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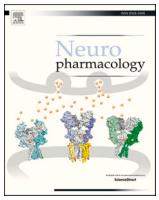
Neuropharmacology 264 (2025) 110228

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm



Sex differences in the antinociceptive effect of codeine and its peripheral but not central metabolism in adult mice

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ARTICLE INFO

Handling Editor: Dr I. Spigelman

Keywords:
Codeine
Antinociception
Pharmacokinetics
Metabolism
Sex differences

ABSTRACT

Codeine is a natural opiate extracted from opium poppy (*Papaver somniferum*) and used to alleviate mild to moderate pain. The analgesic effect of this molecule results from its metabolism into morphine which is an agonist of the mu opioid receptor. Morphine's major metabolite morphine-3-glucuronide induces both thermal and mechanical hypersensitivities while codeine-6-glucuronide has been proposed to be antinociceptive. However, sex differences in codeine antinociceptive effect and pharmacokinetics were barely studied. To this purpose, we injected male and female mice with codeine (2.5, 5, 10, 20 and 40 mg/kg) and thermal hypersensitivity was assessed 30 min after injection using the Tail Immersion Test. Moreover, both peripheral and central metabolism of codeine were evaluated respectively in the blood or pain-related brain structures in the central nervous system. The amounts of codeine and its metabolites were quantified using the isotopic dilution method by liquid chromatography coupled to a mass spectrometer. Our results show that codeine induces a greater antinociceptive effect in males than females mice independently of the estrous cycle. Moreover, major sex differences were found in the peripheral metabolism of this molecule, with higher amounts of pronociceptive morphine-3-glucuronide and less antinociceptive codeine-6-glucuronide in females than in males. Concerning the central metabolism of codeine, we did not find significant sex differences in pain-related brain structures. Collectively, these findings support a greater codeine antinociceptive effect in males than females in mice. These sex differences could be influenced by a higher peripheral metabolism of this molecule in female mice rather than central metabolism.

1. Introduction

The fight against pain is a major social issue. Among the painkillers used to treat mild to moderate pain, codeine (also known as 3-methylmorphine) is widely used despite several side effects, including nausea, constipation, and addiction (Gasche et al., 2004; Tay and Roberts, 2018). Codeine is an alkaloid extracted from poppy latex, and it is considered a Grade II analgesic drug by the World Health Organization (Anekar et al., 2024).

Codeine has no direct analgesic effect because of its weak affinity for the μ opioid receptor (MOR) (Volpe et al., 2011). Codeine metabolism follows a complex pattern, resulting in active and inactive metabolites responsible for its effects (Smith, 2009, 2011) (Fig. 1). In humans, codeine is metabolized into morphine via O-demethylation through the cytochrome p450 (CYP; phase I metabolism) enzyme, CYP2D6. Then, 40–50% of morphine is converted into pronociceptive morphine-3-glucuronide (M3G) (Hasselstrom and Sawe, 1993; Lewis et al., 2010; Lotsch, 2005; Lotsch and Geisslinger, 2001; Roeckel et al.,

Abbreviations: ACN, acetonitrile; AMY, amygdale; AUC, area under the curve; CID, collision gas; CNS, central nervous system; CYP, cytochrome p450; C6G, codeine-6-glucuronide; ED50, half-maximal effective dose; FA, formic acid; IS, internal standard; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; LSC, lumbar spinal cord; MOR, mu-opioid receptor; MPE, maximal possible effect; MRM, multiple reaction monitoring mode; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; PAG, periaqueductal grey; SPE, solid phase extraction; TIT, tail immersion test; UGT, UDP-glucuronosyl-transferase.

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https://doi.org/10.1016/j.neuropharm.2024.110228
Received 19 August 2024; Received in revised form 15 October 2024; Accepted 17 November 2024
Available online 21 November 2024

2017; Smith et al., 1990), and 10% is converted into antinociceptive M6G by UDP-glucuronosyltransferase 2B7 (UGT2B7; phase II metabolism), which is expressed in the liver, intestines, kidneys, and brain cells (Gabel et al., 2022; Hasselstrom and Sawe, 1993; Lewis et al., 2010; Lotsch, 2005; Lotsch and Geisslinger, 2001; Roeckel et al., 2017; Smith et al., 1990). Codeine undergoes the same metabolic pathways in rodents, with some minor differences. For example, CYP2D6 is absent in rodents, but codeine demethylation is performed by its ortholog, CYP2D22 (Singh et al., 2009). Because M6G is not present in rodents due to the lack of UGT2B7 expression, morphine is metabolized only into M3G through UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, and UGT2B36 (Kurita et al., 2017). In addition to M3G, codeine-6-glucuronide (C6G) is present in urine following the injection of codeine (Oguri et al., 1990). Like codeine, C6G has a weak affinity for opioid receptors (Chen et al., 1991; Mignat et al., 1995). Although few studies have reported that C6G may be responsible for the antinociceptive effect of codeine (Pelligrino et al., 1989; Srinivasan et al., 1997; Sullivan et al., 1989), morphine is thought to be the major metabolite responsible for the antinociceptive properties of codeine (Kirchheiner et al., 2007; Vree and Verwey-van Wissen, 1992). Other metabolites, including norcodeine and normorphine, are also present as trace compounds.

CYPs metabolize codeine into morphine. Then, morphine is mainly converted into pronociceptive M3G by UGT.

Morphine binds to MORs located on nociceptors and neurons of the central nervous system (CNS), particularly in areas related to pain, such as the lumbar spinal cord (LSC), periaqueductal grey (PAG), and amygdala (AMY) (Glaum et al., 1994; Jensen and Yaksh, 1986; McGaraughty and Heinricher, 2002). Once activated, MORs promote strong hyperpolarization of neurons, thereby inhibiting nociceptive signals (Fields, 2004).

Codeine antinociception, morphine antinociception, and the development of side effects are influenced by sex in humans and rodents (Cicero et al., 2002; Craft, 2003; Fullerton et al., 2018; Kest et al., 1999; Pleuvry and Maddison, 1980). In addition to female mice requiring more morphine than males to display similar antinociception, female mice also exhibit greater metabolism of morphine into pronociceptive M3G (Gabel et al., 2023), which leads to an imbalance in the pronociception/antinociception ratio (*i.e.,* M3G/morphine) at the periphery and in brain structures involved in the analgesic effect of morphine. However, the mechanisms involved in the pronociceptive effects of M3G are controversial (Eisenstein, 2019; Gabel et al., 2022).

