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Research paper

Biomarkers for prediction of chronic traumatic encephalopathy-like pathology following repeated mild traumatic brain injury in rats are partially sex- and age-dependent

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ABSTRACT

Introduction: Repeated mild traumatic brain injuries (rmTBIs) pose a high risk of developing chronic traumatic encephalopathy (CTE). Since this neurodegenerative disease is diagnosed only post-mortem, new biomarkers for early detection are needed. Although age at injury and biological sex are important factors in many brain pathologies, little is known about their relevance to rmTBI-induced CTE-like consequences. Hence, this study explored how biological sex and age at the time of impact affect progression and changes in biomarker candidates after experimental rmTBI.

Methods: Rats of both sexes, aged 7 weeks (adolescent) or 14 weeks (adult), were subjected to three mTBIs at 5-day intervals. Neurological, behavioral and cognitive impairments, as well as changes in potential plasma and brain biomarkers, were assessed up to 12 weeks post-injury. The generalized estimating equation model was used to compare sexes and age groups.

Results: RmTBI induced ongoing neurological impairment. While no depressive-like behavior was observed, long-term, age-specific changes in anxiety were observed after rmTBI. A short-term increase in plasma p-tau was found after rmTBI only in male and adolescent rats. Plasma neuron-specific enolase (NSE) levels were elevated in adolescent animals at both 2 weeks and 12 weeks post-rmTBI. An increase in brain neurofibrillary tangles (NFT) was detected 12-weeks after rmTBI. Correlation analyses suggested NSE as a prospective biomarker for anhedonia-like behavior, and brain NFT as an indicator of neurological impairment.

Discussion: We found that behavioral outcomes and biomarker changes following rmTBI in rats were both age- and sex-dependent. This information will help in developing translatable diagnostics to guide CTE treatment in clinical settings.

1. Introduction

Traumatic brain injury (TBI) is a major global health issue. A single brain injury event can lead to short-term (acute) neurological and behavioral impairments, as well as persistent neurological symptoms (Graham and Sharp, 2019). When (mild) TBI (mTBI) events are repetitive, cumulative brain microdamage can contribute to progressive neurodegeneration (Juan et al., 2022). Awareness of the long-term sequelae resulting from repetitive head impacts is increasing, particularly among participants in contact and collision sports, military personnel, and victims of physical violence. Sustained effects of repeated mTBI (rmTBI) can lead to persistent cognitive and neurological decline and may result in early-onset dementia due to a tau-based neurodegenerative pathology known as chronic traumatic encephalopathy (CTE) (McKee et al., 2013; Pedrosa et al., 2023). CTE is a tauopathy mainly caused by repeated concussive and subconcussive brain injuries (Graham and Sharp, 2019). Often, diagnosis of CTE is challenging and is currently performed primarily post-mortem. For this reason and for

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generating CTE-preventive treatments, there is a critical need to develop methods and identify translational biomarkers for the early diagnosis of CTE development.

At a physiological level, neurofibrillary tangles (NFTs) composed of (hyper-)phosphorylated tau (p-tau) are observed in the cortex and hippocampus, with notable p-tau accumulation at sulcal depths in irregular cortical spatial patterns (Shively et al., 2021). Both NFT aggregation and the substantial force of impact on the head contribute to neuronal and axonal injury, resulting in neuronal damage and subsequent release of axonal proteins into cerebrospinal fluid and blood (Halicki et al., 2023). While total tau (t-tau) and p-tau released due to NFTs serve as potential biomarkers of progressive neurodegeneration (Mckee and Daneshvar, 2015), other axonal and glial proteins, such as neurofilament light, neuron-specific enolase (NSE), and glial fibrillary acidic protein (GFAP), are being tested as potential plasma biomarkers of early cell damage and inflammatory processes following TBI events (Hiskens et al., 2020).

Despite the extensive research conducted to identify reliable biomarkers for early CTE diagnosis, significant variability in findings remains (Fesharaki-Zadeh, 2023; Majeed et al., 2024). Factors such as biological sex and age at the time of injury are believed to contribute to this variability. Studies on mTBI and concussions suggest a higher incidence and worse outcomes in female compared to male patients (Bazarian et al., 1999; Dick, 2009). Whether CTE incidence is higher in female patients as a consequence of rmTBI is not known as CTE in females is strongly understudied (Suter et al., 2023). However, the variability in research findings related to potential diagnostic biomarkers for CTE may also be influenced by age, as other studies have not consistently supported sex-related differences (Brook et al., 2016). On the other hand, some research has reported sex differences in plasma concentrations of injury biomarkers, such as GFAP, Ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) (Papa et al., 2023), and tau (Clarke et al., 2021). In this context, the roles of biological sex and age in the progression and outcome of TBI-related injuries have been reviewed (Biegon, 2021). Regarding age at injury, some researchers argue that developing brains are more susceptible to concussion-related impairments (Stern et al., 2011), while others have proposed that the age of first exposure does not impact long-term outcomes (Caccese et al., 2020). It might also be, due to higher plasticity, that younger brains better compensate for injury effects than older ones (Johnston et al., 2009). Whether age at first exposure to head injuries, in combination with biological sex, influences the development, progression and severity of rmTBI-induced CTE-like consequences remains an important question to be answered for advancing the field of CTE research.

In this study, we investigated the combined effects of sex and age on the consequences of rmTBI in a rat model of CTE. Using mixed model analyses, we assessed the effects of *injury* alone, *sex x injury* and *age x injury* interactions. Our study examined neurological and behavioral changes, as well as blood and brain biomarker changes. While most investigations focus on the short-term effects of rmTBI, the primary contribution of this study is its analysis of the long-term effects.

2. Methods

2.1. Study design and animals

Animal experiments were conducted following the ARRIVE 2.0 guidelines (Animal Research: Reporting in Vivo Experiments; Essential 10) (Percie du Sert et al., 2020). Ethical approval was obtained from the responsible state authority (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) under reference ID 20–3364, in compliance with the European Communities Council Directive 2010/63/EU. Thirty-two Sprague-Dawley rats (RjHan:SD, n = 16 rats of 13-weeks; n = 16 rats of 6-weeks; equally distributed by sex) were obtained from Janvier. When a new cohort arrived, rats were picked one by one and randomly assigned to either control (sham) or experimental (rmTBI) groups. While sham and rmTBI animals were housed together, they were separated by sex and age. To minimize potential confounders, animal experiments were performed in cohorts composed of 8 animals, having one animal of each experimental group (sham/male/adolescent; sham/female/adolescent; rmTBI/male/adolescent; rmTBI/female/adolescent; sham/male/adult; sham/female/adult; rmTBI/male/adult; rmTBI/female/adult). Additionally, males were housed separately from females, in actively ventilated containers, and disinfection was performed after each procedure, to prevent odor-related bias. Disease induction was performed when animals reached either 14-weeks (adult) or 7-weeks (adolescent) of age. Habituation to the animal room and handling (3×, one time per day) by the experimenter were conducted for at least one week before the start of experimental procedures. Animals were pair-housed in enriched (nesting material and wooden balls) ventilated biocontainment units (Scantainer Allentown, Scanbur, Denmark) under a 14/10-h light/dark cycle, with ad libitum access to standard laboratory food pellets (Altromin 1324 TPF, Altromin, Germany) and autoclaved tap water. Housing conditions included a temperature range of 21.5–23.0 °C and humidity between 45 %–55 %. The study was exploratory in nature and no prior sample calculation was performed. Single datasets of the adult rats included in this study were already separately analyzed and published (Moraga-Amaro et al., 2025). After adding the additional age group (adolescent rats), these data points are now included here in the multi-level analysis regarding both sex and age of the rats. The results of this combined analysis are completely novel and were not published before. No animals or single datasets were excluded post hoc. The experimenter was not blinded to the experimental groups. A priori inclusion criteria were not defined. A priori exclusion criteria are stated in the respective section of each individual outcome parameter. No animals or data points were excluded from this study, so number of animals is constant for each of the measured outcome parameters.

Acute and chronic effects of rmTBI were evaluated during the first 2 weeks and 12 weeks post-injury, respectively. A detailed timeline of experimental procedures is presented in Fig. 1A. Animals were weighed before each sham/mTBI induction session and daily for two consecutive days post-surgery. For rmTBI induction, three sham/mTBI sessions were conducted with 5-day intervals. Neurological severity score (NSS) was assessed at baseline, the day after each surgery, and 12 weeks after the final mTBI. Saccharin preference testing (SPT) was performed at 1, 2, and 12 weeks after rmTBI. Blood samples for biomarker quantification were collected at the specified time points, up to 12 weeks after rmTBI. Anxiety-like behavior and spatial memory were assessed using the open field test (OFT) and Y-maze test, respectively, at week 12 after rmTBI. Following behavioral testing, animals were euthanized, and brain tissues were collected for autoradiography targeting NFTs.

2.2. Repeated mild traumatic brain injury surgery

The CTE model consisted of a series of controlled acceleration impacts performed three times, with a 5-day interval between each injury, as described previously (McAteer et al., 2016; Moraga-Amaro et al., 2025). In brief, animals were anesthetized with 5 % isoflurane for 3 min (induction), and anesthesia was maintained at 2–3 % isoflurane. Eye ointment was used to prevent corneal dehydration, and heating pads were applied to maintain body temperature during the procedure. Hair was shaved and tetracaine (2 % solution) was applied cutaneously. Then, an incision of approximately 1 cm was made from bregma, the skull was exposed, and a 0.25 % bupivacaine solution was applied. A stainless-steel disc (10 mm in diameter, 3 mm in height) was adhered between bregma and lambda using a polyacrylamide adhesive (Pattex, Henkel, Germany). Then, animals were placed on a type E bed foam, with isoflurane supply interrupted for 10–15 s, with the skull positioned directly under a plastic tube containing a 450 g brass weight. For mTBI injury, the brass weight was released from a 1 m height allowing a direct single hit targeting the steel disc. Animals were placed back into anesthesia, the metal disc was removed, and the incision was sutured under

