Central metabolism as a potential origin of sex differences in morphine antinociception but not in the induction of antinociceptive tolerance in mice

Abbreviated title: Morphine central metabolism is potentially driving the sex differences observed in morphine antinociception.

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ABSTRACT

Background and Purpose- In rodents, morphine antinociception is influenced by sex. However, conflicting results have been reported regarding the interaction between sex and morphine antinociceptive tolerance. Morphine is metabolised in the liver and brain into morphine-3-glucuronide (M3G). Sex differences in morphine metabolism and differential metabolic adaptations during tolerance development might contribute to behavioural discrepancies. This article investigates the differences in peripheral and central morphine metabolism after acute and chronic morphine treatment in male and female mice.

Experimental Approach- Sex differences in morphine antinociception and tolerance were assessed using the tail-immersion test. After acute and chronic morphine treatment, morphine and M3G metabolic kinetics in the blood were evaluated using LC-MS/MS. In addition, they were quantified in several central nervous system (CNS) regions. Finally, the blood-brain barrier (BBB) permeability of M3G was assessed in male and female mice.

Key Results- This study demonstrated that female mice showed weaker morphine antinociception and faster induction of tolerance than males. Additionally, female mice showed higher levels of M3G in the blood and several pain-related CNS regions than male mice, whereas lower levels of morphine were observed in these regions. M3G brain/blood ratios after injection of M3G indicated no sex differences in M3G BBB permeability, and these ratios were lower than those obtained after injection of morphine.

Conclusion- These differences are attributable mainly to morphine central metabolism, which differed between males and females in pain-related CNS regions, consistent with weaker morphine antinociceptive effects in females. However, the role of morphine metabolism in antinociceptive tolerance seemed limited.

KEYWORDS

Morphine, M3G, metabolism, sex differences, UDP-glucuronosyltransferase, antinociception, antinociceptive tolerance.

ABBREVIATIONS

ACN, acetonitrile; AUC, area under the curve; AUMC, area under the first moment curve; BBB, blood-brain barrier; CI95, 95% confidence interval; CID, collision gas; Cl/F, clearance over bioavailability; C_{max}, maximal concentration reached over the time course; CNS, central nervous system; D3-morphine, morphine bearing three ^{2}H; D3-M3G, M3G bearing three ^{2}H; ED50, half-maximal effective dose; GRK, G protein-coupled receptor kinase; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LSC, lumbar spinal cord; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; MOR, mu opioid receptor; MPE, maximal possible effect; MRM, multiple reaction monitoring mode; MRP, multidrug resistance associated protein; MRT, mean residence time; NCA, non-compartmental analysis; OB, olfactory bulb; OIH, opioid-induced hyperalgesia; PAG, periaqueductal gray; P-gp, P-glycoprotein; $T_{1/2}$, half-life; UGT, UDP-glucuronosyl-transferase; V_{dss}/F, volume of distribution at steady-state over bioavailability.

What is already known?

- Morphine antinociception and its side effects are influenced by sex
- Morphine metabolism modulates the antinociceptive effect of morphine

What this study adds

- Morphine metabolism takes place in several pain-related brain regions *in vivo*
- Central metabolism of morphine is influenced by sex but remains unchanged during antinociceptive tolerance

Clinical significance

- Sex differences in metabolism must be considered to design more effective analgesic treatments for women
- Understanding sex differences in pain circuits might help to understand opiates side-effects

INTRODUCTION

Pain management has become one of the most prevalent human health issues, with an increasing societal cost. Among painkillers, morphine remains the gold standard to relieve severe pain despite its numerous side effects, including nausea, opioid-induced hyperalgesia (OIH), antinociceptive tolerance, addiction and respiratory depression (Trescot, Datta, Lee, & Hansen, 2008). Morphine antinociception and the development of its side effects are influenced by sex in mammals (Fullerton, Doyle, & Murphy, 2018). In rodents, males show stronger antinociception than females with the same dose of morphine (Cicero, Nock, O'Connor, & Meyer, 2002; Craft, 2003; Kest, Wilson, & Mogil, 1999), whereas human studies have yielded more conflicting results (Aubrun, Salvi, Coriat, & Riou, 2005; Cepeda & Carr, 2003; Cepeda et al., 2003; Comer et al., 2010; Mogil, 2012; Sarton et al., 2000). Several mechanisms have been proposed to explain these sex differences in animal models (Fullerton et al., 2018), although human behavioural discrepancies might depend on other parameters, such as the social context, patient history and presence of comorbidities (Paller, Campbell, Edwards, & Dobs, 2009).

Morphine antinociception relies on morphine binding mainly to μ opioid receptors (MORs) located on neurons of the central nervous system (CNS), especially in regions related to pain such as the lumbar spinal cord (LSC), periaqueductal gray (PAG) and amygdala (Glaum, Miller, & Hammond, 1994; Jensen & Yaksh, 1986; McGaraughty & Heinricher, 2002). Upon activation, these G-protein coupled receptors induce a strong hyperpolarisation of MOR-expressing neurons that inhibits the transmission of nociceptive signals (Fields, 2004). Therefore, morphine levels in pain-related CNS regions, as well as MORs expression and functionality in these structures (*e.g.,* morphine binding affinity or G-protein coupling efficiency), are key factors for morphine-induced antinociception.

Morphine metabolism involves mainly glucuronidation mediated by UDP-glucuronosyltransferase (UGT) expressed in the liver, intestines, kidneys and, to a significant

extent, brain cells (Laux-Biehlmann, Mouheiche, Veriepe, & Goumon, 2013). In humans, the conjugation of a glucuronide moiety by UGT2B7 on the 3-OH or 6-OH group of morphine produces two predominant metabolites: morphine-3-glucuronide (M3G, 60-70%) and morphine-6-glucuronide (M6G, 10%) (Laux-Biehlmann et al., 2013). In addition, UGT1A1, 1A3, 1A6, 1A8, 1A9, and 1A10 also account for M3G production (Stone, Mackenzie, Galetin, Houston, & Miners, 2003). However, in mice, UGT2B7 is absent; therefore, no M6G is produced, while most M3G production is maintained through the action of UGT2B36 (Kurita et al., 2017). M6G has been proposed to be an agonist at MORs, resulting in antinociception (Lotsch & Geisslinger, 2001). Conversely, M3G has been described to antagonise morphine effects. Indeed, studies have reported strong mechanical and thermal hyperalgesia following intraperitoneal, intrathecal, or intracerebroventricular injections of M3G that could block morphine antinociception in rodents (Lewis et al., 2010; M. T. Smith, Watt, & Cramond, 1990). Subsequently, many studies have suggested a role of M3G in the development of morphine-induced OIH and antinociceptive tolerance (Blomqvist et al., 2020; Due et al., 2012; G. D. Smith & Smith, 1995).

Morphine antinociceptive tolerance corresponds to the loss of morphine efficacy over repeated administration and the need for higher doses to achieve sufficient antinociception (Weinsanto et al., 2018). Several pharmacodynamic mechanisms have been previously described to explain this phenomenon and include a loss of functional receptors, impaired MOR desensitisation, resensitisation or recycling and persistent PKC activity (Williams et al., 2013). Interestingly, M3G has been shown to elicit pain probably through binding to the Toll-like receptor 4 (TLR4)/myeloid differentiation protein 2 complex (Due et al., 2012; Lewis et al., 2010), while implications of TLR4 activation in tolerance and OIH have been described (Bai et al., 2014; Eidson, Inoue, Young, Tansey, & Murphy, 2017). However, conflicting results argue in opposite directions (Mattioli et al., 2014; Roeckel et al., 2017).

Taken together, numerous pieces of evidence suggest that morphine and M3G have opposing effects. Interestingly, in mice, sex differences in UGT expression have been reported in the liver and the brain (Buckley & Klaassen, 2007). For instance, UGT1A8 and UGT2B36 expression has been shown to be higher in the female brain. Therefore, the metabolic balance between morphine and M3G in the periphery and the CNS might be influenced by sex in acute and chronic conditions and could participate to the sex differences in morphine antinociception and tolerance. The present article investigates the differences in such metabolic balance in the periphery and the CNS of male and female mice following acute and chronic administration of morphine.