Although evidence of sex-dependent expression of metabolic enzymes has been reported, no study has reported the peripheral and central metabolism of codeine in male and female adult mice. Therefore, the present study aimed to determine whether sex-dependent differences exist in the antinociceptive effects and the peripheral and central metabolism of codeine in adult C57BL/6J mice.

2. Material & methods

2.1. Animals

Experiments were performed with 10 weeks-old male and female C57BL/6J mice (26 ± 4 g and 20 ± 4 g, respectively; JAX:006362; Charles River, L'Arbresle, France). Mice were group housed at five per cage according to the sex with food and water ad libitum, in facilities with controlled temperature (23 °C ± 2 °C) and 20% hygrometry, under a 12-h light/dark cycle (lights on at 7 a.m.). Mice were habituated 1 week to the animal facility and 1 week to the testing environment before procedures. The Chronobiotron (UMS3415) animal facility has an agreement for animal housing and experimentation, delivered by the French veterinary services (D-67-2018-38). Protocols were performed following the European ethical guidelines (EU, 2010/63) and approved by the local ethical committee "Comité d'Ethique en Matière d'Expérimentation Animale de Strasbourg" (CREMEAS, CEEA35) and the French Ministry of Higher Education, Research and Innovation (APAFIS #37039–2022050211068109 v8).

2.2. Behavioural assessment

The tail-immersion-test (TIT) was used to assess both half-maximal effective dose (ED50) of codeine (n = 30 males and n = 30 females, n = 6 per dose of codeine) and the time-course the antinociceptive effect of 10 mg/kg of codeine (n = 6 per sex). Mice were acclimated to the animal housing conditions for 1 week before the manipulation and habituated to be restrained in a grid pocket for 1 additional week. Mice were then tested five times over ten days by measuring the baseline latencies of the tail withdrawal when 2/3 of the tail was immersed in a constant temperature water bath heated at 48 °C. A cut-off was set at 20 s to avoid any tissue damage. When baselines were steady (less than 25% of variation over three consecutive measurements), mice were weighed and received an i.p. injection of 2.5, 5, 10, 20 or 40 mg/kg of codeine (volume of 10 µl/g of animal). The tail-flick reflex latency was measured 30 min after injection of codeine for dose-response curves. Results are expressed as the maximal possible effect (%MPE) according to the following formula:

$$\%\text{MPE} = \frac{(\text{test latency}) - (\text{baseline latency})}{(\text{cut} - \text{off latency}) - (\text{baseline latency})} \times 100$$

2.3. Monitoring of estrous cycle

To determine the stage of the estrous cycle, vaginal smears were performed after the TIT by gently bringing a pipette tip filled with 20 µl of saline solution to the vaginal orifice and flushing back and forth at 4–5 times. Samples were transferred to a microscope slide and spread. Vaginal cytology was evaluated immediately after collection by analyzing the morphology and the proportions of cell types under a microscope (Olympus BX41) using a 10X objective and a photomicrograph of each vaginal smear was taken (Olympus EP50). After air drying, some vaginal smears were stained for 4 min in 0.1% toluidine blue O

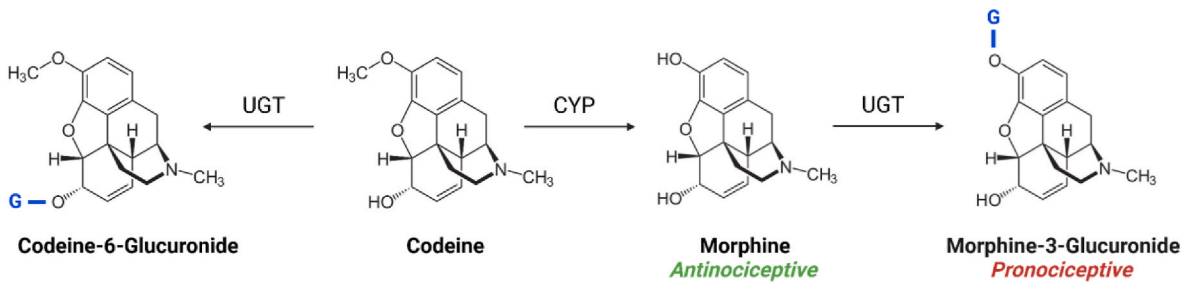

Fig. 1. Simplified codeine metabolism in mice. G: Glucuronide.

then rinsed with distilled water and air dried. The estrous cycle in mice averages 4–5 days and is divided into three stages, assessed using previously established criteria (Byers et al., 2012; Quignon, 2023).

2.4. Peripheral pharmacokinetics of codeine

Peripheral pharmacokinetics of codeine were assessed using a separate group of 12-week-old mice (n = 5 to 6 males and n = 6 to 7 females). All mice were injected with codeine (10 mg/kg) and blood was collected for 3 h to quantify codeine and its metabolites levels. Ratios between metabolites and parent molecules (C6G/Codeine or M3G/Morphine) were also determines (every 10 min for 2 h and every 20 min for the last hour). Intraperitoneal injection was chosen to mimic peripheral administration, which is the most common route of administration of codeine in clinics (per os). A small incision was performed at the end of the tail and 5 µl of blood was collected using a heparinized calibrated capillary (Minicaps End-to-End 5 µl; Hischmann, Eberstadt Germany). Samples were immediately transferred from the capillary in a 1.5 ml microtube containing 4 µl of heparin (0.5 mg/ml). Each tube was vortexed, quickly centrifuged and stored at −80 °C before further analysis.

2.5. Central metabolism of codeine

The central metabolism of codeine was assessed using a separate group of 12-week-old mice (n = 8 to 9 for males and n = 9 to 10 for females). Mice were sacrificed by cervical dislocation 30 min after the i.p. injection of 10 mg/kg of codeine. Blood, brain structures and the lumbar spinal cord (LSC) were collected to quantify codeine and its metabolites levels. M3G/Morphine ratios between M3G/Morphine were also determined. The brain was placed on an ice-cold mouse brain matrix (Bioseb, Vitrolles, France). Razor blades were used to cut the brain into 1 mm-thick slices. A puncher of 1 mm diameter (WPI, Friedberg, Germany) was used to sample the PAG and AMY. The LSC was recovered by hydraulic extrusion as previously described (Richner et al., 2017). All structures were immediately transferred in 1.5 ml microtubes and frozen at −80 °C until further analysis.