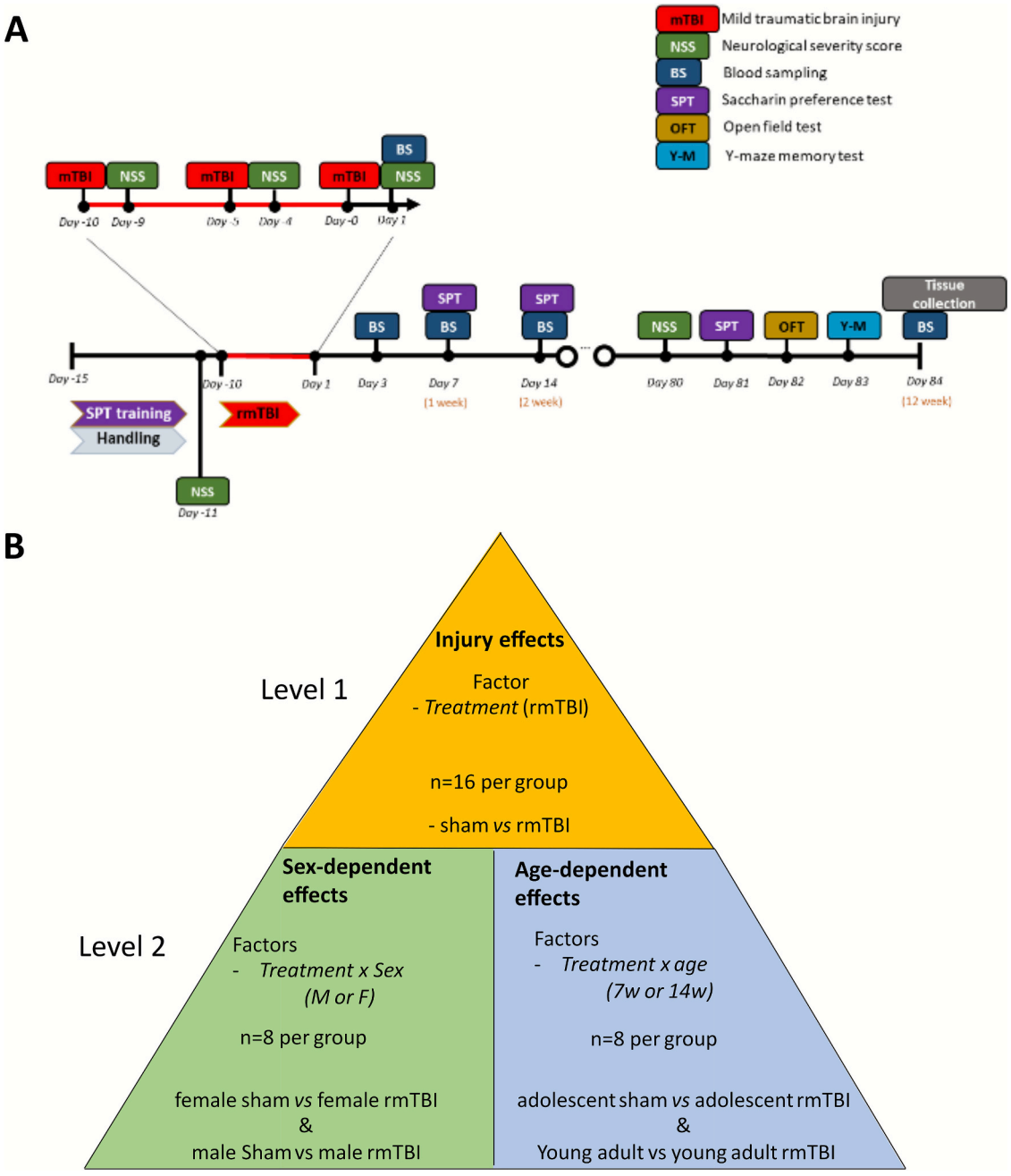

Fig. 1. Experimental timeline and statistical analyses. **A.** Experimental timeline showing the procedures applied to each animal. **B.** Statistical approach (generalized estimating equation model). Injury effects refer to animals merged by sex and age. Sex-dependent effects refer to animals merged by age. Age-dependent effects refer to animals merged by sex.

additional 0.25 % bupivacaine solution. Sham-operated animals received identical treatment, except for the brass weight hit. When isoflurane supply was stopped (29.0 ± 4.6 min after anesthesia induction and, for the mTBI animals, 12.7 ± 2.6 min after the brass weight hit), animals were placed in a supine position in a clean cage, and the time-to-right, starting with the first sign of regain of consciousness (hind- or forepaw movement), was measured.

2.3. Neurological severity score

Neurological function was assessed using a revised neurological severity score (NSS) as described previously (Moraga-Amaro et al., 2025; Yarnell et al., 2016a). This test consisted of the evaluation of 10 different responses, each scored from 0 to 2, with 0 indicating a normal response, and 2 indicating complete impairment. The evaluations were (1) beam walk test (general balance; 1 m long, 2 cm wide, positioned at a height of 29 cm; assessment of whether animals could cross the beam, maintain balance, or fall within 15 s), (2) landing test (assessment of whether rats land evenly on all four paws or flat), (3) tail raise test (assessment of paw extension and body twisting), (4) drag test (assessment of grasping movements in an attempt to slow down the dragging), (5) righting reflex (assessment of the flipping reaction when rats are positioned on their back), (6) ear reflex (assessment of pinna flattening when the auditory meatus is touched with a cotton swab), (7) eyelid closure reflex (assessment of eye blink when a cotton swab approaches the eye), (8) acoustic startle reflex (assessment of startling response to a hand clap), (9) tail reflex (assessment of squeaking when the tail is gently pressed), and (10) paw flexion reflex (assessment of limb

withdrawal reflex after pressing the hind paws). No exclusion criteria were applied in this test.

2.4. Saccharin preference test

Anhedonic-like behavior was assessed using the saccharin preference test (SPT), consisting of a training and a test phase, as described previously (Moraga-Amaro et al., 2025; Moraga-Amaro et al., 2022). In brief, training was performed to adapt animals to consumption of a 0.1 % saccharin solution for three consecutive days (3 h per day during the light phase). Afterwards, rats were offered a choice between a bottle filled with saccharin solution and another one filled with water overnight (test phase). The percentage of saccharin consumption (%) was used as the main outcome measure, calculated as the volume (mL) of saccharin solution consumed divided by the total liquid intake (saccharin + water). For the SPT measurements, animals were isolated during specific time periods. During the training phase, rats were isolated for 3 h per day (during the light phase) for three consecutive days. During the test sessions, animals were isolated overnight at 1, 2 and 12 weeks after rmTBI. Social isolation was restricted to the aforementioned periods to minimize potential effects on outcome measures. Although the effects of social isolation cannot be ruled out, sham animals were also isolated; therefore, we assumed that the observed effects were due to rmTBI and not to social isolation. Exclusion criteria were defined as bottles being emptied due to malfunction. No data points were excluded from this test.

2.5. Open field test

Changes in anxiety-like behavior and locomotion were assessed in the OFT under the same conditions as described before (Moraga-Amaro et al., 2025; Moraga-Amaro et al., 2022). Animals were placed inside a black circular arena (90 cm in diameter, 30 cm in height) and allowed to explore for 5 min. Sessions were video-recorded from above the arena for subsequent offline analysis. Total distance traveled in the arena was used as the outcome measure for locomotion. Total time spent in the center of the arena (defined as the inner 20 cm from the border) was used as the outcome parameter for anxiety-like behavior. Automated offline analyses were performed using the ANY-maze v8.3 software (Ugo Basile, Italy). Exclusion criteria included animals escaping from the OFT arena. No data points were excluded.

2.6. Y-maze test

Long-term spatial memory changes were assessed using the Y-maze test, and video-recorded for offline analysis. This protocol was performed as described previously (Jung et al., 2008; Moraga-Amaro et al., 2025). The Y-maze consisted of three arms (50 × 12 × 24 cm; length x width x height) joined at 120° angles, placed in a room with distinct visual cues on the walls visible to the rats to facilitate spatial navigation. For training sessions, animals were allowed to explore the maze for 15 min, with one of the three arms closed. On the same day, 6 h later, rats were allowed to explore again for 5 min, with all three arms open. The percentage of time spent in the previously closed arm was used as the main outcome measure. All data was analyzed automatically using ANY-maze v8.3 software. Exclusion criteria included animals escaping from the maze. No animals were excluded from this test.

2.7. Plasma biomarker quantification

Approximately 300 μL of blood was collected from the lateral tail vein into EDTA tubes (Microvette® CB 300 K2E, Sarstedt, Germany) at 1 day, 3 days, 1 week, 2 weeks, and 12 weeks after rmTBI. Blood samples were centrifuged (5000 *g* for 3 min) and plasma was extracted and stored at −80 °C until further use. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used for the quantification of total free plasma p-tau and/or phosphorylated microtubule-associated phospho-tau (pMAPT; Elabscience, catalog n° *E*-EL-R1090, Texas, USA), and neuron-specific enolase (NSE; MyBioSource, catalog n° MBS262217, California, USA), as these biomarkers have been reported to be relevant in TBI-related pathologies (Stępniewska et al., 2024). For clarity, plasma "p-tau and pMAPT" is referred to as "p-tau". All procedures were performed according to the manufacturer's instructions. ELISA plates were read at 450 nm using a plate reader (FLUOstar Optima, BGM Labtech, Germany), and plasma p-tau and NSE concentrations were calculated using calibration curves. No exclusion criteria were applied in this test.

2.8. [^{18}F]Florzolotau synthesis

The synthesis of [^{18}F]Florzolotau (florzolotau) was carried out using a TRACERlab FXFN synthesis module (GE Healthcare), following a previously described method with minor modifications (Kawamura et al., 2021). To prevent photoisomerization of both the precursor and radiotracer, the synthesis was conducted in darkness, except during specific stages where monitoring was necessary, such as during HPLC injection. Initially, [^{18}F]F− was produced in a Cyclone 18/9 cyclotron (IBA, Belgium) through proton irradiation of ^{18}O-enriched water via the ^{18}O(p,n)^{18}F nuclear reaction. The resulting [^{18}F]F- was trapped on a preconditioned Sep-Pak® Accell Plus QMA Light cartridge (Waters, Milford, MA, USA) and eluted using a mixture of aqueous potassium carbonate solution (3.5 mg/0.5 mL) and Kryptofix 2.2.2. in acetonitrile (15.0 mg/1.0 mL). The eluate was then transferred to the reaction vessel and dried at 60–120 °C under N_2 flow to remove water and acetonitrile. Subsequently, a solution containing the tosylated precursor (2.0 mg) in anhydrous DMSO (0.4 mL) was added, followed by heating at 120 °C for 15 min for fluorination. Afterwards, hydrochloric acid solution (4 M, 0.5 mL) was introduced, and the reaction mixture was heated at 100 °C for 10 min. Neutralization was achieved by adding sodium acetate solution (1 M, 2.0 mL) to the reaction vessel. The mixture was then purified using HPLC with a Mediterranea Sea C18 column (10 × 250 mm, 5 μm) and acetonitrile/50 mM ammonium acetate (40/60) as the mobile phase under isocratic conditions. The desired fraction was collected, diluted with a sodium ascorbate solution (3 % *w/v*, 25 mL), and reformulated using a C-18 light cartridge (Sep-Pak® Light, Waters, Milford, MA, USA). The elution of the cartridge containing florzolotau was done with pure ethanol (1.5 mL) into the final vial. Chemical and radiochemical purity were assessed by radio-HPLC (Agilent 1200 Series, USA) equipped with a radio detector (Gabi, Elysia-Raytest, Germany) and a UV detector (Agilent Technologies, USA) at a wavelength of 365 nm. Identity verification was done through coelution with the reference standard. The decay-corrected radiochemical yield was 9.7 % with a total synthesis time of 70 min. Radiochemical purity was 96 %, and molar activity was 104.4 GBq/μmol at the conclusion of the synthesis process.