MATERIALS AND METHODS

Animals

Experiments were performed with ten 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 5 per cage according to the sex with a 12 h light-dark cycle, at a temperature of 23°C±2°C and provided with food and water *ad libitum*. All procedures were performed following European directives (2010/63/EU) and were approved by the regional ethics committee and the French Ministry of Agriculture (license No. APAFIS# 23671-2020010713353847v5 and APAFIS#16719-2018091211572566v8 to Y.G.). The ARRIVE Guidelines has been followed for reporting experiments involving animals (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010).

Experimental design

Considering the importance of sex as a biological variable, especially in pain studies (Mogil, 2020), all experiments were conducted according to a 2x2 factorial design to examine the effect of both sex and treatment, as well as their potential interaction. The number of mice used for each experiment was chosen to ensure sufficient statistical power (power of 80% and alpha of 0.05)

(Charan & Kantharia, 2013). In addition, calculations were also based on a pilot study and differences already reported in the literature. All experiments were carried out in a randomised manner for each sex. The experimenter was blinded to the nature of the solutions (saline *vs.* morphine) used for the injections during the behavioural experiments. Due to the technical loss of several blood and lumbar spinal cord samples from male mice, the experiment in which the CNS regions were collected was duplicated only with males, and all structures were extracted. The number of samples for each structure was therefore increased in the male groups (please see the sample numbers indicated in each figure).

Behavioural assessment of morphine antinociceptive effect

The antinociceptive effect of morphine was measured with the tail immersion test. Mice were first habituated to their environmental conditions for a week without any experimental procedures. Then, they were gently handled and habituated to be restrained in a grid pocket for two days. Mice were tested every day by measuring the latency of the tail withdrawal when 2/3 of the tail was immersed in a constant-temperature water bath heated at 47°C. In the absence of response, the cut-off was set at 25 s to avoid tissue damage. The basal thermal nociceptive threshold was determined during two weeks of baseline and considered as steady following three consecutive days of stable measurement prior to the testing phase. Results are expressed as % maximal possible effect according to the following formula:

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

Morphine half-maximal effective dose determination

Morphine half-maximal effective dose (ED50) was determined in males and females using 5 doses of morphine (1 mg/kg, 2.5 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg). Mice were sorted by

sex and randomly assigned to compose groups of 6 mice. On the day of the test, mice were weighed, tested for baseline and injected intraperitoneally (i.p.) with the right dose of morphine (w/v, Francopia, Paris, France; volume of 5 µl/g of mouse) dissolved in NaCl 0.9%. After 30 min, mice were retested to measure morphine-induced antinociceptive effect. This time interval was selected as morphine antinociception is described to peak approximatively 30 min after the injection (Bryant, Eitan, Sinchak, Fanselow, & Evans, 2006; Cicero, Nock, & Meyer, 1996, 1997; Doyle & Murphy, 2018).

Induction of morphine antinociceptive tolerance

To evoke morphine antinociceptive tolerance, mice were weighed and injected i.p. with either 10 mg/kg of morphine (2 mg/ml; volume of 5 µl/g of mouse; w/v, Francopia, Paris, France) dissolved in NaCl 0.9% or with an equal volume of saline solution every morning (light phase at 10 AM) for nine consecutive days. Mice were tested before and 30 min after each injection. On day 10, all mice received an injection of 10 mg/kg of morphine before the final procedure.

Blood collection

On day 10, the tail of the mice were anaesthetised locally with a topical application of lidocaine/prilocaine 5% (Zentiva, Paris, France). After 5 min, a small incision was performed at the end of the tail, and 5 µl of blood were collected using a heparinised calibrated capillary (Minicaps End-to-End 5 µl; Hischmann, Eberstadt Germany). Then, all mice were injected with morphine, and 5 µl of blood were collected every 10 min for 2 hours and every 20 min for the last hour. After each collection, the blood was transferred from the capillary into a micro-tube containing 4 µl of heparin and frozen at -20°C for later analysis. Twelve mice per group were used for the morphine kinetic experiment.

Brain regions and lumbar spinal cord sampling

In a separate group of mice, on day 10, mice were euthanised by cervical dislocation 30 min following the injection of morphine, and the blood and the brain were collected. The blood was transferred to a micro-tube containing 4 µl of heparin and frozen at -20°C for later analysis. Then, the brain was placed on an ice-cold mouse brain matrix. Razor blades were used to cut the brain into 1 mm thick slices. Punchers of 1 mm and 0.5 mm diameters were used to sample the PAG and amygdala, respectively. Olfactory bulbs (OB) were extracted using forceps. For the LSC, hydraulic extrusion was performed as previously described (Richner, Jager, Siupka, & Vaegter, 2017). Structures were directly transferred in micro-tubes and stored at -80°C. OB were collected because high levels of UGTs involved in morphine metabolism are present in the olfactory area (Heydel et al., 2010; Ouzzine, Gulberti, Ramalanjaona, Magdalou, & Fournel-Gigleux, 2014). For each group, 20 males and 13 females were used (see ***Experimental Design*** section).

M3G blood-brain barrier permeability

To investigate whether differences in M3G blood-brain barrier (BBB) permeability exist between males and females, 15 male and 15 female mice were weighed, divided into three groups and injected i.p. with either 10 mg/kg, 20 mg/kg or 40 mg/kg of M3G (w/v, Sigma Aldrich, St. Quentin Fallavier; volume of 5 µl/g of mouse). Mice were euthanised by cervical dislocation 30 min following the injection of M3G, and the blood, the brain regions of interest and the LSC were collected according to the protocol described above.

Sample preparation

Blood **–** On the day of the analysis, blood was thawed, and 10 µl of internal standard (IS; containing 12 pmol of D3-morphine and 10.5 pmol of D3-M3G; Sigma Aldrich) and 100 µl of ice-cold acetonitrile (ACN; Thermo Scientific, San Jose, USA) were added. The samples were

vortexed and centrifuged at 20,000 *g* for 15 min at 4°C. The supernatants were collected, dried under vacuum and suspended in 800 µl of H_2O/0.1% formic acid (v/v; Sigma Aldrich) prior to solid-phase extraction (SPE). HyperSep PGC SPE-cartridges (1cc, 25 mg, Thermo Electron, Villebon-Sur-Yvette, France) were used with a positive pressure manifold (Thermo Electron). Briefly, cartridges were activated with 1 ml of ACN followed by a two-step wash with 2 ml of H_2O/0.1% formic acid (v/v). Then, samples were loaded onto the cartridges and cartridges were dried for a minute under high vacuum. They were subsequently washed with 1 ml of H_2O/0.1% formic acid (v/v) followed by 1 ml of 97.9% H_2O/2% ACN/0.1% formic acid (v/v). Elution was performed with 800 µl of 79.9% H_2O/20% ACN/0.1% formic acid (v/v), and eluates were centrifuged (20,000 *g*, 4°C for 5 min). Supernatants were dried under vacuum and resuspended in 50 µl of H_2O/0.1% formic acid (v/v) prior to LC-MS/MS analysis.

Brain regions and lumbar spinal cord – Samples were sonicated (2 x 5 s, 100 W) in 200 µl of H_2O containing 10 µl of IS (40 pmol of D3-morphine and 60 pmol of D3-M3G). After centrifugation (10 min at 10,000 *g* and 4°C), 10 µl of the supernatants were precipitated with 100 µl of ice-cold ACN for 30 min. Supernatants were dried under vacuum after another centrifugation (15 min at 20,000 *g* and 4°C) and resuspended in 20 µl of H_2O/0.1% formic acid (v/v) prior to LC-MS/MS analysis.

LC-MS/MS instrumentation and analytical conditions

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 x 1 mm, 3.5 µm, flow of 90 µl/min; Agilent, Les Ulis, France) heated at 40°C. LC and MS conditions used are detailed in **Table S1**.

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 optimisation of collision energy and RF Lens parameters were determined manually (for details, see **Table S1**). Qualification and quantification were performed using the multiple reaction monitoring mode (MRM) according to the isotopic dilution method (Ho, Liu, Nichols, & Kumar, 1990).

Data and Statistical Analysis

Design and analysis were done in accordance with the recommendations and requirements on experimental design and analysis in pharmacology published by Curtis *et al.,* in 2018 (Curtis et al., 2018).