2.6. Sample preparation

Blood- Frozen blood was thawed and 10 µl of the internal standard (IS) containing 1.9 pmol of D3-codeine, 5 pmol of D3-morphine, 1.36 pmol of D3-M3G and 47.5 pmol of D3-C6G (Sigma Aldrich, Saint-Quentin-Fallavier, France) were added. Proteins were precipitated with 200 µl of ice-cold acetonitrile (ACN; Thermo Scientific, Villebon-Sur-Yvette, France). The samples were vortexed and centrifuged (20,000 g, 30 min, 4 °C). The supernatants were collected, dried under vacuum and resuspended in 800 µl of H_2O/0.1% formic acid (FA; v/v; Sigma Aldrich) before solid-phase extraction (SPE). HyperSep SPE cartridges (1 cc, 25 mg, Thermo Electron) were used with a positive pressure manifold (Thermo Electron). Briefly, cartridges were activated with 1 ml of ACN 100% followed by a step wash with 2 ml of H_2O/0.1% FA (v/v). Then, samples were loaded onto the cartridges and let to dry for 1 min under a high vacuum. Cartridges were subsequently washed with 1 ml of H_2O/0.1% FA (v/v) followed by 1 ml of 98.9% H_2O/1% ACN/0.1% FA (v/v). Elution was performed with 800 µl of 79.9% H_2O/20% ACN/0.1% FA (v/v). Eluates were centrifuged (20,000 g, 15 min, 4 °C). Supernatants were dried under vacuum and resuspended in 30 µl of H_2O/0.1% FA (v/v).

Tissues- Frozen tissues were sonicated (2 × 5 s; 100 W) in 0.1 mM of ascorbic acid (100 µl for PAG and AMY and 200 µl for LSC). After centrifugation (20,000 g, 30 min, 4 °C), the supernatants were collected and received 10 µl of IS (containing 0.3 pmol of D3-codeine, 0.8 pmol of D3-morphine, 0.2 pmol of D3-M3G and 7.92 pmol of D3-C6G). Samples were precipitated with 1 ml of ice-cold ACN and were centrifuged (20,000 g, 30min, 4 °C). The supernatants were dried under vacuum and suspended in 15 µl of H_2O 99.9%/0.1% FA (v/v) before mass spectrometry analysis.

Dansyl-chloride derivation- Blood and tissue samples were derived using dansyl-chloride derivation to enhance the morphine signal (Lamshoft et al., 2011). Samples were dried under vacuum and suspended with 40 µl of 0.2M bicarbonate ($NaHCO_3$, Sigma Aldrich) and 40 µl of dansyl-chloride (0.85 mg/ml; Sigma Aldrich). Tubes were vortexed, heated at 60 °C for 10 min and then precipitated with 400 µl of ice-cold 99.8% ACN/0.2% FA (v/v). After centrifugation (20,000 g, 30 min, 4 °C), supernatants were collected and dried under vacuum and resuspended in 15 µl of 29.8% H_2O/50% methanol/10% ammonium formate 50 mM/0.2% FA before mass spectrometry (MS) analysis.

2.7. LC-MS/MS instrumentation and analytical conditions

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analyses were performed with a Dionex Ultimate 3000 HPLC system (Thermo Electron) coupled with a triple quadrupole Endura mass spectrometer (Thermo Electron). Xcalibur v4.0 software (RRID:SCR_014593) was used to control the system (Thermo Electron). Samples were loaded onto a ZORBAX SB-C18 column (150 × 1 mm, 3.5 µm, flow of 90 µl min^{-1}; Agilent, Les Ulis, France) heated at 40 °C. Identification of the compounds was based on precursor ions, selective fragment ions and retention times obtained for the heavy counterpart present in the IS. Selection of the monitored transitions and optimization of collision energy and RF Lens parameters were determined manually. Qualification and quantification were performed using the multiple reaction monitoring mode (MRM) according to the isotopic dilution method (Liu et al., 1995). LC and MS conditions used are detailed in Table S1. For tissues and fluids, molecules were quantified using the isotopic dilution method (Liu et al., 1995). Thus, linearity of the intensity/quantity values of the target compounds (morphine, codeine, C6G and M3G) related to a fixed amount of deuterated counterparts (D3-morphine, D3-codeine, D3-C6G, and D3-M3G) were checked to determine the validity of the method. Abscence of contamination of the heavy IS with non-deuterated conterpart was tested. IS (D3-morphine, D3-codeine, D3-C6G, and D3-M3G) were added to tissue extracts. Limits of detection (LOD) for morphine, D3-morphine, codeine, D3-codeine, C6G, D3-C6G, M3G and D3-M3G were typically around 1–50 fmol, depending on the nature of the matrix (Table S2). LOD was defined as the lowest detectable amount of analyte with a signal-to-noise (S/N) ratio >3. Limit of quantification was defined as the lowest detectable amount of analyte with a signal-to-noise (S/N) ratio- > 10 (Table S2). All amounts of opiates measured in samples fit within the standard curve limits, with typical analytical ranges (the range of amounts that can be accurately quantified) from 1 fmol–100 pmol to 600 fmol–100 pmol. Recoveries (extraction efficiency) for morphine, D3-morphine, codeine, D3-codeine, C6G, D3-C6G, M3G and D3-M3G were respectively 31 ± 8%, 33 ± 9%, 92 ± 6% and 94 ± 3%. Accuracy values (defined as the measured amount of analyte vs the theoretical added amount in spiked naive samples) for morphine, D3-morphine, codeine, D3-codeine, C6G, D3-C6G, M3G and D3-M3G were respectively 98 ± 12%, 105 ± 14%, 91 ± 6% and 91 ± 6%. Precision (CV% between repeated injections of the same sample) values were <2% for same-day measurements and <5% for inter-day measurements.

2.8. Determination of peripheral pharmacokinetics

The pharmacokinetics parameters of codeine metabolism were determined by non-compartmental analysis (NCA) using the PKsolver tool described by Zhang et al. (2010). The linear up-log-down trapezoidal rule was used to determine the area under the curve (AUC) of codeine, C6G, morphine and M3G after extrapolation to infinity.