2.9. Autoradiography with [^{18}F]Florzolotau

At the end of the experimental procedures, animals underwent euthanasia via an overdose of chloral hydrate (800 mg/kg, i.p.). Subsequently, brains were harvested and immersed in OCT compound (Tissue-Tek®, Sakura Finetek, USA) before being rapidly frozen using liquid nitrogen. Brains were used to determine possible changes in NFT load following rmTBI. The brains were sectioned coronally (Leica CM3050 S, Leica Microsystems, Germany) at a thickness of 16 μm, and slices at bregma −3.0 ± 0.2 mm were used for the analyses. These sections were mounted onto Superfrost Plus adhesion microscope slides (Epredia, USA) and stored at −20 °C until further processing. For autoradiography, brain sections were thawed and pre-incubated for 15 min in Tris-HCl buffer (50 mM, pH 7.4) supplemented with 1 mM MgCl2, 1 mM CaCl2, 2 mM KCl, and 1 % bovine serum albumin at room temperature. Subsequently, the sections (in triplicate, three slices per

animal) were incubated with 0.5 nM florzolotau in buffer solution for 30 min at room temperature with gentle stirring. Following incubation, the sections were removed from the buffer, washed for 10 min in ice-cold Tris-HCl buffer (pH 7.4, 4 °C), and briefly dipped in ice-cold ultra-pure water. After air-drying over a heating plate (40 °C), the sections were exposed to a phosphor-sensitive plate for 5 min and scanned using a phosphor imager (Amersham Typhoon 5, GE Healthcare, USA) at the highest resolution (10 μm). Due to the number of samples, this procedure was conducted in two batches utilizing the same radiotracer synthesis. Each phosphor plate included a slide containing control tissue (7-week-old female brain homogenate) for normalization. Image quantification was performed using PMOD software (version 4.1; PMOD Technologies LLC, Zürich, Switzerland). A whole-brain contour, together with regional contours for the thalamus, parietal cortex and hippocampus, was delineated on each brain section (Groningen rat brain atlas). Optical density (OD) was calculated and normalized with the OD of the control tissue. The outcome measure is presented as the OD_{ratio}, defined as the ratio of $OD_{sample}/OD_{control\ tissue}$. Each OD value was calculated using triplicate brain slices. Slices of insufficient quality were excluded. No OD values were calculated from fewer than two slices per brain.

2.10. Statistical analysis

All statistical analyses were conducted using SPSS Statistics for Windows (version 22.0; IBM Corp., NY, USA). Graphs were generated using GraphPad Prism (version 8.1; GraphPad Software, CA, USA). The generalized estimating equation (GEE) model, as described and applied previously (Moraga-Amaro et al., 2025), was chosen because it provides higher statistical power compared with ANOVA-type analyses and facilitates the analysis of merged factors and non-normally distributed data (Ma et al., 2012). This model was used for both multifactorial longitudinal and non-longitudinal analyses.

In this study, we tested the influence of the combined factors of "*injury*" (sham or rmTBI), "*sex*" (male or female) and "*age*" (adult or adolescent) on the different outcome measures. Fig. 1B illustrates the different levels of analyses conducted. The factors "*injury*", "*sex*", "*age*", and "*timepoint*" were considered. Three types of pairwise comparisons were conducted: (1) "Injury effects" (data merged/grouped by sex and age, first level), (2) "Sex-dependent effects" (data merged/grouped by age, second level), and (3) "Age-dependent effects" (data merged/grouped by sex, second level). This multifactorial analysis was applied as described previously (Eyolfson et al., 2020). Parameters such as OFT distance traveled, OFT time in center, Y-maze time spent in novel arms, and autoradiography results were treated as non-longitudinal data. For longitudinal data, the factor "*timepoint*" was added to each combination, and pairwise comparisons were calculated using (1) "*injury* x *timepoint*", (2) "*injury* x *sex* x *timepoint*" and (3) "*injury* x *age* x *timepoint*" respectively. Longitudinal data includeding short-term weight gain, time-to-right, NSS, SPT, p-tau in plasma, and NSE in plasma were analyzed accordingly. Wald Chi-square (χ^2) and degrees of freedom (df) are reported for each main effect value. Additionally, correlation analyses were conducted using Spearman's test and expressed as correlation coefficient (r). All results are presented as mean ± SD, and statistical significance was set at $p < 0.05$.

3. Results

3.1. Single and repeated mild traumatic brain injury led to a sex- and age-dependent increase in the time-to-right

To determine the immediate effect of each mTBI on the neurological state of the animals, the time-to-right after the surgery was measured. A general effect of *injury* over *time* in the time-to-right was found (*injury* x *timepoint*: $\chi^2 = 46$; df = 5; $p < 0.001$). In this regard, there was a significant increase (Fig. 2A) in the time-to-right after each of the surgeries in the rmTBI group compared with sham animals (mTBI1: sham = 5.1 ± 3.0 s, rmTBI = 11.5 ± 8.9 s; mTBI2: sham = 5.0 ± 2.7 s, rmTBI = 12.7 ± 9.0 s; mTBI3: sham = 5.2 ± 2.4 s, rmTBI = 9.4 ± 5.0 s; $p < 0.001$ to all). This main effect was also present when analyzing the sex-dependent (*injury* x *sex* x *timepoint*: $\chi^2 = 112$; df = 11; $p < 0.001$) and the age-dependent (*injury* x *age* x *timepoint*: $\chi^2 = 55$; df = 11; $p < 0.001$) effects of the injury. Pairwise comparison of the sex-dependent effect showed an increase in the time-to-right for each of the mTBI surgeries (Fig. 2B), in both female (mTBI1: sham = 3.5 ± 2.4 s, rmTBI = 7.4 ± 4.4 s, $p = 0.013$; mTBI2: sham = 3.4 ± 1.7 s, rmTBI = 7.8 ± 2.9 s, $p < 0.001$; mTBI3: sham = 3.4 ± 1.9 s, rmTBI = 8.3 ± 5.5 s, $p = 0.008$) and male rats (mTBI1: sham = 6.6 ± 2.9 s, rmTBI = 15.6 ± 10.6 s, $p = 0.012$; mTBI2: sham = 6.6 ± 2.6 s, rmTBI = 17.6 ± 10.6 s, $p = 0.002$; mTBI3: sham = 6.5 ± 2.2 s, rmTBI = 10.5 ± 4.5 s, $p < 0.001$). On the other

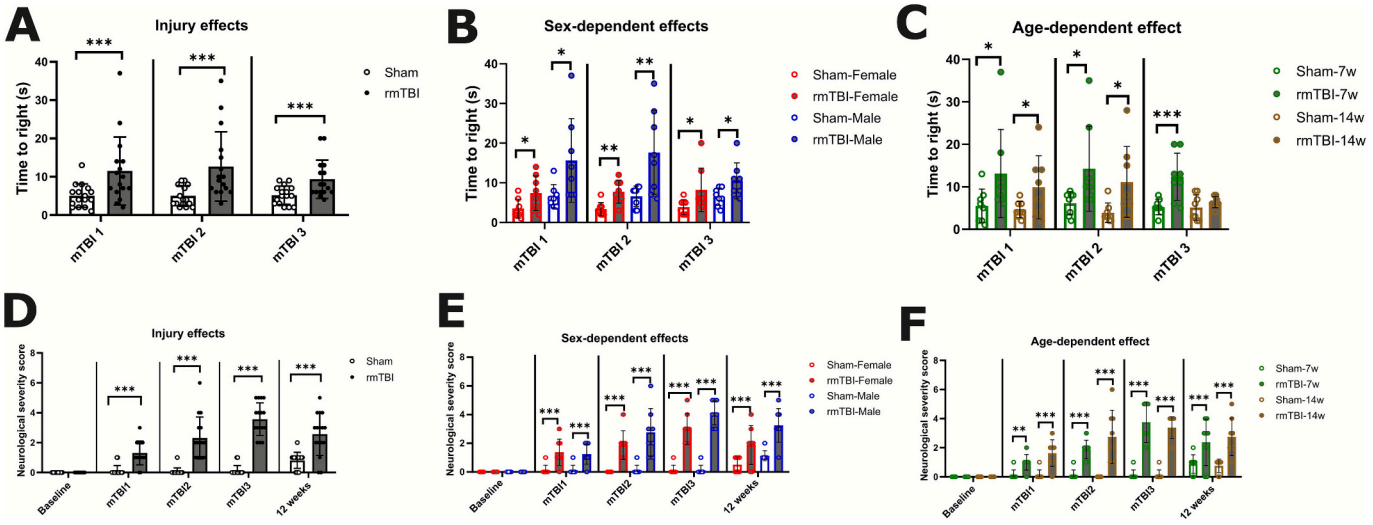

Fig. 2. Short- and long-term effects of rmTBI on neurological state. **A.** Injury effects on the time-to-right. **B.** Sex-dependent effects on the time-to-right. **C.** Age-dependent effects on the time-to-right. **D.** Injury effects on neurological severity score. **E.** Sex-dependent effects on neurological severity score. **F.** Age-dependent effects on neurological severity score. Data are presented as mean ± SD. Statistically significant differences between sham and rmTBI are indicated by asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. *Injury effects* refer to merged data from male, female, adolescent and adult rats. *Sex –dependent effects* refer to merged data from adolescent and adult rats. *Age-dependent effects* refer to merged data from male and female rats.

hand, while age-dependent effects (Fig. 2C) showed this increase at all timepoints for the adolescent rats (mTBI1: sham = 5.5 ± 4.0 s, rmTBI = 13.1 ± 10.4 s, $p = 0.021$; mTBI2: sham = 6.1 ± 2.7 s, rmTBI = 14.3 ± 10.0 s, $p = 0.006$; mTBI3: sham = 5.3 ± 1.8 s, rmTBI = 12.4 ± 5.6 s, $p < 0.001$), these were found in adult rats only after the first and second mTBI surgery (mTBI1: sham = 4.6 ± 1.9 s, rmTBI = 9.9 ± 7.4 s, $p = 0.011$; mTBI2: sham = 3.9 ± 2.3 s, rmTBI = 11.1 ± 8.4 s, $p < 0.001$), but not after the third one (mTBI3: sham = 5.1 ± 3.0 s, rmTBI = 6.4 ± 1.3 s, $p > 0.05$).

3.2. Long-lasting neurological impairments in the neurological severity score due to repeated mild traumatic brain are not exclusively sex- or age-dependent

To determine neurological impairments caused by rmTBI, the NSS was used. No significant differences were found at baseline, for any of the tested comparisons (all $p > 0.05$). A main effect of *injury* over *time* was found (*injury* x *timepoint*: $\chi^2 = 478$; df = 8; $p < 0.001$), reflected as increased neurological impairment (Fig. 2D) after each mTBI surgery (mTBI1: sham = 0.1 ± 0.3, rmTBI = 1.3 ± 0.8; mTBI2: sham = 0.1 ± 0.3, rmTBI = 2.3 ± 1.4; mTBI3: sham = 0.1 ± 0.3, rmTBI = 3.6 ± 1.1; 12 weeks: sham = 0.8 ± 0.5, rmTBI = 2.6 ± 1.4; all $p < 0.001$). This effect was also detected in both sex-dependent (*injury* x *sex* x *timepoint*: $\chi^2 = 518$; df = 14; $p < 0.001$) and age-dependent analyses (*injury* x *age* x *timepoint*: $\chi^2 = 806$; df = 15; $p < 0.001$). Pairwise analyses showed that *injury* increased neurological impairments in both females (mTBI1: sham = 0.1 ± 0.4, rmTBI = 1.4 ± 0.9; mTBI2: sham = 0.0 ± 0.0, rmTBI = 1.9 ± 1.0; mTBI3: sham = 0.1 ± 0.4, rmTBI = 3.0 ± 1.1; 12 weeks: sham = 0.5 ± 0.5, rmTBI = 1.9 ± 1.4; all $p < 0.001$) and males (mTBI1: sham = 0.1 ± 0.4, rmTBI = 1.3 ± 0.7; mTBI2: sham = 0.1 ± 0.4, rmTBI = 2.8 ± 1.7; mTBI3: sham = 0.1 ± 0.4, rmTBI = 4.1 ± 0.8; 12 weeks: sham = 1.1 ± 0.4, rmTBI = 3.3 ± 1.2; all $p < 0.001$) at all timepoints, except at baseline ($p > 0.05$; Fig. 2E). The same pattern was observed in age-dependent analyses (Fig. 2F). Adolescent rats showed increased impairment (mTBI1: sham = 0.1 ± 0.4, rmTBI = 1.0 ± 0.5, $p = 0.003$; mTBI2: sham = 0.1 ± 0.4, rmTBI = 1.9 ± 0.6, $p < 0.001$; mTBI3: sham = 0.1 ± 0.4, rmTBI = 3.8 ± 1.4, $p < 0.001$; 12 weeks: sham = 0.9 ± 0.6, rmTBI = 2.4 ± 1.6, $p < 0.001$), as did adult rats (mTBI1: sham = 0.1 ± 0.4, rmTBI = 1.6 ± 0.9; mTBI2: sham = 0.0 ± 0.0, rmTBI = 2.8 ± 1.8; mTBI3: sham = 0.1 ± 0.4, rmTBI = 3.4 ± 0.7; 12 weeks: sham = 0.8 ± 0.5, rmTBI = 2.8 ± 1.3; all $p < 0.001$).