Statistical analysis was performed using GraphPad Prism 6 Software (RRID: SCR_002798) only for experiments where group size was at least n=5 and composed of independent values. To compare the groups, two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. Normality and variance homogeneity were checked with the D'Agostino & Pearson omnibus normality test and the Levene's test, respectively. If assumptions were violated, a non-parametric approach, the Aligned-Rank Transform (ART) ANOVA, was used (Wobbrock, Findlater, Gergle, & Higgins, 2011). Outliers were included in data analysis and presentation unless a very low amount of morphine in the blood was observed during the whole kinetic suggesting bad injection and when an abnormally high amount of morphine and M3G were found in the CNS regions indicating contamination during sample preparation. Results are presented as mean values ± standard error of the mean (SEM). A p-value <0.05 was considered statistically significant.

Behavioural experiments

Non-linear regression with a 4-parameters logistic equation was used to define the ED_{50} of morphine and the 95% confidence intervals (CI95%) in both males and females. The two fits were compared using a nested-model comparison with the extra sum-of-squares F-test. The same analysis was applied to the data of each mouse of the tolerance experiment to extract the following parameters: the average day at which half of the MPE is left and the Hill slope coefficient. Then, the mean of each parameter was compared using an unpaired t-test with Welch's correction.

Non-compartmental analysis

Pharmacokinetic parameters for morphine and M3G were determined through a non-compartmental analysis (NCA) performed with PKsolver described by Zhang *et al.* in 2010 (Zhang, Huo, Zhou, & Xie, 2010). The λ_z acceptance criteria were set as followed: R adjusted > 0.80, includes ≥ 3 time points, $AUC_{tlast\text{-}inf} \leq 20\%\ AUC_{0\text{-}inf}$. The linear up log down trapezoidal rule was used to determine the AUC of morphine and M3G after extrapolation to infinity.

Brain/Blood ratios

For the comparison between the M3G brain/blood ratio obtained following injection of morphine and M3G, linear regressions were applied and analysed through a nested-model comparison with the extra sum-of-squares F-test.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

RESULTS

Morphine antinociceptive effect and tolerance in males and females

Morphine antinociception and side effects such as antinociceptive tolerance are influenced by sex (Fullerton et al., 2018). To investigate such sex differences, the tail-immersion test was used to assess the antinociceptive effect of morphine and the development of morphine antinociceptive tolerance in male and female C57BL/6J mice. Statistical details are presented in **Table S2**.

As shown in **Figure 1a**, systemic injection of morphine produced dose-dependent antinociceptive effects in both male and female mice. However, as witnessed by the morphine ED_{50}, females required significantly higher amounts of morphine to reach 50% of the MPE than males, suggesting sex differences in the antinociceptive effect of morphine (**Figure 1a**). Interestingly, there were no sex differences in the baseline nociceptive thresholds of the mice.

The protocol for tolerance is provided in **Figure 1b**. Morphine MPE decreased with subsequent injections in both male and female mice (**Figure 1c**). However, females became tolerant to morphine antinociceptive effects significantly earlier than males, as witnessed by the day at which only half of the MPE remained (**Figure 1c**). Interestingly, no significant difference was observed in the Hill slope coefficient between males and females, suggesting that the rate of the tolerance development process was identical in males and females (**Figure 1c**). Nevertheless, it should be noted that males reached the upper cut-off of the tail-immersion test on the first three days of the treatment, and therefore, sex differences in the Hill slope coefficient might have been masked.

Taken together, these results show sex differences in morphine antinociception and in the induction of antinociceptive tolerance.

Peripheral morphine metabolism

Peripheral morphine metabolism contributes to the modulation of morphine and M3G concentrations in the blood. As morphine and M3G are believed to mediate opposite effects,

variations in morphine metabolic balance might contribute to differences in morphine antinociception. Therefore, we next investigated whether peripheral morphine metabolism differed between males and females following acute and chronic administration of morphine (see protocol described in **Figure 2a**). Statistical details are presented in **Table S3**.

To assess sex differences in morphine metabolism, morphine and M3G kinetics and their metabolic ratios over time are depicted in **Figure 2b, c** and **d**, respectively, for control mice. Data obtained for tolerant mice are presented in **Figure 2e, f** and **g**, respectively**.** The associated results obtained from the NCA are represented in **Table 1** and as histograms in **Figure S1**.

The statistical analysis revealed an interaction between sex and chronic morphine treatment on the morphine area under the curve (AUC; **Figure 2h**). Indeed, female control mice showed a higher morphine AUC than female tolerant mice, although this effect was absent in males (see **Table 1**, post hoc analysis). No interaction was noticed on the M3G AUC, but it was significantly lower in male mice than in female mice (**Figure 2i**). No effect of the treatment was observed. Consequently, (***i***) females showed significantly higher metabolic M3G/morphine AUC ratios than males, and (***ii***) tolerant mice also showed a significantly higher metabolic ratio than control mice (**Figure 2j**).

Furthermore, an interaction between sex and chronic treatment was also observed for the morphine maximal concentration reached over the time course, the morphine area under the first moment curve (AUMC) and morphine clearance (**Table 1**). In addition, a trend was observed in the M3G maximal concentration reached over the time course, but it did not reach statistical significance. These interactions were mainly driven by the differences between the control and tolerant female mice, which were not present in male mice (see **Table 1**, post hoc analysis). Nevertheless, there was no interaction with the M3G parameters or the metabolic ratios (**Figure 2j**). This result suggests that peripheral morphine metabolism did not seem to be differentially involved during the induction of antinociceptive tolerance in male and female mice. These interactions are more likely related to changes in morphine absorption and/or clearance.

Interestingly, males displayed significantly higher morphine mean residence time (MRT) and morphine half-life than females, whereas a higher maximal concentration of M3G reached over the time course was observed in females, suggesting slower morphine metabolism in males (**Table 1**). There was no impact of sex on any of the other parameters.

Finally, significantly higher morphine clearance and volume of distribution were observed in tolerant mice compared to control mice, suggesting that chronic morphine treatment might induce adaptations in metabolism and/or transporter expression (**Table 1**). There was no effect of treatment on any M3G parameters.

Taken together, our results indicated that (***i***) female mice displayed much higher peripheral morphine metabolism and had significantly higher levels of M3G than males, and (***ii***) the peripheral metabolism of morphine was increased during the development of antinociceptive tolerance to morphine in mice.

Quantification of morphine and M3G in CNS regions

Morphine antinociception relies mainly on morphine effects in pain-related CNS regions. Additionally, M3G may act in these regions to oppose morphine effects. Consequently, their balance in pain-related CNS regions might modulate morphine antinociception. Thus, we investigated whether sex and chronic morphine treatment, leading to antinociceptive tolerance, influences morphine and M3G levels and their metabolic ratio in the amygdala, PAG and LSC. The OB was also investigated because high levels of UGTs involved in morphine metabolism have been reported in the olfactory area (Heydel et al., 2010; Ouzzine et al., 2014).

Morphine and M3G levels were quantified by LC-MS/MS in the amygdala, PAG, LSC and OB of male and female mice following acute and chronic morphine treatment (see protocol **Figure**

3a). The values obtained with morphine and M3G are reported in **Table 2** and illustrated as histograms in **Figure S2**. Statistical details are presented in **Table S4**.

Amygdala – The analysis revealed that significantly lower levels of morphine and higher levels of M3G were present in the amygdala of female mice compared to male mice (**Table 2**). Therefore, the metabolic ratio between M3G and morphine was significantly lower in male mice than in female mice in this structure (**Figure 3b**). In addition, tolerant mice showed lower levels of morphine and M3G in the amygdala than control mice (**Table 2**). No differences were observed in the metabolic ratio between control and tolerant mice (**Figure 3b**).

PAG – Significantly lower levels of morphine were observed in the PAG of female mice than in the PAG of male mice, whereas no sex differences were observed in M3G levels (**Table 2**). Interestingly, the metabolic ratio between M3G and morphine was significantly lower in male mice than in female mice in the PAG (**Figure 3c**). In addition, no significant difference was observed in the morphine and M3G levels (**Table 2**) or in the M3G/morphine metabolic ratio (**Figure 3c**) between control and tolerant mice.

LSC – Morphine and M3G concentrations were higher in the female LSC than in the male LSC (**Table 2**). However, no sex difference was observed in the metabolic ratio in the LSC (**Figure 3d**). In addition, no significant difference was found in the LSC between control and tolerant mice.