2.9. Statistical analysis

Non-linear regression with a four-parameter logistic equation was used to define the ED50 of codeine and the 95% confidence intervals (95% CI) in both male and female mice. The two fits were compared using a nested-model comparison with the extra sum-of-squares F test. Repeated measures Two-way ANOVA analysis was used for the time-course of the antinociceptive effect of codeine followed by a Sidak multiple comparison test. For the pharmacokinetics study, T-tests were performed on the areas under the curve (AUCs) of all the compounds. When the results did not show homogeneity of variances, a T-test with a Welch correction enabled us to compare the means. When the results did not show normal distribution, a Mann-Whitney *U* test enabled us to compare the means. Results are presented as mean values ± standard error of the mean (SEM). A p value < 0.05 was considered statistically significant. All analyses were performed using GraphPadPrism 8.0.2 software (RRID:SCR_002798).

3. Results

3.1. Antinociceptive effects of codeine

Pain is a personal experience modulated by several factors, such as age (Shackleton et al., 2023), ethnicity (Campbell and Edwards, 2012), and sex (Bartley and Fillingim, 2013). Therefore, the present study determined whether basal differences exist for heat thermal stimuli in male and female C56BL/6J mice. The female mice displayed significantly greater heat thermal sensitivity (1.94 ± 0.10 s) compared to the male mice (3.00 ± 0.16 s) (p value < 0.0001; Fig. 2A). The present study next investigated whether differences in the antinociceptive effects of codeine exist between male and female mice. As shown in Fig. 2B, ip injection of codeine produced a dose-dependent antinociceptive effect 30 min after injection in both male and female mice. This time interval was selected because opiate antinociception peaks approximately 30 min after the injection (Bryant et al., 2006; Cicero et al., 1996; Doyle and Murphy, 2018). Based on the codeine ED50, the female mice required significantly greater amounts of codeine to reach 50% of the MPE compared to the male mice (p value < 0.005). The time course of the antinociceptive effect of 10 mg/kg codeine was assessed, which demonstrated that codeine antinociception was stronger in males than in females at 10 and 30 min after injection (Fig. 2C). Estrous cycle determination in females revealed that 56.67% of the animals were in the diestrus stage (Fig. 2D and E).

3.2. Peripheral metabolism of codeine

Peripheral metabolism in the liver is the main regulator of the blood concentrations of codeine and its metabolites. The sex differences in the antinociceptive effects of codeine may be due to the opposite effects (*i.e.*, pronociceptive and antinociceptive) of codeine metabolites. LC–MS/MS analysis determined the codeine pharmacokinetic parameters in the blood after i.p. administration of 10 mg/kg codeine (Table 1).

Codeine- Codeine blood concentrations differed only slightly at 10 min (Fig. 3A), with more codeine in males (24.08 ± 1.99) than in females (18.12 ± 0.97), which was confirmed by NCA analysis (p value < 0.005, $\mathbf{C}_{max}$; Table 1). Furthermore, pharmacokinetic analysis revealed no significant differences between male and female mice (Table 1).

C6G- The blood concentrations of C6G were up to 40 times lower than those of codeine. The overall kinetic profiles were similar between male and female animals (Fig. 3B).

However, the AUC values indicated that the overall concentration of C6G was 2-fold greater in males than in females (p value < 0.01; Table 2).

Morphine- No significant difference was observed in the morphine

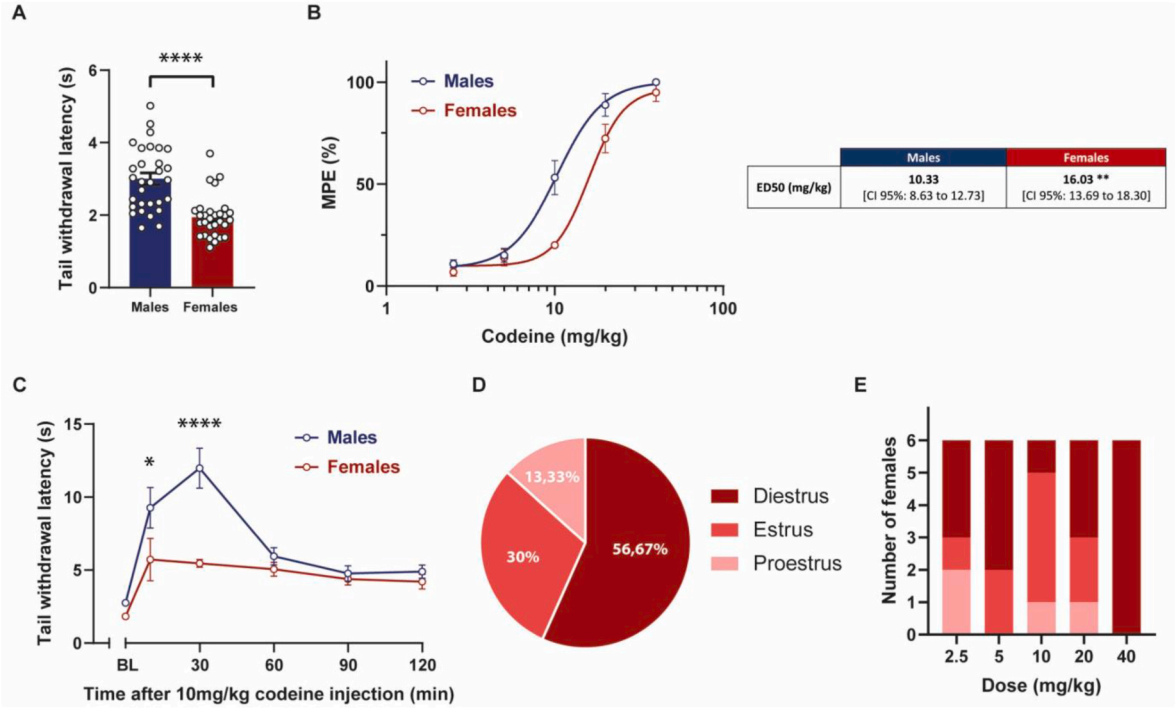

Fig. 2. Antinociceptive effect of codeine in male and female mice. **A)** Baseline latencies of male and female mice in TIT at 48 °C (n = 30 for males and females) Mann-Whitney test. **B)** Dose-response curves for codeine antinociceptive effect in male and female mice using TIT. Antinociception is expressed as % of MPE. Each group received a single dose of codeine (2.5, 5, 10, 20 or 40 mg/kg; n = 6 for all conditions). ED50 were extracted from each fitting and fits were compared with the extra sum-of-the-square F test. **C)** Time-course of the antinociceptive effect of 10 mg/kg of codeine in male and female mice (n = 6). Time-course curves were analysed using Two-way ANOVA (repeated measures mixed model) followed by a Sidak multiple comparison test. **D)** Percentage of estrous cycle stages in females mice used for the dose-response curve and time-course experiments. **E)** Estrous cycle in the each different groups of female mice used for the dose-response curve experiment. *: p-value <0,05; **: p-value <0,005, ****: p-value <0,0001. Data are expressed as Means ± SEM.