3.3. Saccharin consumption was not affected by repeated mild traumatic brain

Changes in sweetened-fluid consumption were assessed by the SPT paradigm at 1, 2, and 12 weeks after rmTBI. A significant effect of *injury* over time was found (*injury* x *timepoint*: $\chi^2 = 15$; df = 5; $p = 0.012$). However, no significant differences between sham and rmTBI groups were observed at any tested timepoint (**Fig. 3A**, 1 week: sham = 62.7 ± 22.6 %, rmTBI = 50.0 ± 27.6 %; 2 weeks: sham = 61.3 ± 23.4 %, rmTBI = 54.7 ± 30.0 %; 12 weeks: sham = 68.6 ± 20.9 %, rmTBI = 66.9 ± 26.9 %; $p > 0.05$ for all comparisons). A main effect was also found for both sex-dependent (*injury* x *sex* x *timepoint*: $\chi^2 = 56$; df = 11; $p < 0.001$) and age-dependent analyses (*injury* x *age* x *timepoint*: $\chi^2 = 79$; df = 111; $p < 0.001$). Yet, no significant differences were detected when comparing sham and rmTBI groups in females (1 week: sham = 67.8 ± 21.9 %, rmTBI = 63.2 ± 30.4 %; 2 weeks: sham = 72.9 ± 17.9 %, rmTBI = 75.2 ± 22.1 %; 12 weeks: sham = 71.3 ± 18.0 %, rmTBI = 81.8 ± 8.9 %; all $p > 0.05$) or in males (1 week: sham = 57.5 ± 23.4 %, rmTBI = 36.8 ± 17.6 %; 2 weeks: sham = 49.7 ± 23.8 %, rmTBI = 34.3 ± 21.1 %; 12 weeks: sham = 65.9 ± 24.4 %, rmTBI = 51.9 ± 31.1 %, all $p > 0.05$) at any timepoint (Fig. 3B). Similarly, no differences were observed between sham and rmTBI groups in adolescent rats (1 week: sham = 57.9 ± 12.8 %, rmTBI = 50.1 ± 28.2 %; 2 weeks: sham = 48.8 ± 20.5 %, rmTBI = 50.9 ± 25.6 %; 12 weeks: sham = 69.9 ± 24.4 %, rmTBI = 76.9 ± 16.1 %; $p > 0.05$ for all comparisons) or in adult rats (1 week: sham = 67.5 ± 29.6 %, rmTBI = 49.9 ± 29.0 %; 2 weeks: sham = 73.4 ± 20.4 %, rmTBI = 58.6 ± 34.7 %; 12 weeks: sham = 67.3 ± 18.2 %, rmTBI = 56.8 ± 32.6 %; all $p > 0.05$) (Fig. 3C).

3.4. Repeated mild traumatic brain induced changes in locomotion and anxiety-related behavior which are age-dependent

Changes in locomotion and anxiety-like behavior were measured in the OFT, represented as total distance traveled and time spent in the center of the arena, respectively. Statistical analyses revealed no significant main effect of *injury* on locomotion (*injury*: $\chi^2 = 2$; df = 1; $p > 0.05$). Accordingly, no differences in distance traveled were found between sham and rmTBI animals (Fig. 4A: sham = 24.5 ± 6.7 m, rmTBI = 27.5 ± 8.2 m; $p > 0.05$). While no sex-dependent effect (*injury x sex*: $\chi^2 = 3$; df = 3; $p > 0.05$) was detected, a significant age-dependent effect was observed (*injury x age*: $\chi^2 = 10$; df = 3; $p = 0.023$). Between-group comparisons showed no differences between sham and rmTBI within each sex (Fig. 4B: female: sham = 25.0 ± 7.8 m, rmTBI = 26.6 ± 3.8 m; male: sham = 24.1 ± 5.8 m, rmTBI = 28.4 ± 11.2 m; $p > 0.05$ for all comparisons). In contrast, adolescent animals showed increased locomotion after rmTBI (Fig. 4C; sham = 22.2 ± 4.0 m, rmTBI = 31.2 ± 8.6 m, $p = 0.04$), whereas no difference was observed in adult rats (sham = 26.8 ± 8.2 m, rmTBI = 23.8 ± 6.1 m, $p > 0.05$).

No main *injury* effects were found in the time spent in the center (*injury*: $\chi^2 = 0$; df = 1; $p > 0.05$). Sham and rmTBI animals did not differ (Fig. 4D, sham = 64.0 ± 27.7 s, rmTBI = 63.5 ± 43.4 s; $p > 0.05$). Similarly, no sex-dependent effect was detected (*injury x sex*: $\chi^2 = 2$; df = 3; $p > 0.05$), but a significant age-dependent effect was found (*injury x age*: $\chi^2 = 10$; df = 3; $p = 0.015$). Between-group comparisons showed no differences between sham and rmTBI within each sex (Fig. 4E; female: sham = 70.3 ± 34.0 s, rmTBI = 57.6 ± 26.6 s; male: sham = 57.8 ± 19.8 s, rmTBI = 69.5 ± 57.0 s; $p > 0.05$ for all comparisons). By contrast, age-specific effects were evident (Fig. 4F): adolescent rats showed

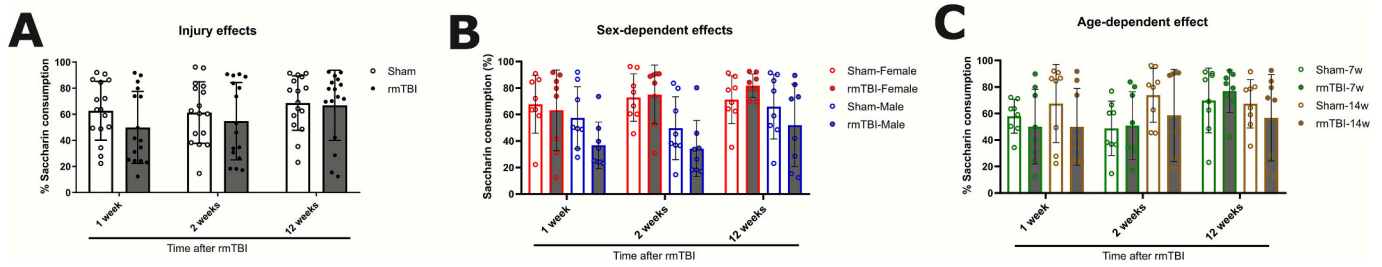

Fig. 3. Short- and long-term effects of rmTBI on saccharin consumption. **A.** Injury effects on saccharin consumption at different timepoints. **B.** Sex-dependent effects on saccharin consumption at different timepoints. **C.** Age-dependent effects on saccharin consumption at different timepoints. Data are presented as mean ± SD. *Injury effects* refer to merged data from male, female, adolescent and adult rats. *Sex –dependent effects* refer to merged data from adolescent and adult rats. *Age-dependent effects* refer to merged data from male and female rats.

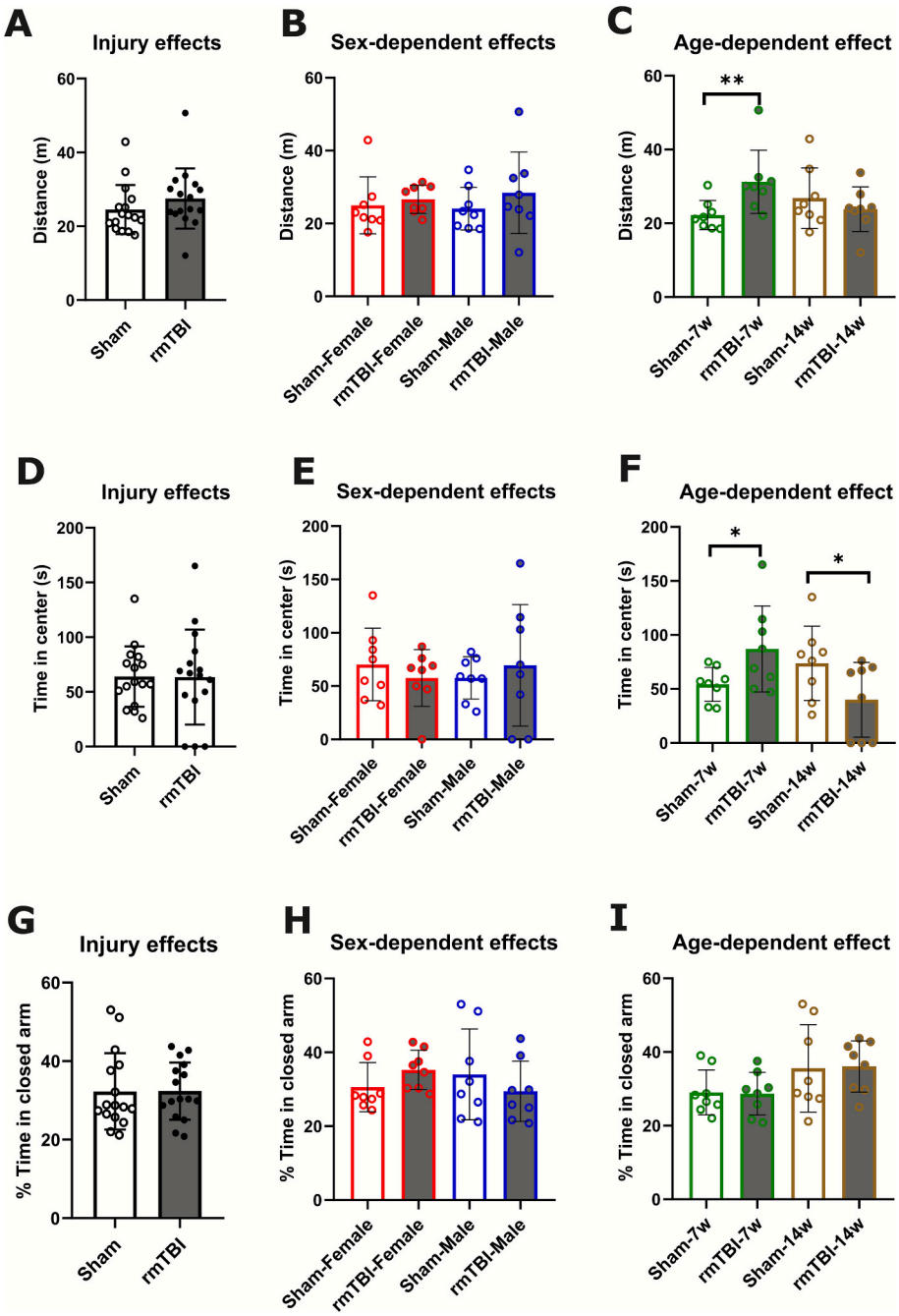

Fig. 4. Long-term effects (12 weeks post third injury) of rmTBI on behavior in the OFT and Y-maze test. **A.** Injury effects on the distance traveled in the open field. **B.** Sex-dependent effects on the distance traveled in the open field. **C.** Age-dependent effects on the distance traveled in the open field. **D.** Injury effects on the time spent in the center of the open field. **E.** Sex-dependent effects on the time spent in the center of the open field. **F.** Age-dependent effects in the time spent in the center of the open field. **G.** Injury effects on the time spent in the closed arm of the Y-maze. **H.** Sex-dependent effects on the time spent in the closed arm of the Y-maze. **I.** Age-dependent effects on the time spent in the closed arm of the Y-maze. Data are presented as mean ± SD. Statistically significant differences between sham and rmTBI are indicated by asterisks: $*p < 0.05$; $**p < 0.01$. *Injury effects* refers to merged data from male, female, adolescent and adult rats. *Sex –dependent effects* refer to merged data from adolescent and adult rats. *Age-dependent effects* refer to merged data from male and female rats.

increased time spent in the center after rmTBI (sham = 54.3 ± 15.7 s, rmTBI = 87.1 ± 39.8 s; $p = 0.013$), whereas adult rats showed decreased time (sham = 73.8 ± 34.3 s, rmTBI = 40.0 ± 34.6 s; $p = 0.018$).

