration (see Supplementary Figure 1).

This study underscores the importance of considering both dosage and frequency in behavioural research, as different regimens can yield varying effects. The observed changes in behaviour with daily dosing could inform future studies examining semaglutide's effects in models with additional variables or health conditions[14,15] such as obesity[8,16], neurodegenerative diseases[17–19], or addiction[6,7].

A key limitation of this study is the lack of a weight-matched control group (e.g., mice on a calorie-restricted diet), which would help determine whether the observed behavioural changes are directly due to semaglutide's central effects or simply a consequence of weight loss. While the weekly injection group, which experienced some weight loss but did not exhibit behavioural changes, partially addresses this concern, the difference in weight loss between the groups remains a potential confounding factor. Additionally, the dose of semaglutide used in this study (0.1 mg/kg) may have allowed for behavioural effects to be observed with daily dosing but not with weekly injections. This dose was chosen to be conservative in assessing the drug's impact on behaviour, but it is possible that a higher dose could reveal more consistent effects across different dosing regimens.

In conclusion, the daily administration of semaglutide in healthy male C57BL/6JRj mice results in notable behavioural effects, including increased jumping activity, reduced nestlet shredding, decreased female urine sniffing, and lower immobility in the forced swimming test. Future research should build upon these findings to explore the full range of behavioural impacts and underlying mechanisms associated with semaglutide treatment.