OB – Significantly lower levels of morphine and M3G were present in the OB of female mice compared to male mice (**Table 2**). The metabolic ratios were significantly lower in males than in females (**Figure 3e**). In addition, tolerant mice showed significantly lower levels of morphine than control mice in the OB. Moreover, a trend was observed for M3G in the same direction (**Table 2**). However, there was no effect of the treatment on the M3G/morphine metabolic ratio, although a trend was reported (**Figure 3e**).

Taken together, these results indicate sex differences in morphine and M3G levels, as well as in their metabolic ratio, in pain-related brain regions 30 min after the injection of morphine. However, the induction of morphine tolerance did not modify the metabolic ratio. These results suggest a limited effect of tolerance on the balance between morphine and M3G in the analysed regions. Furthermore, sex was not implicated in the differences between control and tolerant mice, as witnessed by the absence of any interactions between the two factors.

Morphine and M3G brain/blood ratios

To investigate the origin of the differences in morphine and M3G levels and metabolic ratios in the different groups of mice, we determined whether these differences (***i***) are the consequence of the differences observed in peripheral metabolism, (***ii***) rely on differences in M3G BBB permeability, and/or (***iii***) are dependent on the central metabolism of morphine.

First, we established brain/blood ratios to investigate whether the differences in morphine and M3G concentrations found in the CNS regions were based on those found in the blood. The left and middle panels of **Figure 4** illustrate the brain/blood ratios calculated for morphine and M3G following an i.p. injection of morphine. Statistical details are presented in **Table S5**.

Amygdala – The analysis revealed no significant difference in morphine brain/blood ratios (**Figure 4a**). However, an interaction between sex and chronic morphine treatment was observed on the M3G brain/blood ratios in the amygdala (**Figure 4b**). Post hoc analysis indicated significantly higher M3G brain/blood ratios in female control mice than in male control mice, while significantly higher M3G brain/blood ratios were found between control and tolerant males. This last difference was observed only in males, resulting in the interaction.

PAG – Moreover, significantly higher morphine (**Figure 4d**) and M3G (**Figure 4e**) brain/blood ratios were observed in the PAG of males compared to females. Unexpectedly, tolerant mice also tended to show higher morphine brain/blood ratios than control mice. However, this effect did not

reach statistical significance (**Figure 4d**). In addition, no effect of chronic morphine treatment was observed on the M3G brain/blood ratios in the PAG (**Figure 4e**).

LSC – Females showed higher morphine brain/blood ratios in the LSC than males (**Figure 4g**). Conversely, no difference was noticed in the M3G brain/blood ratios (**Figure 4h**). In addition, no effect of chronic treatment on the brain/blood ratios was observed in the LSC.

OB – No sex difference was noticed in the morphine brain/blood ratios in the OB (**Figure 4j**), although males showed significantly higher M3G brain/blood ratios than females (**Figure 4k**). Furthermore, chronic treatment did not influence the morphine or M3G brain/blood ratios in the OB.

Overall, the M3G brain/blood ratios were 10-fold lower than those for morphine, suggesting that M3G is less BBB permeant than morphine, as already reported in the literature (Bickel, Schumacher, Kang, & Voigt, 1996; Xie, Hammarlund-Udenaes, de Boer, & de Lange, 1999).

Taken together, these results suggested that the sex differences in morphine and M3G levels observed in the CNS regions between males and females do not necessarily reflect the differences found in the blood. Morphine and/or M3G BBB permeability or central metabolism of morphine could be partially responsible for such differences. However, it appeared that chronic morphine treatment had a rather limited influence on the brain/blood ratios, suggesting that the differences in morphine and M3G levels observed in the CNS regions between control and tolerant mice might reflect those observed in the blood.

M3G BBB permeability in males and females

As the main differences observed in the M3G brain/blood ratios differed by sex, we evaluated to what extent the BBB permeability for M3G differed between males and females. Different doses of M3G were injected into naïve male and female mice. Then, the levels of M3G were quantified

in the blood and brain regions of interest. The M3G brain/blood ratios in each CNS structure as a function of the M3G blood concentrations after i.p. injections of increasing concentrations of M3G are illustrated in **Figure 4c, 4f, 4i and 4l**. Statistical details are presented in **Table S6**.

As shown in **Figure 4**, there were no significant differences in M3G BBB permeability in any structure analysed when the M3G brain/blood ratios were plotted as a function of the M3G blood concentration in female and male mice (red and blue lines, respectively; **Figure 4c**, **4f**, **4i**, **4l**). In addition, the BBB permeability of M3G seemed to be relatively linear with increasing doses of M3G (**Figure S3**).

Central metabolism of morphine

We next hypothesized that the differences observed in M3G brain/blood ratios in the CNS regions relied on the central metabolism of morphine, which could differ between male and female mice. Hence, we evaluated whether the M3G brain/blood ratios found in each CNS region after injection of morphine (**Figure 4b** for amygdala**, 4e** for PAG**, 4h** for LSC**, 4k** for BO) were different from those obtained after injection of M3G (**Figure 4c** for amygdala**, 4f** for PAG**, 4i** for LSC**, 4 l** for BO). As each mouse showed different concentrations of M3G in the blood, we performed linear regression analysis for the M3G brain/blood ratio as a function of the M3G blood concentrations observed following the injection of morphine. Then, for each CNS region, we compared the obtained regression fit with its associated M3G BBB permeability analysis to evaluate whether significant central metabolism exists and whether it is different between male and female mice. Statistical details are presented in **Table S6**.

As shown in **Figure 4**, based on the M3G concentrations found in the blood of each mouse, the M3G brain/blood ratios obtained in the amygdala after the injection of morphine were significantly higher than those obtained after the injection of M3G in female mice (**Figure 4b and 4c**) but not in male mice. In addition, it appeared that female mice displayed significantly higher M3G

brain/blood ratios than male mice in this region. These results indicated that morphine was metabolised into M3G directly in the CNS and that such metabolism differed between male and female mice. In contrast, male mice showed significantly more robust central morphine metabolism in the OB than females (**Figure 4k and 4l**). However, the M3G brain/blood ratios reported in the PAG were unexpectedly low, and there were no differences in the LSC.

Taken together, these results suggested that morphine is metabolised within the CNS *in vivo* in key areas related to pain. In addition, sex differences were observed in the central metabolism of morphine, which may contribute to the behavioural differences observed in the antinociceptive effects of morphine between male and female mice.

DISCUSSION

Sex differences in morphine antinociceptive effects

The sex differences in morphine antinociceptive effects observed in our experiment were consistent with the ED50 values reported in the literature (Cicero et al., 1997; Craft, Stratmann, Bartok, Walpole, & King, 1999; Doyle & Murphy, 2018). Most of the studies using rodents have shown that morphine elicits weaker antinociception in females, but the origin of this sex disparity remains controversial.

Indeed, sex differences have been observed in key pharmacodynamic processes involved in morphine antinociception. For instance, higher expression of MORs in the PAG and the locus coeruleus has been observed in male mice than in female mice (Guajardo, Snyder, Ho, & Valentino, 2017; Loyd, Morgan, & Murphy, 2008). This differential expression in the PAG seems to be essential to elicit sex differences in morphine antinociception (Loyd, Wang, & Murphy, 2008). Sex differences in MOR splicing and trafficking have also been observed, although these mechanisms require further investigation in order to understand the extent to which they participate

to sex differences in morphine antinociception (Enman, Reyes, Shi, Valentino, & Van Bockstaele, 2019; Liu et al., 2018). On the other hand, no or only minor sex differences have been observed in morphine binding affinity and MOR-mediated G protein activation (Kepler et al., 1991; Peckham, Barkley, Divin, Cicero, & Traynor, 2005; Selley et al., 2003). Concomitantly, other mechanisms have also been proposed, including organisational and activational differences (Cicero et al., 2002), functional differences in the recruited pain circuit (Loyd & Murphy, 2014), dimorphism in glial cell activation (Doyle, Eidson, Sinkiewicz, & Murphy, 2017), and a potential role for drug metabolism (Soldin & Mattison, 2009).