3.5. Spatial memory was not affected by repeated mild traumatic brain

Spatial long-term memory was assessed using the Y-maze test, where the percentage of time spent in the novel arm was used as the outcome measure. No main effect of injury was found (*injury*: $\chi^2 = 0$; df = 1; p > 0.05) in the percentage of time animals spent on the previously closed arm, with pairwise comparisons showing no differences between sham and rmTBI animals (Fig. 4G, sham = 32.3 ± 9.7 %, rmTBI = 32.4 ± 7.3 %; p > 0.05). No sex-dependent effect was observed (*injury x sex*: $\chi^2 = 6$; df = 3; p > 0.05), and no injury-related differences were detected within males or females (Fig. 4H, female: sham = 30.6 ± 6.7 %, rmTBI = 35.3 ± 5.3 %; male: sham = 34.0 ± 12.3 %, rmTBI = 29.5 ± 8.1 %; all p > 0.05). In contrast, a significant age-dependent effect was found (*injury x age*: $\chi^2 = 11$; df = 3; $p = 0.014$). However, pairwise comparisons

revealed no effect of injury in either adolescent or adult rats (Fig. 4I, adolescent: sham = 29.0 ± 6.1 %, rmTBI = 28.7 ± 5.8 %; adult: sham = 35.6 ± 11.9 %, rmTBI = 36.1 ± 7.0 %; all $p > 0.05$).

3.6. Early plasma p-tau concentration following repeated mild traumatic brain is sex- and age-dependent

Plasma p-tau concentration was measured at multiple timepoints. A significant effect of *injury* was found (*injury* x *timepoint*: $\chi^2 = 42$; df = 9; $p < 0.001$). However, no significant differences between sham and rmTBI animals were detected at any individual timepoint (Fig. 5A,1 day: sham = 120.4 ± 89.8 pg/mL, rmTBI = 171.8 ± 119.9 pg/mL; 3 days: sham = 107.1 ± 100.8 pg/mL, rmTBI = 162.5 ± 104.2 pg/mL; 7 days: sham = 114.4 ± 101.3 pg/mL, rmTBI = 106.5 ± 46.1 pg/mL; 14 days: sham = 150.3 ± 81.9 pg/mL, rmTBI = 161.9 ± 105.9 pg/mL; 12 weeks: sham = 81.6 ± 83.7 pg/mL, rmTBI = 101.9 ± 118.8 pg/mL; $p > 0.05$ for all comparisons). At the next level of analysis, significant sex-dependent (*injury* x *sex* x *timepoint*: $\chi^2 = 154$; df = 19; $p < 0.001$) and age-dependent effects were observed (*injury* x *age* x *timepoint*: $\chi^2 = 444$; df = 19; $p < 0.001$). For sex-dependent effects, plasma p-tau was significantly increased in males at 3 days post-rmTBI (Fig. 5B; 3 days: sham = 78.1 ± 54.5 pg/mL, rmTBI = 163.9 ± 85.9 pg/mL, $p = 0.008$). No other comparisons between sham and rmTBI were significant in males (1 day: sham = 94.0 ± 48.5 pg/mL, rmTBI = 117.2 ± 53.3 pg/mL; 7 days: sham = 80.2 ± 59.7 pg/mL, rmTBI = 90.0 ± 56.6 pg/mL; 14 days: sham = 119.6 ± 44.8 pg/mL, rmTBI = 124.8 ± 53.8 pg/mL; 12 weeks: sham = 68.5 ± 50.3 pg/mL, rmTBI = 72.3 ± 81.3 pg/mL; all $p > 0.05$). Similarly, no significant differences were observed in females (1 day: sham = 146.9 ± 115.4 pg/mL, rmTBI = 226.5 ± 145.3 pg/mL; 3 days: sham = 136.0 ± 131.2 pg/mL, rmTBI = 161.0 ± 126.0 pg/mL; 7 days: sham

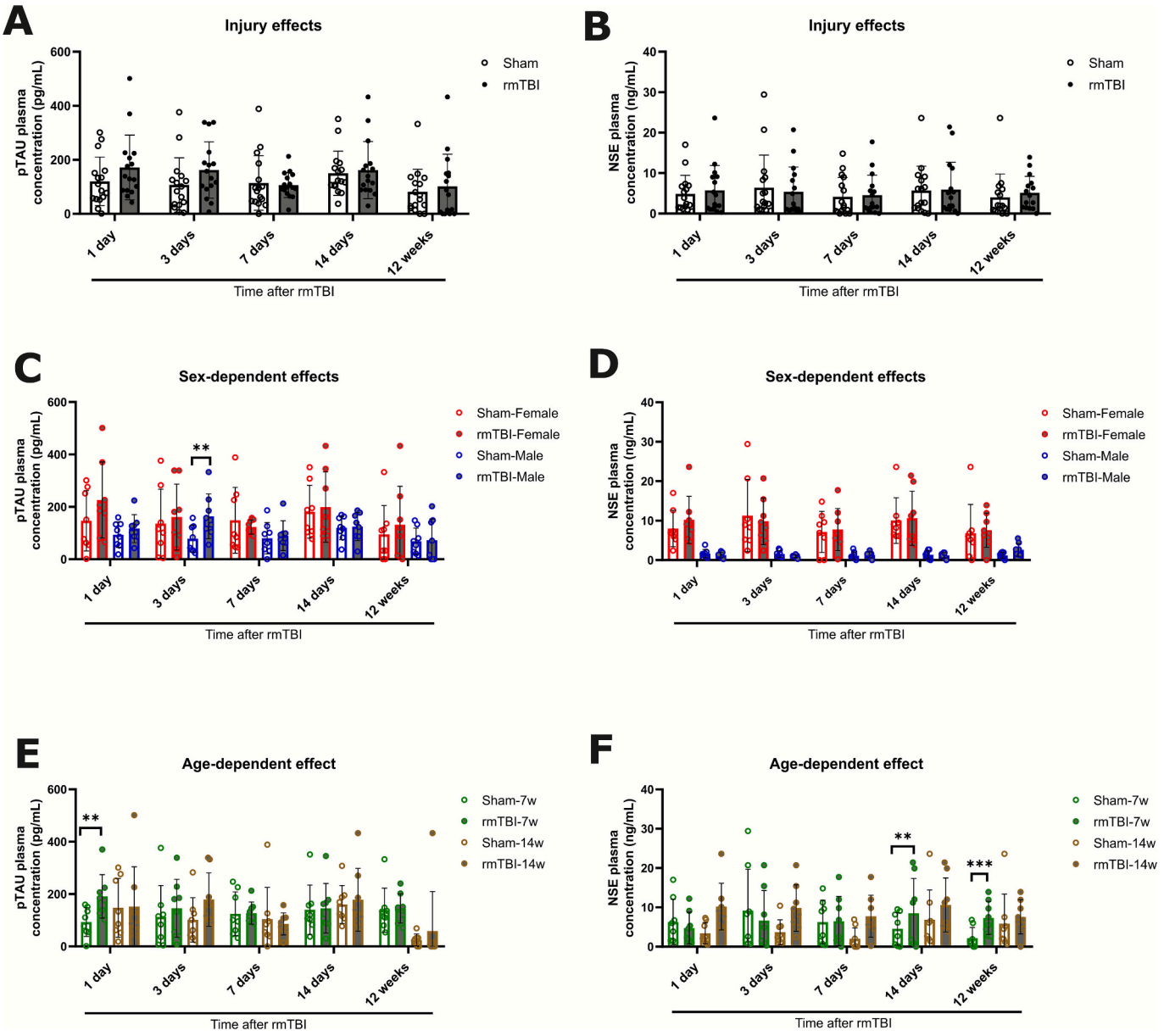

Fig. 5. Changes in plasma biomarkers following rmTBI are sex- and age-dependent. **A.** Injury effects on plasma p-tau concentration. **B.** Sex-dependent effects on plasma p-tau concentration. **C.** Age-dependent effects on plasma p-tau concentration. **D.** Injury effects on plasma NSE concentration. **E.** Sex-dependent effects on plasma NSE concentration. **F.** Age-dependent effects on plasma NSE concentration. Data are presented as mean ± SD. Statistically significant differences between sham and rmTBI are indicated by asterisks: $^{**}p < 0.01$, $^{***}p < 0.001$. *Injury effects* refer to merged data from male, female, adolescent and adult rats. *Sex –dependent effects* refer to merged data from adolescent and adult rats. *Age-dependent effects* refer to merged data from male and female rats.

= 148.6 ± 125.5 pg/mL, rmTBI = 123.0 ± 27.1 pg/mL; 14 days: sham = 181.1 ± 101.0 pg/mL, rmTBI = 199.1 ± 134.1 pg/mL; 12 weeks: sham = 94.8 ± 109.9 pg/mL, rmTBI = 131.6 ± 147.1 pg/mL; all p > 0.05). For age-dependent effects, an increase in plasma p-tau was found in adult rats at day 1 post-rmTBI (**Fig. 5C**, 1 day: sham = 93.3 ± 55.0 pg/mL; rmTBI = 181.1 ± 76.5 pg/mL; $p = 0.003$). No other significant injury-related changes were observed in adolescent rats (3 days: sham = 114.6 ± 106.4 pg/mL, rmTBI = 145.7 ± 110.7 pg/mL; 7 days: sham = 120.3 ± 75.0 pg/mL, rmTBI = 127.4 ± 42.4 pg/mL; 14 days: sham = 140.9 ± 85.5 pg/mL, rmTBI = 145.5 ± 94.9 pg/mL; 12 weeks: sham = 142.9 ± 74.9 pg/mL, rmTBI = 145.2 ± 55.7 pg/mL; $p > 0.05$ for all comparisons) or in adult rats at any timepoint (1 day: sham = 147.6 ± 112.1 pg/mL; rmTBI = 152.4 ± 151.7 pg/mL; 3 days: sham = 101.5 ± 85.0 pg/mL, rmTBI = 179.2 ± 101.7 pg/mL; 7 days: sham = 104.2 ± 121.4 pg/mL, rmTBI = 85.6 ± 42.0 pg/mL; 14 days: sham = 160.5 ± 72.5 pg/mL, rmTBI = 178.4 ± 120.0 pg/mL; 12 weeks: sham = 24.8 ± 22.3 pg/mL, rmTBI = 58.7 ± 151.2 pg/mL; p > 0.05 for all comparisons).