Table 1
Pharmacokinetics parameters obtained from the NCA for codeine in the blood.

Parameters	Codeine	
	Males (n = 6)	Females (n = 7)
C_{max} (nmol.mL^{-1})**	24.08 ± 1.99	18.12 ± 0.97
$AUC_{0\text{-}180}$ (nmol.min.mL^{-1})	327.9 ± 20.35	305.8 ± 29.27
$AUC_{0\text{-}inf}$ (nmol.min.mL^{-1})	349.1 ± 22.72	354.4 ± 38.95
$T_{1/2}$ (min)	44.60 ± 4.18	44.77 ± 6.64
MRT (min)	58.87 ± 1.33	71.63 ± 10.44
Cl/F ((mg/kg)/(nmol/mL)/min))	0.03 ± 0.002	0.03 ± 0.004
Vz/F ((mg/kg)/(nmol/mL))	1.85 ± 0.14	1.82 ± 0.19

C_{max}: maximal concentration, AUC: area under the curve, $T_{1/2}$: half-life, MRT:, mean residence time, Cl/f: clearance, Vz/f: volume of distribution. Cl/f and Vz/f are relative to bioavailability. Data were analysed by *t*-test or Mann-Whitney test. **: p-value <0,005.

concentration between male and female mice throughout the kinetics (Fig. 3C) or between the AUC values (p value = 0,8625; Table 2). The levels of morphine were close to the C6G concentrations. Compared with the C6G concentrations, lower concentrations of morphine were detected in the blood 3 h after codeine injection.

M3G- Higher concentrations of M3G were detected in the blood of female mice than in that of male mice (Fig. 3D). According to the AUC values, the M3G concentrations were approximately 1.5 times greater in females than in males (p value < 0.001; Table 2).

C6G/codeine ratio- The metabolic ratio of C6G/codeine throughout the kinetic period was greater in males than in females (p value < 0.05; Fig. 3E). The statistical analysis of the AUC values of the ratios confirmed significantly greater ratios in males than in females (p value < 0.05; Fig. 3F).

M3G/morphine ratio- Neither ratios observed throughout the kinetic (Fig. 3G) nor the AUCs of the ratios (Fig. 3H) significantly differ between male and female mice (p-value = 0.0758).

3.3. Central metabolism of codeine

Because of its hydrophobic nature, codeine easily crosses the blood–brain barrier (BBB) (Oldendorf et al., 1972; Xie and Hammarlund-Udenaes, 1998). Therefore, the present study evaluated whether sex-dependent metabolism of codeine occurs in the CNS by targeting different brain structures implicated in pain modulation (*i.e.*, PAG, AMY, and LSC) (Fields, 2004; Gabel et al., 2023). After quantification, we found that codeine, morphine, and M3G were present within the PAG (Fig. 4A–D), AMY (Fig. 4E–H), and LSC (Fig. 4I–L), whereas C6G was below the LOD.

In contrast to blood analyses, sex differences in M3G quantities were limited to the LSC in the CNS (p value < 0.005; Fig. 4K). However, the M3G/morphine ratios (Fig. 4L) did not differ between male and female animals.

To assess the central amount of the targeted molecules without peripheral blood contamination, a ratio between the central concentration and the blood concentration was calculated in the PAG, AMY, and LSC in the same animals (brain/blood ratios of codeine, morphine, and M3G; Fig. 5). No differences in the brain/blood ratios for each structure were observed between male and female mice.

Table 2
Blood pharmacokinetics parameters obtained from the NCA for codeine metabolites in the blood. C_{max}: maximal concentration, AUC: area under the curve, MRT: mean residence time. *Data were analysed by t-test or Mann-Whitney test. *: p-value < 0,05, ***: p-value < 0,0005, ****: p-value < 0,0001.*

Parameters	Codeine-6-Glucuronide		Morphine		Morphine-3-Glucuronide	
	Males	Females	Males	Females	Males	Females
C_{max} (nmol. mL^{-1})	0.55 ± 0.08	0.39 ± 0.02	0.34 ± 0.05	0.37 ± 0.07	12.41 ± 0.76 ****	23.91 ± 1.44
$AUC_{0\text{-}t}$ (nmol. min. mL^{-1})	16.55 ± 3.76 *	8.171 ± 0.48	29.92 ± 6.48	31.33 ± 11.06	1040 ± 123.20 ***	1645 ± 78.48
$AUC_{0\text{-}inf}$ (nmol. min. mL^{-1})	36.75 ± 4.18 *	23.72 ± 1.39	59.30 ± 14.74	41.56 ± 10.60	1332 ± 152.20 *	1761 ± 95.16
MRT (min)	6.87 ± 2.80	6.82 ± 2.78	228.6 ± 48.13	118.30 ± 19.48	130.20 ± 23.49 *	73.01 ± 6.19