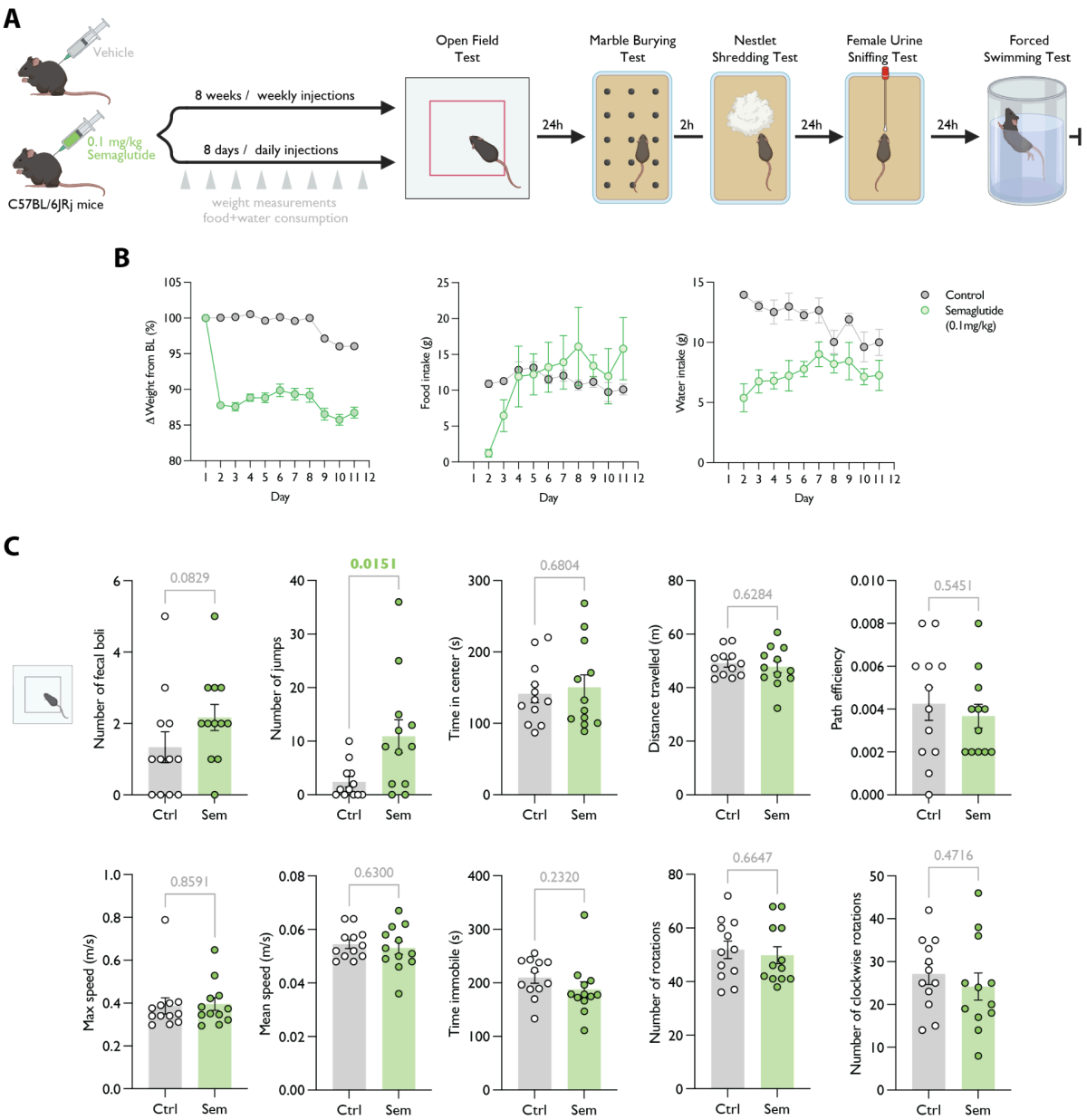

Figure 1. Daily semaglutide administration in healthy mice causes significant weight loss and increases jumping behaviour in the open field test. (A) Experimental design. (B) Daily weight difference, food and water consumption. Weight is measured per animal (n=12 per group); food and water consumption are measured per cage as animals are group-housed, with 3 animals per cage (n=4 per group). (C) Open Field Test results. No statistical differences were detected in any of the tested variables with the exception of jumping behaviour, which was higher in the treated group. N=12 per group; Mean±SEM; p-values per comparison (statistically significant p-values are highlighted in bold and green).