3.7. Long-term repeated mild traumatic brain -induced changes in NSE plasma concentrations are age-dependent

Plasma NSE concentration was measured as a biomarker of cellular damage in the brain. A significant main effect of injury was found (*injury* x *timepoint*: $\chi^2 = 34$; df = 9; $p < 0.001$), which did not translate into differences between sham and rmTBI rats at any individual timepoint (**Fig. 5D**, 1 day: sham = 4.9 ± 4.6 ng/mL, rmTBI = 5.7 ± 6.2 ng/mL; 3 days: sham = 6.4 ± 8.0 ng/mL, rmTBI = 5.4 ± 6.1 ng/mL; 7 days: sham = 4.1 ± 4.8 ng/mL, rmTBI = 4.5 ± 5.0 ng/mL; 14 days: sham = 5.7 ± 6.0 ng/mL, rmTBI = 5.9 ± 6.8 ng/mL; 12 weeks: sham = 4.0 ± 5.8 ng/mL, rmTBI = 5.1 ± 4.1 ng/mL; $p > 0.05$ for all comparisons). At the second level, significant effects were found for both sex-dependent (*injury* x *sex* x *timepoint*: $\chi^2 = 4894$; df = 19; p < 0.001) and age-dependent (*injury* x *age* x *timepoint*: $\chi^2 = 169$; df = 19; p < 0.001) analyses. Pairwise analyses revealed no significant sex-dependent differences (Fig. 5E) in female rats (1 day: sham = 8.0 ± 4.6 ng/mL, rmTBI = 10.2 ± 6.0 ng/mL; 3 days: sham = 11.3 ± 6.0 ng/mL, rmTBI = 9.8 ± 5.9 ng/mL; 7 days: sham = 7.2 ± 5.2 ng/mL, rmTBI = 7.8 ± 5.3 ng/mL; 14 days: sham = 10.0 ± 5.8 ng/mL, rmTBI = 10.6 ± 6.9 ng/mL; 12 weeks: sham = 6.8 ± 7.3 ng/mL, rmTBI = 7.6 ± 4.3 ng/mL; all p > 0.05) or in male rats (1 day: sham = 1.7 ± 1.0 ng/mL, rmTBI = 1.3 ± 0.7 ng/mL; 3 days: sham = 1.5 ± 0.8 ng/mL, rmTBI = 1.0 ± 0.3 ng/mL; 7 days: sham = 1.1 ± 1.0 ng/mL, rmTBI = 1.3 ± 0.8 ng/mL; 14 days: sham = 1.4 ± 1.1 ng/mL, rmTBI = 1.2 ± 0.7 ng/mL; 12 weeks: sham = 1.2 ± 0.8 ng/mL, rmTBI = 2.6 ± 1.6 ng/mL; all p > 0.05). By contrast, age-dependent effects were observed (Fig. 5F). Adolescent rats exposed to rmTBI showed significantly higher plasma NSE concentrations at 14 days (14 days: sham = 4.5 ± 4.1 ng/mL, rmTBI = 8.5 ± 8.8 ng/mL; $p = 0.015$) and 12 weeks (12 weeks: sham = 2.2 ± 2.7 ng/mL, rmTBI = 7.3 ± 4.2 ng/mL; $p < 0.001$) compared with sham. No other significant sham vs rmTBI differences were detected at earlier timepoints in adolescent rats (1 day: sham = 6.3 ± 5.8 ng/mL, rmTBI = 1.3 ± 0.7 ng/mL; 3 days: sham = 9.1 ± 10.6 ng/mL, rmTBI = 1.0 ± 0.3 ng/mL; 7 days: sham = 6.3 ± 5.6 ng/mL, rmTBI = 1.3 ± 0.8 ng/mL; $p > 0.05$ for all comparisons) or in adult rats (1 day: sham = 3.4 ± 2.7 ng/mL, rmTBI = 10.2 ± 6.0 ng/mL; 3 days: sham = 3.7 ± 3.2 ng/mL, rmTBI = 9.8 ± 5.9 ng/mL; 7 days: sham = 2.0 ± 2.7 ng/mL, rmTBI = 7.7 ± 5.3 ng/mL; 14 days: sham = 6.9 ± 7.6 ng/mL, rmTBI = 10.6 ± 6.9 ng/mL; 12 weeks: sham = 5.8 ± 7.5 ng/mL, rmTBI = 7.6 ± 4.3 ng/mL; all p > 0.05).

3.8. Long-term repeated mild traumatic brain -induced brain NFT increase is sex- and age-dependent

Autoradiography with florzolotau in brain slices was performed to determine changes in NFT load at 12 weeks after rmTBI. At the whole-brain level, a significant main effect of *injury* was found (*injury*: $\chi^2 = 9$; df = 1; $p = 0.003$), corresponding to increased NFT in rmTBI animals (Fig. 6A; sham = 1.8 ± 0.4, rmTBI = 1.9 ± 0.2, p = 0.003). This effect was also observed in sex-dependent (*injury* x *sex*: $\chi^2 = 12$; df = 3; $p = 0.006$) and age-dependent analyses (*injury* x *age*: $\chi^2 = 12$; df = 3; $p = 0.007$). Both females (sham = 1.6 ± 0.5, rmTBI = 1.8 ± 0.3, $p = 0.035$) and males (sham = 1.8 ± 0.2, rmTBI = 2.0 ± 0.2, $p = 0.030$) showed increased brain NFT (Fig. 6B). With respect to age, NFT increases were detected only in adult rats (sham = 1.7 ± 0.5, rmTBI = 1.9 ± 0.3, $p < 0.001$), but not in adolescents (Fig. 6C; sham = 1.9 ± 0.1, rmTBI = 1.9 ± 0.2, $p > 0.05$). In addition, sham adults had significantly lower NFT levels compared with sham adolescents ($p = 0.005$).

At the regional level, significant *injury* effects were found in the thalamus (*injury*: $\chi^2 = 10$; df = 1; $p = 0.002$), parietal cortex (*injury*: $\chi^2 = 10$; df = 1; p = 0.002) and hippocampus (*injury*: $\chi^2 = 8$; df = 1; p = 0.005), reflecting increased NFT in rmTBI rats (thalamus: Fig. 6D; sham = 1.3 ± 0.1, rmTBI = 1.5 ± 0.1, p = 0.002; (parietal cortex: Fig. 6G; sham = 1.3 ± 0.1, rmTBI = 1.4 ± 0.1, p = 0.002; hippocampus: Fig. 6J; sham = 1.3 ± 0.2, rmTBI = 1.4 ± 0.1, p = 0.005). In the thalamus, both sex- (*injury* x *sex*: $\chi^2 = 12$; df = 3; $p = 0.009$) and age-dependent effects (*injury* x *age*: $\chi^2 = 17$; df = 3; p < 0.001) were found. Females (sham = 1.3 ± 0.2, rmTBI = 1.4 ± 0.1, $p = 0.044$) and males (sham = 1.4 ± 0.1, rmTBI = 1.5 ± 0.2, $p = 0.014$) both exhibited increased NFT (Fig. 6E), but this effect was limited to adults (sham = 1.3 ± 0.2, rmTBI = 1.5 ± 0.1, p < 0.001) and not present in adolescent rats (Fig. 6F sham = 1.4 ± 0.1, rmTBI = 1.4 ± 0.1, p > 0.05). In the parietal cortex, a sex-dependent effect was detected: males (sham = 1.3 ± 0.1, rmTBI = 1.4 ± 0.1, p = 0.007) but not females (sham = 1.2 ± 0.2, rmTBI = 1.3 ± 0.1, p > 0.05) displayed increased NFT density (Fig. 6H). Age-dependent effects (Fig. 6I) were also present, with adult rats showing higher NFT (sham = 1.2 ± 0.2, rmTBI = 1.4 ± 0.1, p < 0.001) but no change in adolescent rats (sham = 1.4 ± 0.1, rmTBI = 1.4 ± 0.1, p > 0.05). A similar pattern was observed in the hippocampus. A sex-dependent effect was found, with NFT increases in males (sham = 1.3 ± 0.2, rmTBI = 1.4 ± 0.1, $p = 0.040$) but not in females (sham = 1.3 ± 0.2, rmTBI = 1.5 ± 0.1, p > 0.05) (Fig. 6K). Age-dependent analysis revealed increased NFT in adults (sham = 1.2 ± 0.2, rmTBI = 1.4 ± 0.1, $p = 0.001$), but not in adolescents (Fig. 6L; sham = 1.4 ± 0.1, rmTBI = 1.4 ± 0.1, p > 0.05). Additionally, sham adult rats exhibited decreased NFT compared with sham adolescents in all analyzed brain regions (thalamus, p = 0.001; parietal cortex, $p = 0.002$; hippocampus, p = 0.002).

3.9. Brain and plasma biomarker correlations with neurological state and saccharin consumption after repeated mild traumatic brain are sex- and age-dependent

To determine possible relationships between plasma and brain biomarkers with neurological severity and saccharin consumption in the SPT, Spearman's correlation analyses were performed. Analyses were restricted to rmTBI animals in order to evaluate correlations due to *injury* alone, as well as sex- and age-dependent effects.

At the injury level (Fig. 7A), plasma NSE concentration at 2 weeks post-rmTBI showed a strong inverse correlation with NSS at 12 weeks ($r = -0.72$, p = 0.002), and a positive correlation with plasma NSE at 12 weeks ($r = 0.66$, $p = 0.003$). Moreover, plasma NSE at 12 weeks correlated with several outcome measures, including NSS at 12 weeks ($r = -0.57$, $p = 0.019$), SPT at 12 weeks ($r = 0.55$, $p = 0.022$), and plasma p-tau at 12 weeks (r = 0.55, $p = 0.014$).