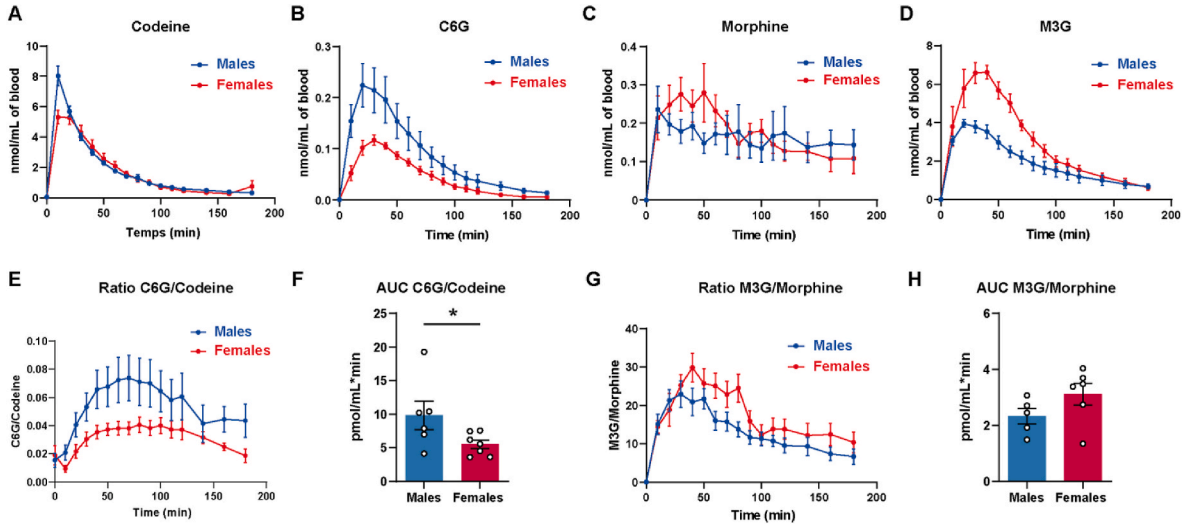

Fig. 3. Pharmacokinetics of codeine and its metabolites in male and female mice. **A-D)** Blood kinetic profile of codeine and codeine-metabolites over 180 min. **E)** Ratio C6G/codeine over 180 min and **F)** associated AUC analysis. **G)** Ratio M3G/Morphine over 180 min and **H)** associated AUC analysis. Kinetics for codeine, C6G and M3G were performed on n = 5/6 males and n = 6/7 females. Kinetics for morphine were performed on n = 5 males and n = 6 females Non-compartemental analysis. T-test or Welch *t*-test analysis for the AUC; *, p-value <0.05. Data are expressed as means ± SEM.

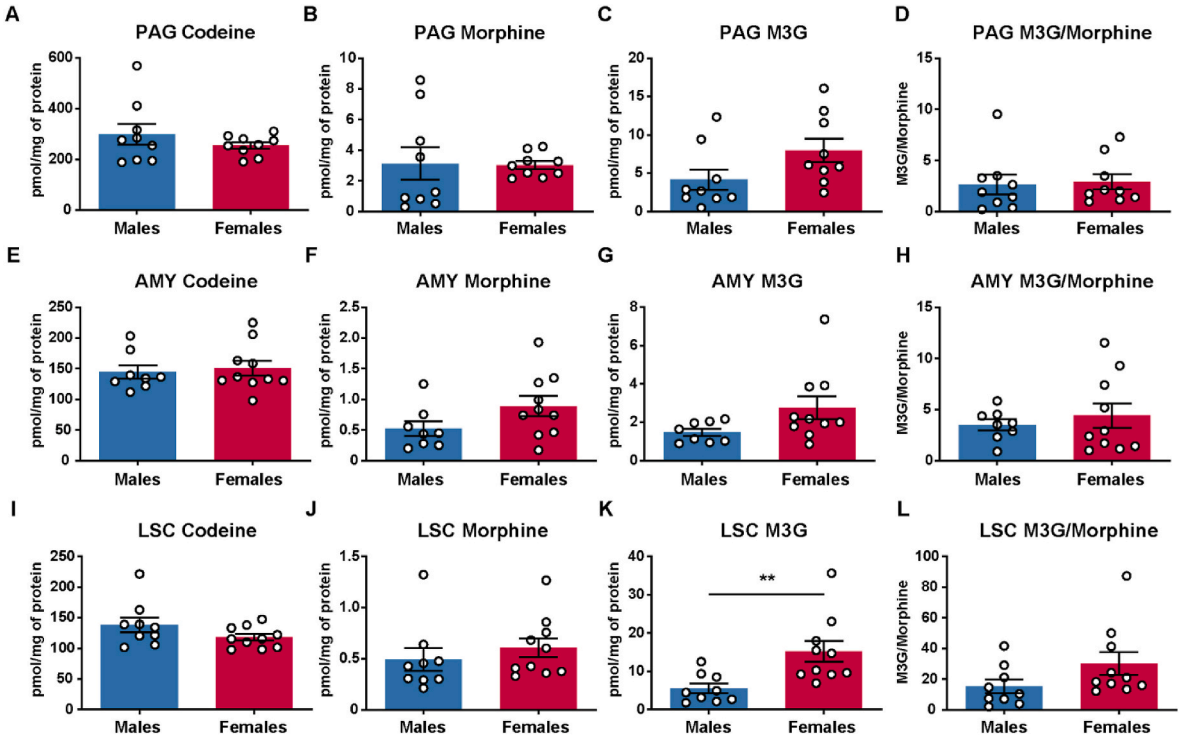

Fig. 4. Central metabolism of codeine in male and female mice. **A-D)** Quantification of codeine, morphine, M3G and M3G/Morphine ratios at in periaqueductal grey (PAG), **E-H)** in bilateral amygdala (AMY) and **I-L)** in lumbar spinal cord (LSC). n = 9 males and n = 9 females for PAG. n = 8 males and n = 10 females for AMY. n = 9 males and females for LSC. T-test or Mann-Whitney analysis. Data are expressed as means ± SEM. **: p-value <0,005.