Influence of sex on peripheral metabolism of morphine

The morphine and M3G concentrations found in the blood of control mice were consistent with the higher potency of morphine observed in male C57BL/6J mice (Kest et al., 1999). Female mice showed higher morphine metabolism, as witnessed by the 2-fold higher M3G/morphine ratio observed at several time points. These results were consistent with the differences observed after intravenous (i.v.) injection (South, Edwards, & Smith, 2009). Interestingly, no difference in morphine glucuronidation by hepatic microsomes was observed between male and female rats (Rush, Newton, & Hook, 1983). In addition, sex differences have previously been shown in the distribution of glucuronide metabolites (Bond, Medinsky, Dent, & Rickert, 1981). Taken together, it is possible that the distribution in the body and excretion of morphine and M3G differ between males and females, leading to higher concentrations of M3G in the blood of female mice. Although sex differences in mouse peripheral metabolism of morphine were observed in our experiment, Sarton *et al.* in 2000 did not observe any sex differences in morphine, M3G and M6G levels in the plasma of healthy volunteers (Sarton et al., 2000). These species differences are likely due to differences in UGT and/or transporter expression. Nevertheless, experiments performed mainly in rats have shown that differences in the M3G/morphine plasma ratio might play a role in the sex

differences observed in morphine antinociception (Baker & Ratka, 2002; Craft, 2003; G. D. Smith & Smith, 1995).

Sex differences in central metabolism of morphine in pain-related CNS regions

Sex differences in morphine antinociception might rely on the central metabolism of morphine. Indeed, *in vitro* studies have shown the capability of brain homogenates and glial cells to metabolise morphine into M3G in both rodents and humans (Wahlstrom, Winblad, Bixo, & Rane, 1988; Yamada et al., 2003). In addition, UGTs involved in morphine metabolism are expressed in the brain of humans and rodents (Buckley & Klaassen, 2007; Kutsuno et al., 2015; Shelby, Cherrington, Vansell, & Klaassen, 2003). Several reports in humans have indirectly suggested the existence of morphine central metabolism (Goudas et al., 1999; Sandouk, Serrie, Scherrmann, Langlade, & Bourre, 1991; M. T. Smith, Wright, Williams, Stuart, & Cramond, 1999). Even though M3G displays low BBB permeability (Bickel et al., 1996), we showed here that higher levels of M3G were present in several CNS regions following peripheral injection of morphine compared with after peripheral injection of M3G, consistent with the data reported for the brains of guinea pigs (Murphey & Olsen, 1994).

Consequently, our results suggest that morphine metabolism occurs in some areas of the CNS *in vivo*. Interestingly, the M3G brain/blood ratios were higher in females than in males, at least in the amygdala, although no sex difference in M3G BBB permeability was observed. Furthermore, even though the M3G brain/blood ratios observed in the PAG after the injection of morphine were unexpectedly lower than those reported following the injection of M3G, it should be noted that the M3G half-life reported after the injection of M3G is approximately 30 min (Handal, Grung, Skurtveit, Ripel, & Morland, 2002). In contrast, we reported a MRT for M3G between 45 min and 55 min following morphine injection. Therefore, the total amount of M3G present in the blood before quantification in the CNS regions is likely much higher after administration of M3G than

after administration of morphine. Morphine glucuronides brain kinetics are delayed compared to those observed in the blood (M. Barjavel, Sandouk, Plotkine, & Scherrmann, 1994; Stain et al., 1995; Xie, Bouw, & Hammarlund-Udenaes, 2000) and as we showed that the BBB permeability of M3G increases proportionally with its blood concentration, the central metabolism of morphine is probably underestimated in our experiment.

In agreement with these statements, the lower morphine levels found in the PAG and amygdala of female mice are consistent with their lower response to morphine in light of the role of these structures in morphine-induced antinociception (Jensen & Yaksh, 1986; McGaraughty & Heinricher, 2002). Moreover, several studies have described the neuroexcitatory and pronociceptive effects of M3G following intrathecal and intracerebroventricular injections (Bartlett, Cramond, & Smith, 1994; Lewis et al., 2010). Alternatively, Peckmann *et al.* in 2006 reported a higher ED50 in female rats than in males for several opiates that produce 3-glucuronide metabolites (Peckham & Traynor, 2006). Hence, M3G and other 3-glucuronide metabolites might act as excitatory signals, and M3G levels found in the CNS might modulate morphine antinociception in mice. However, conflicting results have been reported suggesting no pronociceptive effects of M3G (Ouellet & Pollack, 1997; Penson et al., 2000; Swartjes et al., 2012). The M3G/morphine ratios were remarkably higher in the PAG and amygdala of female mice compared to male mice. It is worth noting that there were surprisingly no differences in the M3G/morphine ratios in the LSC. Importantly, the antinociceptive effect of morphine following s.c. injection in male rats has been correlated with the M3G/morphine ratio found in the cortical extracellular fluid in a microdialysis study (M. J. Barjavel, Scherrmann, & Bhargava, 1995).

Taken together, our results indicate that the metabolism of morphine occurs in the CNS *in vivo* and modulates morphine and M3G levels in some pain-related CNS regions. Therefore, it might modulate morphine antinociception. Importantly, this central metabolism is influenced by sex in C57BL/6J mice and might therefore participate in the sex differences in morphine antinociception.

In the brain, the expression of UGT1A8 and UGT2B36, two enzymes involved in M3G production (Kurita et al., 2017; Stone et al., 2003), is higher in female than in male mice (Buckley & Klaassen, 2007). Interestingly, UGT expression has been shown to be modulated by sex hormones (Strasser, Smid, Mashford, & Desmond, 1997) and both androgen receptors and estrogen receptor α modulate the expression of several UGT isoforms (Bao et al., 2008; Cho, Ning, Zhang, Rubin, & Jeong, 2016). Although morphine antinociception has been shown to be influenced by the estrous cycle in females (Krzanowska & Bodnar, 1999; Loyd, Morgan, et al., 2008; Stoffel, Ulibarri, & Craft, 2003), several reports indicate the implication of the organisational rather than activational effects of steroid hormones in the sex differences observed in morphine antinociception (Cataldo et al., 2005; Cicero et al., 2002). Future studies will investigate whether sex hormones are involved in the sex differences observed in central metabolism of morphine and to what extent these differences contribute to the behavioural contrast observed in morphine antinociception.

It is worth noting that in the PAG, lower morphine brain/blood ratio were observed in females than in males suggesting either sex differences in the BBB permeability of morphine or in the central metabolism of morphine. Morphine has been shown to be a substrate of the P-glycoprotein (P-gp) that limits its distribution in the brain (Callaghan & Riordan, 1993; Hamabe et al., 2007). In addition, multidrug resistance associated proteins (MRPs) have also been shown to be involved in morphine transport in the brain (Su & Pasternak, 2013). Therefore, sex differences in these transporter expressions could also explain the differences observed in morphine quantities in the PAG between males and females (Soldin, Chung, & Mattison, 2011). It is however unlikely that sex differences in morphine BBB permeability or hepatic metabolism might fully explain the differences observed in morphine antinociception, considering that sex differences in response to morphine were observed following direct i.c.v. injections (Kest et al., 1999).

Sex differences in antinociceptive tolerance to morphine

We observed significant sex differences in the development of antinociceptive tolerance to morphine. However, the rate at which the tolerance developed remained the same between males and females, as witnessed by the absence of differences in the Hill slope coefficients. These results suggest that, in our paradigm, the sex disparities observed in the development of morphine tolerance might be due to the sex differences in the initial morphine effectiveness rather than to sex-specific mechanisms involved in the development of tolerance. Such findings are corroborated by a study in which sex differences in acute morphine potency were controlled during the assessment of tolerance (Barrett, Cook, Terner, Craft, & Picker, 2001). Nonetheless, a sexual dimorphism in antinociceptive tolerance has been previously documented, although strong discrepancies exist in the literature (Craft et al., 1999; Kest, Sarton, & Dahan, 2000). Importantly, in our experiment, sex differences in the Hill-slope coefficient might have been hidden as male mice reached the cut-off of the test for at least the first three days of the tolerance protocol. Indeed, if males were allowed to reach higher latencies, the Hill-slope coefficient could change.

Peripheral metabolism during antinociceptive tolerance to morphine

We observed significant interactions at day 10 between sex and treatment in the maximal concentration, AUC, AUMC and clearance of morphine. However, there was no interaction in the reported parameters for M3G, the metabolic ratios obtained in the blood, or any condition tested in the CNS, except for the M3G brain/blood ratios in the amygdala. These results suggest that the rapid induction of antinociceptive tolerance in females might not be related to sex-specific mechanisms involving morphine metabolism into M3G.