Sex-dependent analyses revealed different patterns (Fig. 7B-C). In females, plasma NSE measured at 2 weeks showed a negative correlation with SPT at 2 weeks ($r = -0.86$, $p = 0.005$) and with NSS at 12 weeks ($r = -0.68$, $p = 0.033$), but a positive correlation with plasma NSE at 12 weeks ($r = 0.76$, $p = 0.018$). Plasma NSE at 12 weeks correlated negatively with SPT at 12 weeks ($r = -0.81$, $p = 0.011$). Additional correlations included plasma p-tau at 12 weeks with NSS after 1 day ($r = -0.64$, $p = 0.049$), with plasma p-tau at 2 weeks ($r = 0.78$, $p = 0.015$),

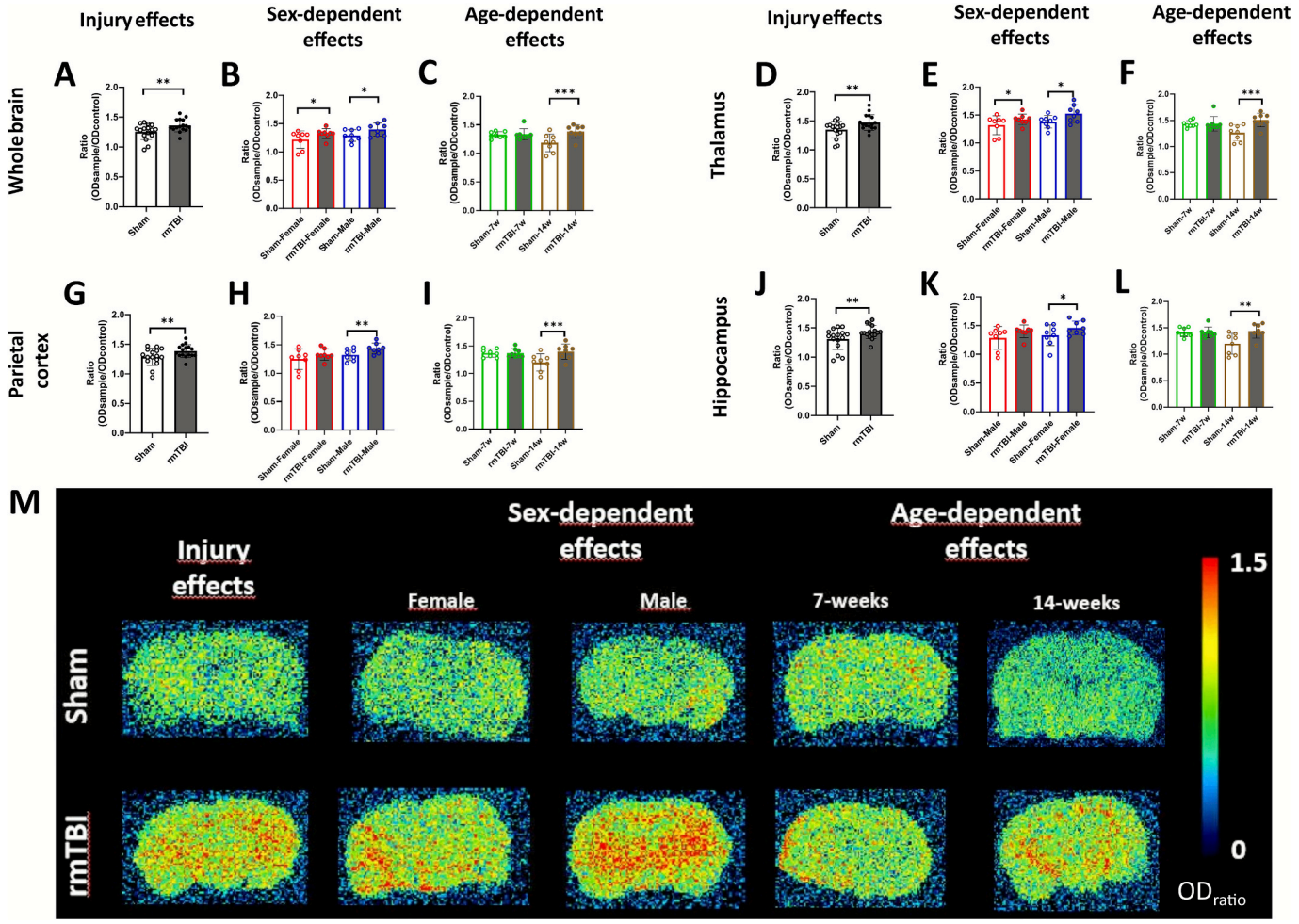

Fig. 6. Brain NFT increases 12 weeks post-rmTBI. **A.** Injury effects on NFTs in the whole brain. **B.** Sex-dependent effects on NFTs in the whole brain. **C.** Age-dependent effects on NFTs in the whole brain. **D.** Injury effects on NFTs in the thalamus. **E.** Sex-dependent effects on NFTs in the thalamus. **F.** Age-dependent effects on NFTs in the thalamus. **G.** Injury effects on NFTs in the parietal cortex. **H.** Sex-dependent effects on NFTs in the parietal cortex. **I.** Age-dependent effects on NFTs in the parietal cortex. **J.** Injury effects on NFTs in the hippocampus. **K.** Sex-dependent effects on NFTs in the hippocampus. **L.** Age-dependent effects on NFTs in the hippocampus. **M.** Representative autoradiography images of NFTs in brain slices at section level corresponding to approximately bregma −3.0 mm. Each brain section corresponds to the slice belonging to the experimental group which showed the closest value to the group average. Ratios correspond to the $OD_{sample}/OD_{control\ tissue}$. Data are presented as mean ± SD. Statistically significant differences between sham and rmTBI are indicated by asterisks: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. *Injury effects* refer to merged data from male, female, adolescent and adult rats. *Sex –dependent effects* refer to merged data from adolescent and adult rats. *Age-dependent effects* refer to merged data from male and female rats.

and with brain NFT after 12 weeks ($r = -0.71$, $p = 0.030$). Furthermore, brain NFT also correlated positively with NSS at 1 day ($r = 0.85$, $p = 0.006$). By contrast, in males (Fig. 7C), the only significant correlations were found between plasma NSE at 12 weeks and SPT at 12 weeks ($r = 0.71$, $p = 0.029$), plasma p-tau at 2 weeks ($r = 0.88$, $p = 0.004$), and plasma NSE at 2 weeks with SPT at 12 weeks (r = −0.81, p = 0.011).

Age-dependent analyses also revealed distinct results (Figs. 7D–E). In adolescents, plasma NSE at 2 weeks correlated negatively with NSS at 12 weeks ($r = -0.88$, p = 0.022) and positively with plasma NSE at 12 weeks ($r = 0.69$, $p = 0.035$). In adults, more correlations were observed: plasma NSE at 2 weeks correlated with both SPT at 12 weeks (r = 0.69, p = 0.035) and plasma p-tau at 12 weeks ($r = 0.75$, $p = 0.021$), while plasma NSE at 12 weeks also correlated with SPT at 12 weeks (r = 0.69, p = 0.035). Additionally, brain NFT correlated strongly with NSS at 1 day ($r = 0.82$, p = 0.011), plasma p-tau at 12 weeks showed a strong negative correlation with NSS at 12 weeks (r = −0.86, $p = 0.007$), and a SPT at 2 weeks correlated positively with SPT at 12 weeks ($r = 0.74$, $p = 0.023$).

4. Discussion

This study aimed to determine the influence of injury, sex and age on the acute and long-term effects of rmTBI in a model of CTE, examining neurological, behavioral and physiological outcomes in rats, with a focus on identifying potential global and individual early biomarkers associated with the severity of neurobehavioral impairments. We demonstrate that the rmTBI model used in this study successfully induced both acute and long lasting (up to 12 weeks post-injury) neurological impairments, which manifested as increased time-to-right and elevated NSS, and which were not influenced by sex or age. Regarding long-term behavioral impairments, while no apparent changes in consumption of saccharin solution were observed across factors, an exclusively age-dependent effect was found in anxiety-like behavior. In addition, while no significant changes were observed in plasma biomarkers such as NSE and p-tau when analyzing the effects of injury alone, our results indicated early changes in plasma p-tau specifically in males and in adolescent rats, as well as changes in NSE in adolescent rats, suggesting that early biomarkers are both sex- and age-dependent. Furthermore, increased brain NFTs were observed at 12

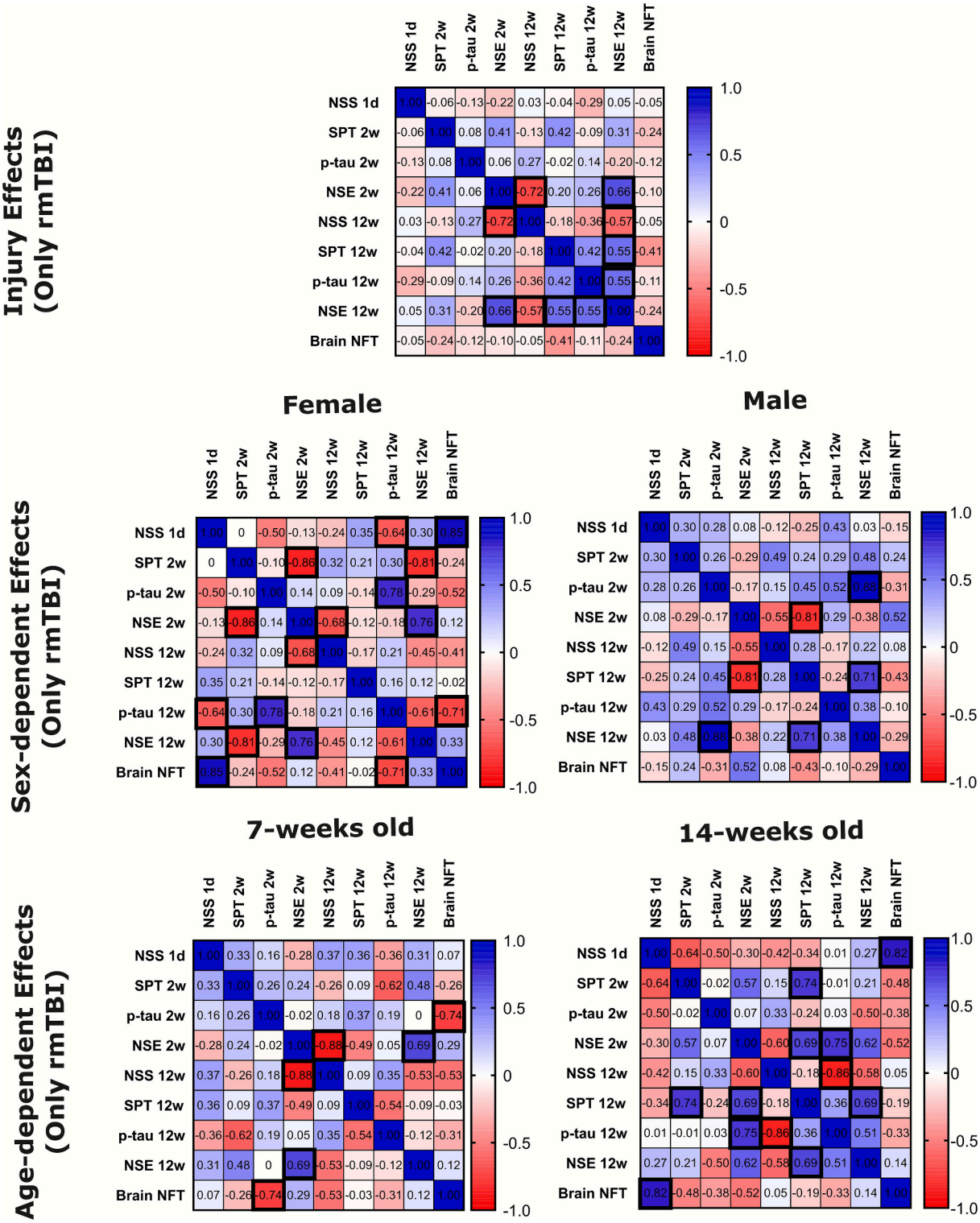

Fig. 7. Spearmann's correlation maps. Correlation map showing positive (in blue) and negative (in red) correlations between different parameters. **NSS-1d** refers to the neurological severity score at 1 day after rmTBI. **NSS-12w** refers to the neurological severity score at 12 weeks after rmTBI. **SPT-2w** refers to the saccharin preference test 2 weeks after rmTBI. **SPT-12w** refers to the saccharin preference test 12 weeks after rmTBI. **p-tau-2w** refers to the plasma p-tau measured 2 weeks after rmTBI **p-tau-12w** refers to the plasma p-tau measured 12 weeks after rmTBI. **NSE-2w** refers to the plasma NSE measured 2 weeks after rmTBI. **NSE-12w** refers to the plasma NSE measured 12 weeks after rmTBI. **Brain NFT** refers to the florzolotau binding 12 weeks after rmTBI. The color scale indicates the correlation coefficient (r) between two variables. The intensity of the blue and red colors reflects the strength of the correlation, with darker red indicating stronger positive correlations and darker blue indicating stronger negative correlations. All squares with highlighted borders are statistically significant ($p < 0.05$). *Injury effects* refer to merged data from male, female, adolescent, and adult rats. *Sex –dependent effects* refer to merged data from adolescent and adult rats. *Age-dependent effects* refer to merged data from male and female rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

weeks post rmTBI, influenced by injury, sex and age. Interestingly, correlation analyses suggest that while NSE appears to be a promising candidate for early global diagnosis of CTE, p-tau may be more accurate in predicting future CTE severity in females.