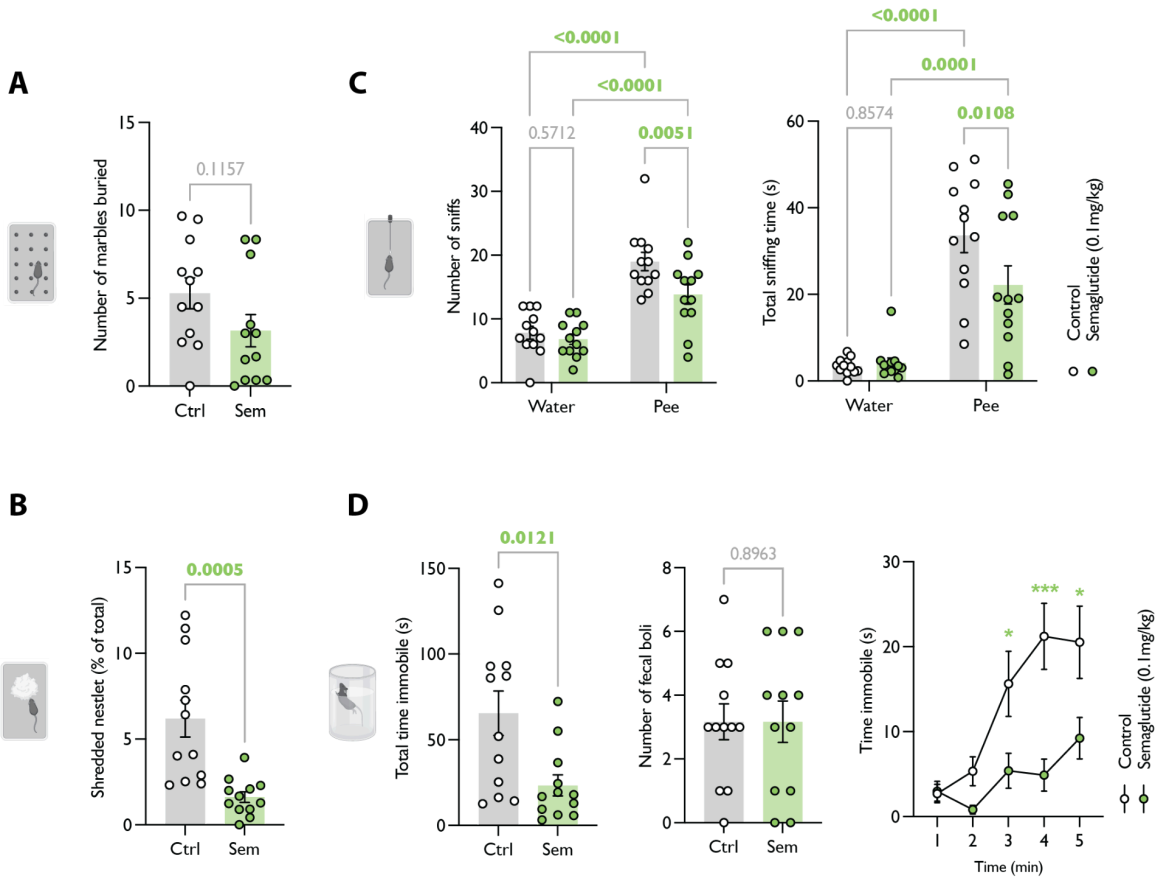

Figure 2. Daily semaglutide administration in healthy mice causes changes in the Female Urine Sniffing Test, Nestlet Shredding Test and Forced Swimming Test, but not in the Marble Burying Test. (A) Marble Burying Test results. (B) Nestlet Shredding Test results. (C) Female Urine Sniffing Test (FUST) results. (D) Forced Swimming Test results. In all graphs, n=12 per group; Mean±SEM; p-values per comparison (statistically significant p-values are highlighted in bold and green).

Methods

Animals

Male C57BL/JRj mice were purchased from Janvier Labs at 7 weeks old, and used in experiments at 8-18 weeks of age. Mice were group housed in cages of 3, and provided with ad libitum access to food (regular chow) and water. Mice were housed under regular lighting conditions (12h light cycle, lights on at 6:00 AM). All injections, weighing procedures, and behavioural experiments were performed between 8:00 AM and 2:00 PM.

Behaviour

Open Field Test

Mice were placed in an arena (45 x 45 x 45 cm) made of polycarbonate with a paper flooring (30% Ethanol was used to clean between mice, and paper flooring renovated for each mouse). The arena was placed inside of a noise cancelling box (80 x 60 x 62 cm). Mice were allowed to explore the arena for 15 min. Mice were tracked and standard measurements of speed, zone visits and distance travelled etc. were automatically scored by Anymaze software. Faecal boli were manually counted at the end of the test. Jumping was manually counted offline based on the recorded videos.

Marble Burying Test

Standard cages with flat lids, containing one layer of 5 cm of bedding, were used to position a grid of 5x4 standard glass toy marbles (blue, 15 mm diameter, 5.2 g) evenly spaced throughout the cage. Mice were individually placed in the cage and left undisturbed (cage inside of a noise-cancelling box) for 30 mins. After the test,

mice were removed and cages were photographed to evaluate (manually and semi-automatically) the number of marbles buried. See Supplementary Material (Supplementary Figure 2) for a discussion on marble burying quantification.

Nestlet Shredding Test

Mice were individually placed in cages with flat lids containing a layer of bedding and one new cotton nestlet, and left undisturbed in the cage in a noise-isolated box for 30 min. Nestlets were weighed before and after the test; weight differences are reported as shredding %.

Female Urine Sniffing Test

In order to determine the estrous stage of the females used to harvest the pee samples used in the test, vaginal cell samples (25 ul, in sterile saline solution) were taken from females, stained with cresyl violet (0.1%), and assessed under the microscope to identify estrous stage. Pee from females (from the same genetic background as the males) in estrus was harvested on the test day. Samples of 40 ul of pee were pipetted into cotton swabs and kept in closed test tubes under a fume hood until the testing phase. Pee samples were taken from the same batch for all tested mice in one group (two groups of n=12 mice).