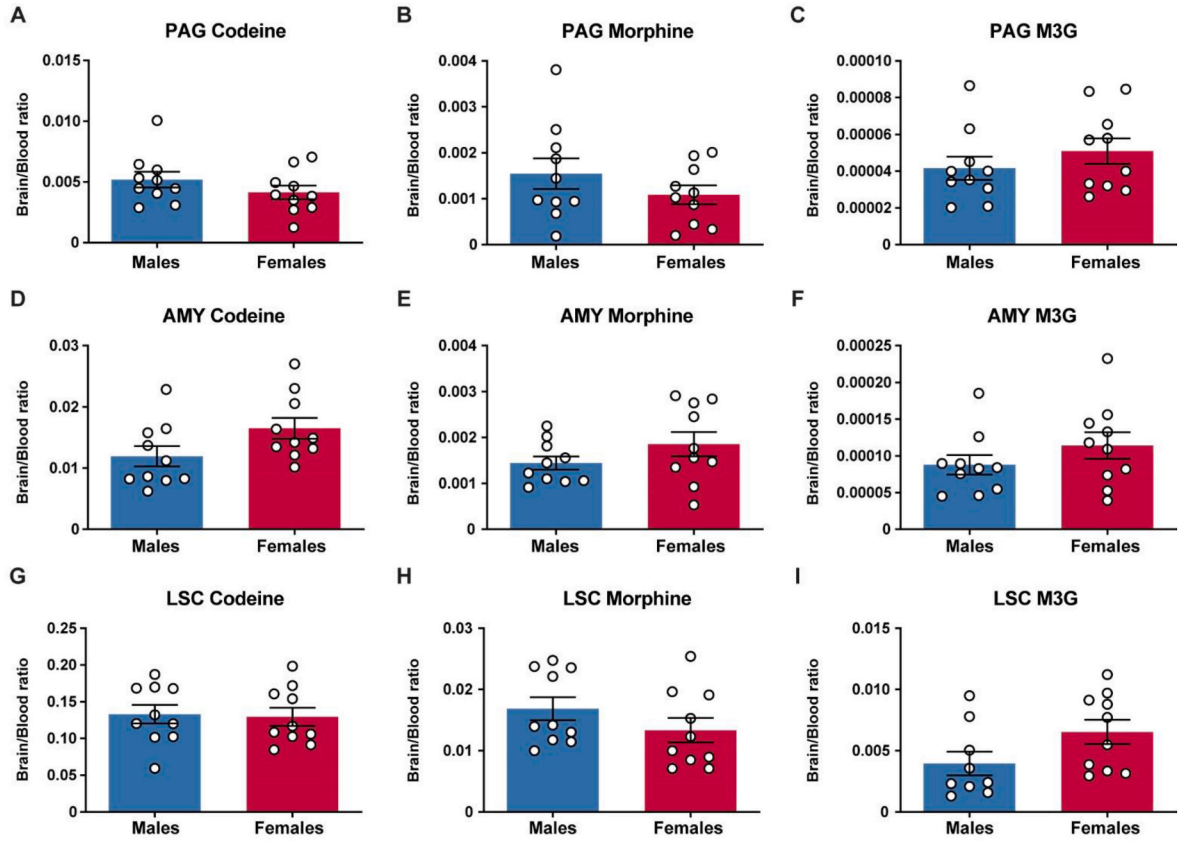

Fig. 5. Brain/Blood ratios in CNS structures in male and female mice. A-C) Brain/Blood ratio at 30 min in periaqueductal grey (PAG), **D-F)** in bilateral amygdala (AMY) and **G-I)** in lumbar spinal cord (LSC). n = 9 males and n = 9 females for PAG. n = 8 males and n = 10 females for AMY. n = 9 males and females for LSC. T-test or Mann-Whitney analysis. Data are expressed as means ± SEM.

4. Discussion

4.1. Antinociceptive effect of codeine

The present study identified greater heat thermal sensitivity in females than in males (Fig. 2A), which agreed with other studies (Hurley and Adams, 2008). Because codeine has poor affinity for MOR, it may not induce direct antinociception through the binding of this receptor following its administration. One hypothesis is that the antinociceptive effect of codeine relies on its conversion into morphine. Given that morphine antinociception is modulated by sex in rodents (Fullerton et al., 2018; Gabel et al., 2023), the present study evaluated whether codeine is also modulated by sex. With the present experimental conditions (i.p. injection, TIT, at 48 °C), ED50 values of 10.33 mg/kg and 16.03 mg/kg were calculated for males and females respectively. Moreover, there was low variability in TIT among females in the time-course experiments, even though the females used in the experiment were not in the same stage of the estrous cycle (Fig. 2E). These findings suggested that the estrous cycle has no effect or a limited effect on codeine antinociception in females. Notably, depending on the study, the ED50 of codeine is highly variable. These differences may be explained by the route of administration, mouse strains, or behavioral tests used (Lichtman, 1998; Milo et al., 2006; Miranda et al., 2013). Nevertheless, the present results were consistent with those of previous studies, showing that morphine antinociception is 30% more effective in male mice than in female mice (Gabel et al., 2023; Juni et al., 2008; Kest et al., 1999, 2000b). The sex-dependent antinociceptive effect of codeine may be explained by the differences in M3G blood concentration due to the pronociceptive effect of this metabolite (Gabel et al., 2023; Klimas and Mikus, 2014; Roeckel et al., 2017). Finally, the animals were naïve in the present study, and decreased antinociceptive effects of opiates in chronic pain have been reported. This may be explained by increased levels of metabolic enzymes, such as CYPs, which may amplify opiate metabolism.

4.2. Codeine, C6G, morphine, and M3G in the blood

The present results suggested that mice convert most of the codeine into M3G (through a first conversion into morphine) and, to a lesser extent, C6G (Fig. 3). Furthermore, the differences in the concentrations of these metabolites observed between males and females indicated sex-dependent peripheral metabolism, which may explain the antinociceptive differences. Moreover, the peak concentration of morphine reached 300 nM in the blood, which is within the affinity range of the MOR (*i.e.,* Ki of 1–20 nM) for antinociception (Mignat et al., 1995). Because no sex difference in morphine blood levels was observed, peripheral morphine may not be involved in the sex-dependent antinociceptive effect. However, because morphine was transformed into pronociceptive M3G to a greater extent in females (Fig. 3), this metabolite may decrease morphine antinociception to a greater extent in females than in males (Lewis et al., 2010; Roeckel et al., 2017).

4.3. Sex differences in UGT expression

In rodents, sex differences in UGT expression have been reported in the liver and kidney, which may explain the sex differences in M3G and C6G blood levels reported in the present study (Buckley and Klaassen, 2007). Moreover, UGT1A isoforms are generally more highly expressed in females than in males, whereas the opposite is found for UGT2B members (Buckley and Klaassen, 2007). Sex hormones may drive these differences, as they have been shown to directly induce or repress some UGT isoforms (Muraca and Fevery, 1984; Strasser et al., 1997). Finally, even though most metabolism occurs in the liver, the expression of CYP and UGT has also been reported in the brain, suggesting potential metabolism in the CNS (Berczi, 1998; Buckley and Klaassen, 2007; Sakakibara et al., 2016; Suleman et al., 1998; Wahlstrom et al., 1988).