While the results discussed in the following paragraphs both align with and contradict previous findings in the literature, it is important to note that most prior studies examined the effects of rmTBI over a period of days up to a maximum of 5 weeks, whereas our measurements extended to 12-weeks post-injury. Additionally, the species (rats or mice) and the age at which repetitive impacts were administered varied across the studies, which likely influences behavioral outcomes. Consequently, it is difficult to directly compare our findings with the literature, and further study of longer-term consequences of rmTBI is needed to confirm our results, ideally in another relevant animal model.

In this study, we applied a CTE model based on rmTBI as previously used (Moraga-Amaro et al., 2025) and originally described by McAteer and colleagues (McAteer et al., 2016), which simulates head projectile impacts being characteristic for contact sports. To assess neurological impairment severity, we measured the time-to-right following each mTBI surgery and applied the revised NSS test as developed by Yarnell and colleagues (Yarnell et al., 2016a). Regardless of sex and age, we observed a general increase in time-to-right after surgery due to rmTBI across all factors analyzed, consistent with previous rmTBI studies (Massé et al., 2024). In line with prior descriptions of this model in rodents (Jacotte-Simancas et al., 2023; Kahriman et al., 2023; Meconi et al., 2018; Moraga-Amaro et al., 2025; Qin et al., 2018), we observed acute increased neurological impairment in the NSS. However, we also observed that these effects were long-lasting, persisting for at least 12 weeks. While these findings were largely independent of age or sex, the long-term neurological impairments were more pronounced in males than in females.

Due to the multifactorial influence on the outcomes of rmTBI, studies focusing on the identification of specific biomarkers for a more personalized diagnosis and treatment are crucial in this field. In this study, we focused on the temporal changes in plasma p-tau and NSE, as these biomarkers have previously been shown to increase after mTBI and rmTBI (Agoston et al., 2022; Halicki et al., 2023; Mavroudis et al., 2022; Stępniewska et al., 2024). We found no significant effects of injury alone on plasma p-tau or NSE at any of the tested time points. However, sex- and age-dependent effects were observed, in line with previous reports on sex differences in blood biomarkers (Papa et al., 2023). Short-term increases in plasma p-tau after rmTBI were found only in males at 3 days post-injury and only in adolescent animals, 1 day post-injury. Regarding plasma NSE, while no sex-dependent effects were found, long-term increases in plasma NSE were observed in adolescent animals at both 14 days and 12 weeks after rmTBI. It is important to note that our results support the notion of sex- and age- related differences in these blood biomarkers, with several significant differences observed between males and females, as well as between adolescent and adult animals. Further studies are needed to determine whether p-tau can serve as an early biomarker for CTE, while NSE may be useful as a late-stage biomarker.

Furthermore, higher brain NFTs were observed at 12 weeks post-injury, with effects due to injury, sex and age. Long-term increases in rodent brain NFT have been previously reported (McAteer et al., 2016; Puangmalai et al., 2024). In all brain areas analyzed, NFTs were increased by injury alone. When analyzing sex-dependent effects, both males and females showed increased brain NFTs at the whole brain level and in the thalamus. However, in the parietal cortex and the hippocampus, this effect was exclusively observed in males. Regarding age-dependent effects, increased NFTs were only observed in adult animals, and not in adolescent ones. To our knowledge, no clinical studies have explored the potential of NFT PET imaging to investigate this biomarker in CTE. Further studies with florzolotau are needed to evaluate its potential in the diagnosis of CTE using brain imaging techniques.

Here, we conducted behavioral screening for long-term anxiety- and depression-related changes and cognitive impairment due to rmTBI. Previous studies on rodent models of rmTBI and depressive-like behavior in animal models showed mixed results: one study reported no sex-related differences in the forced swim test (FST) (Liu et al., 2024), two studies found increased immobility in the FST only in females (McCorkle et al., 2022; Wright et al., 2017), and one observed decreased motivation in the splash test only in males (Liu et al., 2024). One possible explanation for these differences are the time intervals between rmTBI and behavioral testing. While McCorkle and colleagues and Wright and colleagues found differences at 7 days and 14 days post-injury, Liu and colleagues tested the FST at 9 weeks post injury (Liu et al., 2024; McCorkle et al., 2022; Wright et al., 2017). In this regard, we found no apparent anhedonic-like behavior in either male or female rats in the saccharin preference test, which aligns with McCorkle and colleagues' findings in the SPT (McCorkle et al., 2022). Nonetheless, male rats consumed significantly less saccharin than females, indicating a sex difference in test performance. Furthermore, injury did not affect saccharin consumption in either adolescent or adult rats, however adult rats drank significantly less saccharin-containing water than adolescent ones at 12 weeks post-injury.

Preclinical studies on the effects of rmTBI on anxiety-like behavior in rodents have shown diverse results, too. For instance, anxiety changes independent of sex have been reported, with some studies showing increased anxiety (Eyolfson et al., 2022; Liu et al., 2024; Wright et al., 2017) and others showing decreased anxiety instead (Schwab et al., 2021) at different timepoints due to rmTBI. Moreover, one study showed that rmTBI increased anxiety only in males (Leung et al., 2023), while no studies have demonstrated the same effect specifically in females. Our results showed no overall effect of injury on anxiety, which aligns with previous studies indicating no sex effects in rodent models of anxiety (Jacotte-Simancas et al., 2023; Meconi et al., 2018). However, we observed an age effect: adolescent rats exhibited higher locomotion with increased time spent in the center of the open field, while adult rats exhibited anxiety-like behavior in terms of decreased time spent in the center. To our knowledge, only two studies have reported age- and injury-dependent effects in anxiety, both showing increased anxiety in adolescent mice, but there the animals were much earlier after rmTBI, i.e. 2 days or 3 weeks post-injury (Eyolfson et al., 2022; Mannix et al., 2017).

Regarding memory impairment, studies examining the influence of sex on rodent rmTBI-induced memory deficits have also found sex-dependent differences. Memory impairments have been observed in males in the Morris water maze (Schwab et al., 2021) and only in males in object-based memory tasks (Eyolfson et al., 2022; McCorkle et al., 2022), while in the fear-conditioning paradigm, impairments were found only in females (Leung et al., 2023). Other studies reported memory impairments in both sexes in object-based memory tests (Wright et al., 2017) and in the Barnes maze paradigm (Meconi et al., 2018). In terms of age-related effects, Eyolfson et al. reported that only adolescent mice exhibited memory impairments (Eyolfson et al., 2022), while Mannix et al. found similar impairments in both adolescent and young adult mice in the Morris water maze following rmTBI (Mannix et al., 2017). In our study, no memory deficits were detected in the Y-maze test, as there were no significant differences between sham and rmTBI animals at any factor level, indicating the absence of both sex-dependent and age-dependent effects.

Interestingly, when correlating the different parameters analyzed in this study, sex- and age-dependent effects became more evident. Several inverse correlations were found between plasma NSE and SPT in both males and females, yet only in adults, not in adolescent rats. This suggests that NSE may serve as an age-dependent biomarker for predicting future mood impairments. In addition, early NSE levels correlated with NSS at 12 weeks post-injury in the adolescent rat group, alluding to the potential use of this biomarker as a predictor of long-term neurological impairments in this subgroup. These results are in line with findings in children following TBI, where higher blood NSE levels are associated

with worse outcomes (Żurek and Fedora, 2012). Conversely, while blood p-tau has been shown to be a biomarker of TBI severity (Edwards et al., 2023; Lange et al., 2024; Rubenstein et al., 2024; Sass et al., 2021), we only observed correlations in the female and adult subgroups. Furthermore, brain NFT increases at 12 weeks post-injury were correlated with neurological impairments only in female and adult rats. Interestingly, it has been suggested that disease outcomes following rmTBI in females, but not in males, are age dependent in mice (Ferguson et al., 2017) and are thought to be relevant risk factors in humans (King, 2014), with brain NFTs being one of the main hallmarks. These findings indicate that further studies are needed to better understand the differential use of biomarkers for CTE detection and treatment.

While animal models of CTE are crucial for developing methods to diagnose this disease early, there are some limitations associated with their use. In this study, we employed the acceleration-free weight model, which simulates a head impact from a projectile (such as an American football ball). However, there are numerous other TBI models that simulate different types of injuries, and the results may vary depending on the model used. Therefore, it is necessary to identify common and differential biomarkers across brain injury models to improve therapeutic strategies. Likewise, although our study comprised heterogeneous groups, potentially masking the effects of individual factors, it may better reflect the complexity of clinical studies.

5. Conclusions

In our study, we identified sex- and age-dependent differences in the progression and outcomes of short- and long-term effects of rmTBI in rats. We used a combination of factors to analyze heterogeneous experimental groups organized by sex or age to investigate their influence in rmTBI. This study demonstrated the short-term effects of rmTBI, successfully inducing neurological impairments across all experimental group combinations. Interestingly, neither short- nor long-term changes in depressive behavior, nor long-term memory impairments were observed in any of the groups studied. However, long-term changes in anxiety-like behavior were found only in adult rats, suggesting a sex-dependent effect in this area. In addition, both sex- and age-dependent changes in plasma p-tau and NSE were observed, indicating p-tau as an early biomarker, and NSE as a late biomarker for CTE. Brain NFT was present in nearly all groups, however, regional brain analyses suggest higher NFT accumulation in males and adult rats. Additionally, our findings propose plasma NSE as a biomarker of behavioral outcomes, and brain NFT as a marker of protracted neurological impairments in CTE, in a sex- and age-dependent manner. Together, these findings support the use of homogeneous multifactorial groups to advance the research on CTE pathophysiology and promote individualized diagnosis and treatment of this condition, helping to avoid underrepresentation of specific patient subgroups.

CRediT authorship contribution statement

Rodrigo Moraga-Amaro: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Oscar Moreno:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Jordi Llop:** Writing – review & editing, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Marion Bankstahl:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization. **Jens P. Bankstahl:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (OpenAI) in order to improve the readability and language of the manuscript. After using this tool, the authors and a native speaker reviewed and edited the content as needed and the authors take full responsibility for the content of the published article.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.(Jens P Bankstahl reports financial support was provided by German Research Foundation. Jordi Llop reports financial support was provided by Spanish Research Agency. Oscar Moreno reports financial support was provided by Spanish Research Agency. Jordi Llop reports equipment, drugs, or supplies was provided by Aprinoia Therapeutics Inc. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.)

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Data availability

The data that support the findings of this study are available at Doi: 10.26068/mhhrpm/20250613-000.

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Glossary

CTE: Chronic traumatic encephalopathy
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
GEE: Generalized estimating equations
GFAP: Glial fibrillary acid protein
mTBI: Mild traumatic brain injury
NFTs: Neurofibrillary tangles
NSE: Neuro-specific enolase
NSS: Neurological severity score
OD: Optical density
OFT: Open field test
pMAPT: Phosphorylated microtubule-associated phospho-tau
p-tau: Hyperphosphorylated tau
rmTBI: Repeated mild traumatic brain injury
SPT: Saccharin preference test
t-tau: Total tau