The test followed several phases as described in[12], administered during one day: (1) Habituation to a cotton swab without any liquid on it for 1 h; (2) Exposure to a cotton swab with water for 3 min; (3) Waiting time without a cotton swab in the cage for 45 min; (4) Exposure to a cotton swab with pee for 3 min. In stages 2 and 4, the sniffing was measured manually based on video recordings of the mice's behaviour performed with Anymaze with a camera placed above the cage.

Data for the weekly injection group was excluded from this test, as the control animals in this group did not perform as expected according to the test's assumptions[12]. Consequently, the data was deemed non-valid and not included in the final analysis.

Forced Swimming Test

Mice were placed in cylindrical containers (30 cm tall, 17 cm diameter) containing room-temperature water (up to a measure of 15.5 cm from the bottom of the container) for 5 minutes. Mice were tracked automatically with a side-camera with Anymaze to detect immobility episodes. Mice were sacrificed after finishing the test.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism. Data are represented as Mean ± SEM. Group comparisons were made using either unpaired t-tests or Mann-Whitney U tests, depending on the data distribution. For repeated measures, two-way repeated measures ANOVA was applied, followed by post-hoc tests where appropriate. Significance was set at $p < 0.05$. For all tests, assumptions such as normality and sphericity were assessed, and non-parametric tests were used if assumptions were violated.

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Author contributions

AM* conceived the idea, planned the experiments, did behavioural experiments, analysed the data, wrote the manuscript and prepared the figures. SA did behavioural experiments. AM did data analysis and developed the marble counting semi-automatic method. All authors contributed to the experimental design planning. All authors read and approved the manuscript.