4.4. Codeine, C6G, morphine, and M3G in the CNS

Codeine is a hydrophobic compound that easily crosses the BBB to reach the CNS (Oldendorf et al., 1972; Viscusi and Viscusi, 2020; Xie and Hammarlund-Udenaes, 1998). The present study demonstrated central metabolism of codeine in the CNS, which agreed with the previously described expression of CYP and UGT (Ouzzine et al., 2014; Suleman et al., 1998). As morphine and C6G (Srinivasan et al., 1997) may be responsible for the antinociceptive effects of codeine, the levels of these metabolites and M3G were quantified after a single administration of 10 mg/kg codeine in male and female mice (Fig. 4). There were no sex differences in the PAG and AMY, but higher M3G levels were detected in the LSC of females (Fig. 4K). Therefore, these findings suggested that the central metabolism of codeine is influenced only by sex in the LSC, even though previous studies have reported sex differences in CYP and UGT expression within the brain (Buckley and Klaassen, 2007; Renaud et al., 2011).

The CNS quantification of codeine metabolites may be due to the distribution of the metabolites from the blood into the CNS rather than central metabolism. However, major glucuronide metabolites, such as M3G and C6G, are highly hydrophilic and are less prone to cross the blood–brain barrier compared to morphine. Moreover, the present study identified significant sex differences in M3G and C6G blood concentrations but no differences in the CNS. Therefore, unless there are sex differences in the distribution of these metabolites into the CNS, counterbalancing the peripheral metabolism of codeine, it is unlikely that the LC−MS/MS quantification in the PAG, AMY, and LSC is due to distribution rather than central metabolism.

4.5. Influence of sex hormones

The significant role of sex hormones in pain perception and antinociception/analgesia has been extensively documented in rodents and humans (Packiasabapathy and Sadhasivam, 2018). Such differences can rely on drugs that can be differentially metabolized between sexes (Schwartz, 2003; Soldin and Mattison, 2009). Differences in antinociception may also result from MOR expression and regulation under the influence of sex hormones (Loyd and Murphy, 2014). From an anatomical point of view within the CNS, it has also been described that male and female pain-modulating pathways also influence the antinociceptive response (Ghazisaeidi et al., 2023). However, contradictory reports have reported that sex has no influence on the antinociceptive/analgesic effects of opiates (Kest et al., 1999, 2000a).

4.6. Transposition to humans

In humans, associations between CYP2D6 and the response to opioids have been shown to be stronger in women than in men (Lopes et al., 2020). However, apparent CYP2D6 activity is highly variable, independent of the menstrual cycle, sex hormones, or phenotype (Labbe et al., 2000). In addition, higher CYP3A4 activity has been detected in women than in men (Parkinson et al., 2004; Scandlyn et al., 2008; Tanaka, 1999; Yang et al., 2012). Conversely, higher activity of CYP2D6 has been reported in men. In addition to sex-dependent expression, a wide interindividual variability in CYP2D6 exists due to genetic polymorphisms in humans. Up to 6% of the population is deficient in CYP2D6 (Gaedigk et al., 2017) and is more sensitive to codeine-induced analgesia. Alternatively, CYP2D6 ultrarapid metabolizers (1.4–21.2% of the population) are poorly responsive to codeine (Tay and Roberts, 2018).

Unlike CYP enzymes, UGT enzymes are less prone to genetic polymorphisms, even though some have been reported in the literature (Chen et al., 2023; Miners et al., 2002). A study in humans has revealed that a polymorphism in UGT2B7 amino acid 268 has no significant effect on the metabolism of morphine into M3G (Bhasker et al., 2000). However, conflicting results have shown that the common single-nucleotide

polymorphisms of UGT1A1, UGT1A9, and UGT2B7, as well as the dimers between these UGTs, affect the generation of M3G and M6G in a region-specific manner by altering their intrinsic clearance (Yang et al., 2017).

Only a few studies have investigated sex differences in UGT expression in humans (Gallagher et al., 2010; Mukai et al., 2015). Men exhibit a 4-fold higher UGT2B17 expression level compared to women, which is accompanied by increased glucuronidation activity in men (Gallagher et al., 2010). Other human studies have also demonstrated that UGT2B15-mediated oxazepam glucuronidation is faster in men than in women (Court et al., 2002, 2004; Greenblatt et al., 1980).

5. Conclusion

Although codeine has been used for more than a hundred years, sex differences in the metabolism of codeine and effects have been poorly investigated in rodents. The present results demonstrated that codeine has a stronger antinociceptive effect in adult male C57BL6/J mice than in adult female C57BL6/J mice. A pharmacokinetic study revealed increased levels of pronociceptive M3G in the blood of females. Further analysis revealed for the first time the presence of C6G in the blood of mice, with higher concentrations found in males. In contrast to the periphery, no sex differences were present in the central metabolism of codeine in supraspinal pain-related structures, in contrast to the lumbar spinal cord. Taken together, these results suggested that the sex differences in the antinociceptive effect of codeine may be related to its peripheral rather than its central metabolism.

CRediT authorship contribution statement

Volodya Hovhannisyan: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Abdel-Karim Berkati:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Marine Simonneaux:** Writing – review & editing, Methodology, Investigation. **Florian Gabel:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Virginie Andry:** Methodology, Investigation. **Yannick Goumon:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Funding

This work was funded by INSERM, CNRS, University of Strasbourg (Unistra and CoRTecS; DIALOG 2024 Ref.: RBA/SCC/JLA/DHA/N° 2023-464m and RBA/SCC/JLA/DHA/N° 2023-464m), French Ministère Délégué à la Recherche et à l'Enseignement Supérieur (Ph.D. fellowship to V.H., A.-K.B., and M.S) and SFRI STRAT'US (ANR-20-SFRI-0012 PhD extension to V.H.). We thank the following research programs of excellence for their support: FHU Neurogenycs, French National Research Agency (ANR) through the Programme d'Investissement d'Avenir (V.H. and A.-K.B.; contract ANR-17- EURE-0022, EURIDOL graduate school of pain) and Ligue contre le Cancer (JMG/SP-n° 01M.2020). This work of the Interdisciplinary Thematic Institute NeuroStra, as part of the ITI 2021-2028 program of the University of Strasbourg, CNRS and lnserm, was supported by ldEx Unistra (ANR-10-IDEX-0002) under the framework of the French Program Investments for the Future. We also thank the FRC Neurodon - Rotary-Espoir en Tête 2022 for financial support to the SMPMS.

Declaration of competing 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2024.110228.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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