We observed that tolerant mice had lower levels of morphine in the blood than control mice. The M3G/morphine ratios were increased in the blood of tolerant mice, suggesting that chronic morphine injections increased morphine metabolism. Interestingly, the mRNAs of metabolic enzymes implicated in testosterone metabolism are upregulated in the liver following acute

morphine administration (Aloisi et al., 2010). Therefore, morphine might directly or indirectly regulate metabolic enzyme and transporter expression, hence modulating its own metabolism, distribution and/or excretion. It is, however, unlikely that these differences in M3G metabolic pathway play a major role in morphine tolerance, as tolerant male mice show the same metabolic ratio as control females.

Interestingly, in rats, although the metabolism of morphine into M3G is predominant, a significant proportion of morphine is converted into normorphine, an antinociceptive metabolite, which is readily converted into normorphine-3-glucuronide described to possess pronociceptive effects (Evans & Shanahan, 1995; Lasagna & De Kornfeld, 1958; G. D. Smith & Smith, 1998). Therefore, it is possible that the morphine chronic treatment induces metabolic adaptations of this side pathway resulting in an increase of morphine metabolism into normorphine that could participate in morphine tolerance (South, Wright, Lau, Mather, & Smith, 2001). This hypothesis is supported by the increase of morphine clearance after morphine chronic treatment in females, although no differences was observed in any M3G pharmacokinetic parameters. Therefore, the apparent decrease in morphine levels in tolerant mice may result from metabolic adaptations in the normorphine pathway. Further investigation in which normorphine and normorphine-3-glucuronide are quantified is required.

Central metabolism in pain-related CNS regions during antinociceptive tolerance to morphine

The quantification in the blood was consistent with the decreased levels of morphine observed in the amygdala and OB. However, morphine was still present and should have continued to produce an antinociceptive effect. Furthermore, M3G levels remained unchanged or even decreased in the region tested, eliminating a potential increase in its pronociceptive effect due to higher concentrations in the CNS. Finally, the M3G/morphine ratio in the analysed CNS regions

did not differ between control and tolerant mice, excluding a potential role for central metabolism of morphine into M3G in morphine tolerance.

In contrast to these pharmacokinetic findings, pharmacodynamic processes have been shown to be involved in morphine tolerance. For instance, enhanced MOR desensitisation has been observed in the PAG and locus coeruleus after chronic administration of morphine (Dang & Williams, 2004; Ingram, Macey, Fossum, & Morgan, 2008). In addition, impaired recovery from desensitisation has also been observed after chronic morphine exposure and was absent in β-arrestin-2 knockout animals (Connor, Bagley, Chieng, & Christie, 2015; Quillinan, Lau, Virk, von Zastrow, & Williams, 2011), in agreement with behavioural studies suggesting the involvement of the G protein-coupled receptor kinase (GRK)-arrestin system in the tolerance phenomenon (Bohn, Gainetdinov, Lin, Lefkowitz, & Caron, 2000; Bohn, Lefkowitz, & Caron, 2002). To what extent these mechanisms are involved in the sex differences observed in the antinociceptive tolerance to morphine remains to be investigated.

CONCLUSION

In conclusion, our results showed sex differences in morphine antinociception, tolerance and metabolism in mice. Female mice displayed lower antinociception following the administration of morphine, consistent with (***i***) higher levels of M3G found in the blood and (***ii***) lower levels of morphine and greater levels of M3G found in some pain-related CNS regions. The differences observed in these regions were related to sex differences in the central metabolism of morphine.

In addition, morphine tolerance appeared earlier during the protocol in females, although this effect seemed to not be influenced by sex. Moreover, tolerant mice showed lower concentrations of morphine in the blood and higher M3G/morphine metabolic ratios. However, globally, no changes were observed in the CNS regions of tolerant mice.

All these data support that morphine hepatic and central metabolism are related to the observed sex differences in morphine antinociception in C57BL/6J mice, but their role in antinociceptive tolerance seems to be relatively limited.

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

COMPETING INTERESTS - The authors declare that they have no competing interests.

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REFERENCES

Alexander, S. P., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., et al. (2021). THE CONCISE GUIDE TO PHARMACOLOGY 2021/22: Introduction and Other Protein Targets. *Br J Pharmacol, 178 Suppl 1*, S1-S26. doi:10.1111/bph.15537

Aloisi, A. M., Ceccarelli, I., Fiorenzani, P., Maddalena, M., Rossi, A., Tomei, V., et al. (2010). Aromatase and 5-alpha reductase gene expression: modulation by pain and morphine treatment in male rats. *Mol Pain, 6*, 69. doi:10.1186/1744-8069-6-69

Aubrun, F., Salvi, N., Coriat, P., & Riou, B. (2005). Sex- and age-related differences in morphine requirements for postoperative pain relief. *Anesthesiology, 103*(1), 156-160. doi:10.1097/00000542-200507000-00023

Bai, L., Zhai, C., Han, K., Li, Z., Qian, J., Jing, Y., et al. (2014). Toll-like receptor 4-mediated nuclear factor-kappaB activation in spinal cord contributes to chronic morphine-induced analgesic tolerance and hyperalgesia in rats. *Neurosci Bull, 30*(6), 936-948. doi:10.1007/s12264-014-1483-7

Baker, L., & Ratka, A. (2002). Sex-specific differences in levels of morphine, morphine-3-glucuronide, and morphine antinociception in rats. *Pain, 95*(1-2), 65-74. doi:10.1016/s0304-3959(01)00376-1

Bao, B. Y., Chuang, B. F., Wang, Q., Sartor, O., Balk, S. P., Brown, M., et al. (2008). Androgen receptor mediates the expression of UDP-glucuronosyltransferase 2 B15 and B17 genes. *Prostate, 68*(8), 839-848. doi:10.1002/pros.20749

Barjavel, M., Sandouk, P., Plotkine, M., & Scherrmann, J. M. (1994). Morphine and morphine metabolite kinetics in the rat brain as assessed by transcortical microdialysis. *Life Sci, 55*(16), 1301-1308. doi:10.1016/0024-3205(94)90069-8

Barjavel, M. J., Scherrmann, J. M., & Bhargava, H. N. (1995). Relationship between morphine analgesia and cortical extracellular fluid levels of morphine and its metabolites in the rat: a microdialysis study. *Br J Pharmacol, 116*(8), 3205-3210. doi:10.1111/j.1476-5381.1995.tb15125.x

Barrett, A. C., Cook, C. D., Terner, J. M., Craft, R. M., & Picker, M. J. (2001). Importance of sex and relative efficacy at the mu opioid receptor in the development of tolerance and cross-tolerance to the antinociceptive effects of opioids. *Psychopharmacology (Berl), 158*(2), 154-164. doi:10.1007/s002130100821

Bartlett, S. E., Cramond, T., & Smith, M. T. (1994). The excitatory effects of morphine-3-glucuronide are attenuated by LY274614, a competitive NMDA receptor antagonist, and by midazolam, an agonist at the benzodiazepine site on the GABAA receptor complex. *Life Sci, 54*(10), 687-694. doi:10.1016/0024-3205(94)00552-4

Bickel, U., Schumacher, O. P., Kang, Y. S., & Voigt, K. (1996). Poor permeability of morphine 3-glucuronide and morphine 6-glucuronide through the blood-brain barrier in the rat. *J Pharmacol Exp Ther, 278*(1), 107-113.

Blomqvist, K. J., Viisanen, H., Ahlstrom, F. H. G., Jokinen, V., Sidorova, Y. A., Suleymanova, I., et al. (2020). Morphine-3-glucuronide causes antinociceptive cross-tolerance to morphine and increases spinal substance P expression. *Eur J Pharmacol, 875*, 173021. doi:10.1016/j.ejphar.2020.173021

Bohn, L. M., Gainetdinov, R. R., Lin, F. T., Lefkowitz, R. J., & Caron, M. G. (2000). Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature, 408*(6813), 720-723. doi:10.1038/35047086

Bohn, L. M., Lefkowitz, R. J., & Caron, M. G. (2002). Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. *J Neurosci, 22*(23), 10494-10500. doi:22/23/10494

Bond, J. A., Medinsky, M. A., Dent, J. G., & Rickert, D. E. (1981). Sex-dependent metabolism and biliary excretion of [2,4-14C] dinitrotoluene in isolated perfused rat livers. *J Pharmacol Exp Ther, 219*(3), 598-603.