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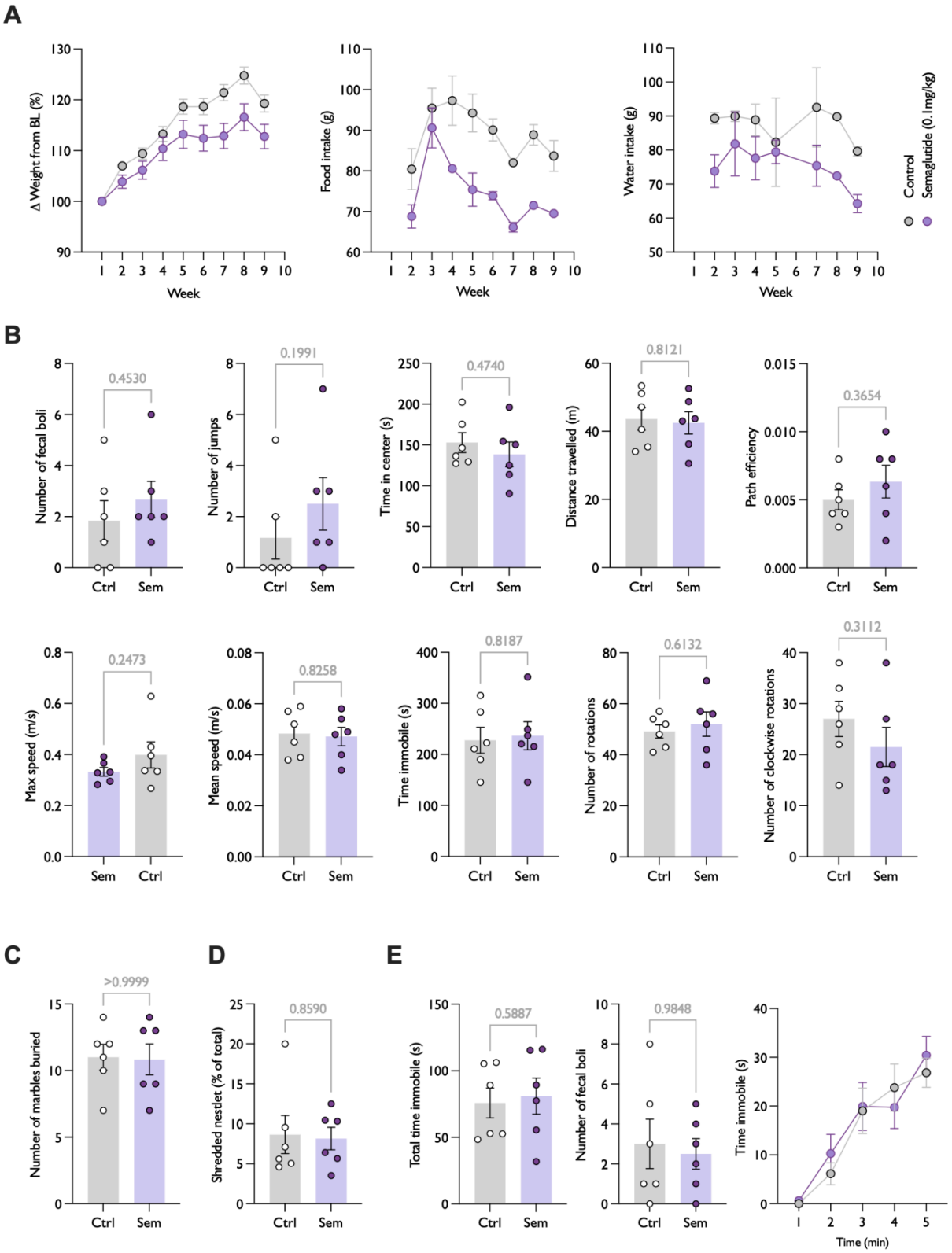
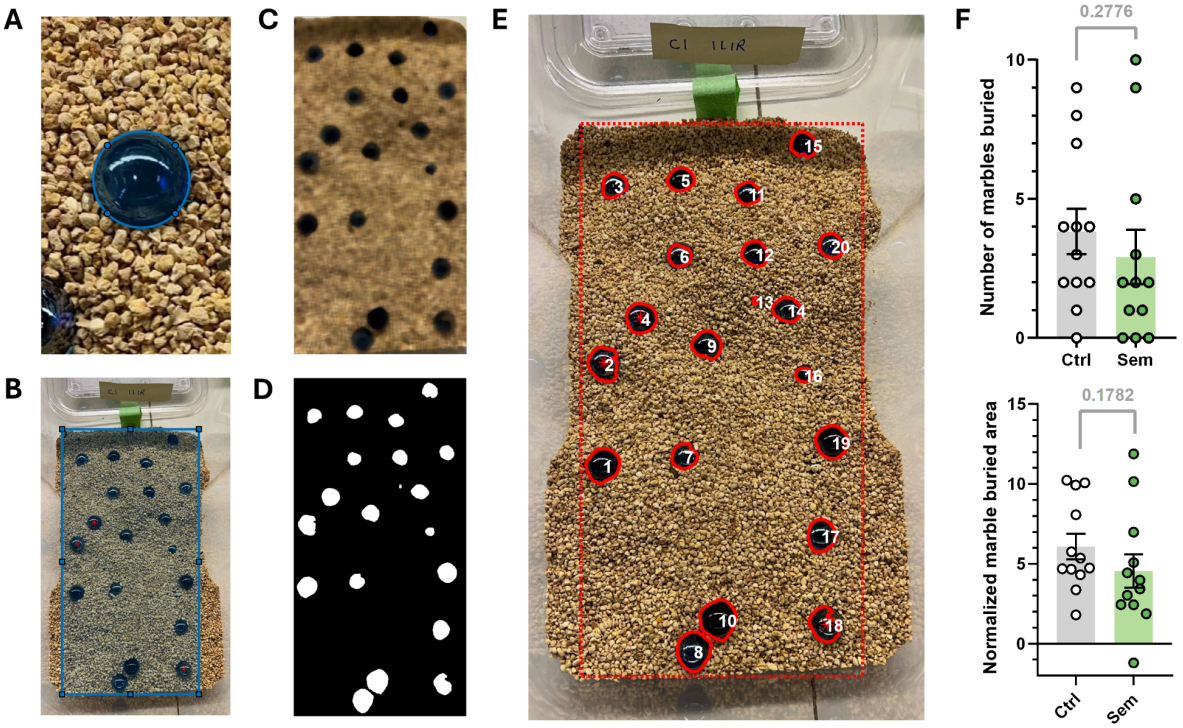
Supplementary Material