Bryant, C. D., Eitan, S., Sinchak, K., Fanselow, M. S., & Evans, C. J. (2006). NMDA receptor antagonism disrupts the development of morphine analgesic tolerance in male, but not female C57BL/6J mice. *Am J Physiol Regul Integr Comp Physiol, 291*(2), R315-326. doi:10.1152/ajpregu.00831.2005

Buckley, D. B., & Klaassen, C. D. (2007). Tissue- and gender-specific mRNA expression of UDP-glucuronosyltransferases (UGTs) in mice. *Drug Metab Dispos, 35*(1), 121-127. doi:10.1124/dmd.106.012070

Callaghan, R., & Riordan, J. R. (1993). Synthetic and natural opiates interact with P-glycoprotein in multidrug-resistant cells. *J Biol Chem, 268*(21), 16059-16064.

Cataldo, G., Bernal, S., Markowitz, A., Ogawa, S., Ragnauth, A., Pfaff, D. W., et al. (2005). Organizational manipulation of gonadal hormones and systemic morphine analgesia in female rats: effects of adult ovariectomy and estradiol replacement. *Brain Res, 1059*(1), 13-19. doi:10.1016/j.brainres.2005.08.003

Cepeda, M. S., & Carr, D. B. (2003). Women experience more pain and require more morphine than men to achieve a similar degree of analgesia. *Anesth Analg, 97*(5), 1464-1468. doi:10.1213/01.ane.0000080153.36643.83

Cepeda, M. S., Farrar, J. T., Baumgarten, M., Boston, R., Carr, D. B., & Strom, B. L. (2003). Side effects of opioids during short-term administration: effect of age, gender, and race. *Clin Pharmacol Ther, 74*(2), 102-112. doi:10.1016/S0009-9236(03)00152-8

Charan, J., & Kantharia, N. D. (2013). How to calculate sample size in animal studies? *J Pharmacol Pharmacother, 4*(4), 303-306. doi:10.4103/0976-500X.119726

Cho, S. J., Ning, M., Zhang, Y., Rubin, L. H., & Jeong, H. (2016). 17beta-Estradiol up-regulates UDP-glucuronosyltransferase 1A9 expression via estrogen receptor alpha. *Acta Pharm Sin B, 6*(5), 504-509. doi:10.1016/j.apsb.2016.04.005

Cicero, T. J., Nock, B., & Meyer, E. R. (1996). Gender-related differences in the antinociceptive properties of morphine. *J Pharmacol Exp Ther, 279*(2), 767-773.

Cicero, T. J., Nock, B., & Meyer, E. R. (1997). Sex-related differences in morphine's antinociceptive activity: relationship to serum and brain morphine concentrations. *J Pharmacol Exp Ther, 282*(2), 939-944.

Cicero, T. J., Nock, B., O'Connor, L., & Meyer, E. R. (2002). Role of steroids in sex differences in morphine-induced analgesia: activational and organizational effects. *J Pharmacol Exp Ther, 300*(2), 695-701. doi:10.1124/jpet.300.2.695

Comer, S. D., Cooper, Z. D., Kowalczyk, W. J., Sullivan, M. A., Evans, S. M., Bisaga, A. M., et al. (2010). Evaluation of potential sex differences in the subjective and analgesic effects of morphine in normal, healthy volunteers. *Psychopharmacology (Berl), 208*(1), 45-55. doi:10.1007/s00213-009-1703-4

Connor, M., Bagley, E. E., Chieng, B. C., & Christie, M. J. (2015). beta-Arrestin-2 knockout prevents development of cellular mu-opioid receptor tolerance but does not affect opioid-withdrawal-related adaptations in single PAG neurons. *Br J Pharmacol, 172*(2), 492-500. doi:10.1111/bph.12673

Craft, R. M. (2003). Sex differences in opioid analgesia: "from mouse to man". *Clin J Pain, 19*(3), 175-186. doi:10.1097/00002508-200305000-00005

Craft, R. M., Stratmann, J. A., Bartok, R. E., Walpole, T. I., & King, S. J. (1999). Sex differences in development of morphine tolerance and dependence in the rat. *Psychopharmacology (Berl), 143*(1), 1-7. doi:10.1007/s002130050911

Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., et al. (2018). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. *Br J Pharmacol, 175*(7), 987-993. doi:10.1111/bph.14153

Dang, V. C., & Williams, J. T. (2004). Chronic morphine treatment reduces recovery from opioid desensitization. *J Neurosci, 24*(35), 7699-7706. doi:10.1523/JNEUROSCI.2499-04.2004

Doyle, H. H., Eidson, L. N., Sinkiewicz, D. M., & Murphy, A. Z. (2017). Sex Differences in Microglia Activity within the Periaqueductal Gray of the Rat: A Potential Mechanism Driving the Dimorphic Effects of Morphine. *J Neurosci, 37*(12), 3202-3214. doi:10.1523/JNEUROSCI.2906-16.2017

Doyle, H. H., & Murphy, A. Z. (2018). Sex-dependent influences of morphine and its metabolites on pain sensitivity in the rat. *Physiol Behav, 187*, 32-41. doi:10.1016/j.physbeh.2017.11.030

Due, M. R., Piekarz, A. D., Wilson, N., Feldman, P., Ripsch, M. S., Chavez, S., et al. (2012). Neuroexcitatory effects of morphine-3-glucuronide are dependent on Toll-like receptor 4 signaling. *J Neuroinflammation, 9*, 200. doi:10.1186 / 1742-2094-9-200

Eidson, L. N., Inoue, K., Young, L. J., Tansey, M. G., & Murphy, A. Z. (2017). Toll-like Receptor 4 Mediates Morphine-Induced Neuroinflammation and Tolerance via Soluble Tumor Necrosis Factor Signaling. *Neuropsychopharmacology, 42*(3), 661-670. doi:10.1038/npp.2016.131

Enman, N. M., Reyes, B. A. S., Shi, Y., Valentino, R. J., & Van Bockstaele, E. J. (2019). Sex differences in morphine-induced trafficking of mu-opioid and corticotropin-releasing factor receptors in locus coeruleus neurons. *Brain Res, 1706*, 75-85. doi:10.1016/j.brainres.2018.11.001

Evans, A. M., & Shanahan, K. (1995). The disposition of morphine and its metabolites in the in-situ rat isolated perfused liver. *J Pharm Pharmacol, 47*(4), 333-339. doi:10.1111/j.2042-7158.1995.tb05805.x

Fields, H. (2004). State-dependent opioid control of pain. *Nat Rev Neurosci, 5*(7), 565-575. doi:10.1038/nrn1431

Fullerton, E. F., Doyle, H. H., & Murphy, A. Z. (2018). Impact of sex on pain and opioid analgesia: a review. *Curr Opin Behav Sci, 23*, 183-190. doi:10.1016/j.cobeha.2018.08.001

Glaum, S. R., Miller, R. J., & Hammond, D. L. (1994). Inhibitory actions of delta 1-, delta 2-, and mu-opioid receptor agonists on excitatory transmission in lamina II neurons of adult rat spinal cord. *J Neurosci, 14*(8), 4965-4971.

Goudas, L. C., Langlade, A., Serrie, A., Matson, W., Milbury, P., Thurel, C., et al. (1999). Acute decreases in cerebrospinal fluid glutathione levels after intracerebroventricular morphine for cancer pain. *Anesth Analg, 89*(5), 1209-1215.