Supplementary Figure 1. No behavioural effects were detected when administering Semaglutide on a weekly schedule. (A) Weekly weight difference, food and water consumption. Weight is measured per animal (n=6 per group); food and water consumption are measured per cage as animals are group-housed, with 3 animals per cage (n=2 per group). (B) Open Field Test results. No statistical differences were detected in any of the tested variables. (C) Marble Burying Test results. No statistical differences were detected. (D) Nestlet Shredding Test results. No statistical differences were detected. (E) Forced Swimming Test results. No statistical differences were detected. In all graphs, n=6 per group; Mean±SEM; p-values per comparison (statistically significant p-values are highlighted in bold and purple).

Supplementary Method 1. Comparison between marble burying scoring between experimenters, and automatic area quantification

To quantify the marble-burying test, a custom semi-automated algorithm was developed in MATLAB (MathWorks, R2019b). Initially, the average size of a marble was estimated by manually creating circular regions of interest (ROIs) surrounding unburied marbles, and then averaging the resulting ROIs' areas (Suppl. Figure 2A). This estimation was later used to quantify the number of marbles buried and normalise the total surface of unburied marbles, enabling comparison between different pictures. Subsequently, the area corresponding to the cage floor containing marbles was manually selected by creating a rectangular ROI (Suppl. Figure 2B). The algorithm was only applied to this ROI to prevent objects, reflections, or shadows outside the cage or on the cage's walls from being misclassified as marbles. A 2-D median filter was then applied to the ROI to mitigate the specular highlights on the marbles that could be identified as holes and sudden changes in contrast in the bedding material that could be falsely detected as marbles (Suppl. Figure 2C), followed by image binarization through colour thresholding (Suppl. Figure 2D). The latter step was performed by inspecting the ROI for pixels where the three channels (R, G and B) were below a chosen threshold and replacing them with white values, whereas the remaining pixels were replaced with black values. The optimal neighbourhood size for the filtering step and the threshold for the image binarization are dependent on the image size and the experimental setup (e.g., marble colour, bedding material, light conditions, etc.). Therefore, it is recommended to optimise these values by visually inspecting the results obtained for a few cases and fixing them for all images. In our case, optimal results were obtained with a neighbourhood size of 55 pixels and a threshold of 50 on a 0-255 scale. Morphological operations were then performed on the binary image to fill out holes resulting from specular highlights on the marbles and to correct for underestimation of the marbles' edges resulting from the filtering step. This involved creating a disk-shaped structuring element with a radius of 10 pixels and applying morphological closing, followed by dilation to a new disk-shaped structuring element with a radius of 4 pixels. Finally, the marbles' boundaries were detected (Suppl. Figure 2E), and the number of marbles buried as well as the normalised marble buried area were computed (Suppl. Figure 2F). This was achieved through the calculation of the number of marbles with less than 1/3 of the area unburied (i.e., at least 2/3 of the area buried) and the subtraction of the area covered by unburied marbles (normalised to a single marble area) from the total number of marbles in the cage, respectively. The code will be made publicly available upon article publication.



Supplementary Figure 2. Semi-automated algorithm for quantification of marble-burying test. (A) Example of a circular ROI defined around a marble to estimate the average marble area. (B) Example of a rectangular ROI defined to delimit the region of the cage floor containing marbles. (C) A 2D median filter is applied to the ROI defined in B. This allows for the smoothing of the image of the bedding material and for the removal of specular highlights on the marbles. (D) Image binarization of C. (D) Marbles' boundaries obtained from the binarized image shown in D (red solid lines around the marbles) are overlaid to the original picture. The analysed ROI is depicted by a red dashed rectangle. A unique number is assigned to each detected marble. Marbles used to estimate the average marble area are marked with an asterisk. (F) Results obtained for the semi-automatic quantification of the marble-burying test. The statistics show the p-values obtained by applying a Student's t-test.