Guajardo, H. M., Snyder, K., Ho, A., & Valentino, R. J. (2017). Sex Differences in mu-Opioid Receptor Regulation of the Rat Locus Coeruleus and Their Cognitive Consequences. *Neuropsychopharmacology, 42*(6), 1295-1304. doi:10.1038/npp.2016.252

Hamabe, W., Maeda, T., Kiguchi, N., Yamamoto, C., Tokuyama, S., & Kishioka, S. (2007). Negative relationship between morphine analgesia and P-glycoprotein expression levels in the brain. *Journal of Pharmacological Sciences, 105*(4), 353-360. doi:10.1254/jphs.FP0071287

Handal, M., Grung, M., Skurtveit, S., Ripel, A., & Morland, J. (2002). Pharmacokinetic differences of morphine and morphine-glucuronides are reflected in locomotor activity. *Pharmacol Biochem Behav, 73*(4), 883-892. doi:10.1016/s0091-3057(02)00925-5

Heydel, J. M., Holsztynska, E. J., Legendre, A., Thiebaud, N., Artur, Y., & Le Bon, A. M. (2010). UDP-glucuronosyltransferases (UGTs) in neuro-olfactory tissues: expression, regulation, and function. *Drug Metab Rev, 42*(1), 74-97. doi:10.3109/03602530903208363

Ho, Y. S., Liu, R. H., Nichols, A. W., & Kumar, S. D. (1990). Isotopic Analog as the Internal Standard for Quantitative-Determination - Evaluation of Mass-Spectra of Commonly Abused Drugs and Their Deuterated Analogs. *Journal of Forensic Sciences, 35*(1), 123-132.

Ingram, S. L., Macey, T. A., Fossum, E. N., & Morgan, M. M. (2008). Tolerance to repeated morphine administration is associated with increased potency of opioid agonists. *Neuropsychopharmacology, 33*(10), 2494-2504. doi:10.1038/sj.npp.1301634

Jensen, T. S., & Yaksh, T. L. (1986). Comparison of antinociceptive action of morphine in the periaqueductal gray, medial and paramedial medulla in rat. *Brain Res, 363*(1), 99-113. doi:10.1016/0006-8993(86)90662-1

Kepler, K. L., Standifer, K. M., Paul, D., Kest, B., Pasternak, G. W., & Bodnar, R. J. (1991). Gender effects and central opioid analgesia. *Pain, 45*(1), 87-94. doi:10.1016/0304-3959(91)90168-W

Kest, B., Sarton, E., & Dahan, A. (2000). Gender differences in opioid-mediated analgesia: animal and human studies. *Anesthesiology, 93*(2), 539-547. doi:10.1097/00000542-200008000-00034

Kest, B., Wilson, S. G., & Mogil, J. S. (1999). Sex differences in supraspinal morphine analgesia are dependent on genotype. *J Pharmacol Exp Ther, 289*(3), 1370-1375.

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *J Pharmacol Pharmacother, 1*(2), 94-99. doi:10.4103/0976-500X.72351

Krzanowska, E. K., & Bodnar, R. J. (1999). Morphine antinociception elicited from the ventrolateral periaqueductal gray is sensitive to sex and gonadectomy differences in rats. *Brain Res, 821*(1), 224-230. doi:10.1016/s0006-8993(98)01364-x

Kurita, A., Miyauchi, Y., Ikushiro, S. I., Mackenzie, P. I., Yamada, H., & Ishii, Y. (2017). Comprehensive Characterization of Mouse UDP-Glucuronosyltransferase (Ugt) belonging to the Ugt2b Subfamily: Identification of Ugt2b36 as the Predominant Isoform Involved in Morphine Glucuronidation. *J Pharmacol Exp Ther, 361*(2), 199-208. doi:jpet.117.240382 [pii] 10.1124/jpet.117.240382

Kutsuno, Y., Hirashima, R., Sakamoto, M., Ushikubo, H., Michimae, H., Itoh, T., et al. (2015). Expression of UDP-Glucuronosyltransferase 1 (UGT1) and Glucuronidation Activity toward Endogenous Substances in Humanized UGT1 Mouse Brain. *Drug Metab Dispos, 43*(7), 1071-1076. doi:10.1124/dmd.115.063719

Lasagna, L., & De Kornfeld, T. J. (1958). Analgesic potency of normorphine in patients with postoperative pain. *J Pharmacol Exp Ther, 124*(3), 260-263.

Laux-Biehlmann, A., Mouheiche, J., Veriepe, J., & Goumon, Y. (2013). Endogenous morphine and its metabolites in mammals: history, synthesis, localization and perspectives. *Neuroscience, 233*, 95-117. doi:10.1016/j.neuroscience.2012.12.013

Lewis, S. S., Hutchinson, M. R., Rezvani, N., Loram, L. C., Zhang, Y., Maier, S. F., et al. (2010). Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1beta. *Neuroscience, 165*(2), 569-583. doi:S0306-4522(09)01651-0 10.1016/j.neuroscience.2009.10.011

Liu, A., Zhang, H., Qin, F., Wang, Q., Sun, Q., Xie, S., et al. (2018). Sex Associated Differential Expressions of the Alternatively Spliced Variants mRNA of OPRM1 in Brain Regions of C57BL/6 Mouse. *Cell Physiol Biochem, 50*(4), 1441-1459. doi:10.1159/000494644

Lotsch, J., & Geisslinger, G. (2001). Morphine-6-glucuronide: an analgesic of the future? *Clin Pharmacokinet, 40*(7), 485-499. doi:10.2165/00003088-200140070-00001

Loyd, D. R., Morgan, M. M., & Murphy, A. Z. (2008). Sexually dimorphic activation of the periaqueductal gray-rostral ventromedial medullary circuit during the development of tolerance to morphine in the rat. *Eur J Neurosci, 27*(6), 1517-1524. doi:10.1111/j.1460-9568.2008.06100.x

Loyd, D. R., & Murphy, A. Z. (2014). The neuroanatomy of sexual dimorphism in opioid analgesia. *Exp Neurol, 259*, 57-63. doi:10.1016/j.expneurol.2014.04.004

Loyd, D. R., Wang, X., & Murphy, A. Z. (2008). Sex differences in micro-opioid receptor expression in the rat midbrain periaqueductal gray are essential for eliciting sex differences in morphine analgesia. *J Neurosci, 28*(52), 14007-14017. doi:10.1523/JNEUROSCI.4123-08.2008

Mattioli, T. A., Leduc-Pessah, H., Skelhorne-Gross, G., Nicol, C. J., Milne, B., Trang, T., et al. (2014). Toll-like receptor 4 mutant and null mice retain morphine-induced tolerance, hyperalgesia, and physical dependence. *PLoS One, 9*(5), e97361. doi:10.1371/journal.pone.0097361

McGaraughty, S., & Heinricher, M. M. (2002). Microinjection of morphine into various amygdaloid nuclei differentially affects nociceptive responsiveness and RVM neuronal activity. *Pain, 96*(1-2), 153-162. doi:10.1016/s0304-3959(01)00440-7

Mogil, J. S. (2012). Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nat Rev Neurosci, 13*(12), 859-866. doi:10.1038/nrn3360

Mogil, J. S. (2020). Qualitative sex differences in pain processing: emerging evidence of a biased literature. *Nat Rev Neurosci, 21*(7), 353-365. doi:10.1038/s41583-020-0310-6

Murphey, L. J., & Olsen, G. D. (1994). Diffusion of morphine-6-beta-D-glucuronide into the neonatal guinea pig brain during drug-induced respiratory depression. *J Pharmacol Exp Ther, 271*(1), 118-124.

Ouellet, D. M., & Pollack, G. M. (1997). Effect of prior morphine-3-glucuronide exposure on morphine disposition and antinociception. *Biochem Pharmacol, 53*(10), 1451-1457. doi:10.1016/s0006-2952(97)00086-5

Ouzzine, M., Gulberti, S., Ramalanjaona, N., Magdalou, J., & Fournel-Gigleux, S. (2014). The UDP-glucuronosyltransferases of the blood-brain barrier: their role in drug metabolism and detoxication. *Frontiers in Cellular Neuroscience, 8*doi:10.3389/Fncel.2014.00349

Paller, C. J., Campbell, C. M., Edwards, R. R., & Dobs, A. S. (2009). Sex-based differences in pain perception and treatment. *Pain Med, 10*(2), 289-299. doi:10.1111/j.1526-4637.2008.00558.x

Peckham, E. M., Barkley, L. M., Divin, M. F., Cicero, T. J., & Traynor, J. R. (2005). Comparison of the antinociceptive effect of acute morphine in female and male Sprague-Dawley rats using the long-lasting mu-antagonist methocinnamox. *Brain Res, 1058*(1-2), 137-147. doi:10.1016/j.brainres.2005.07.060

Peckham, E. M., & Traynor, J. R. (2006). Comparison of the antinociceptive response to morphine and morphine-like compounds in male and female Sprague-Dawley rats. *J Pharmacol Exp Ther, 316*(3), 1195-1201. doi:10.1124/jpet.105.094276

Penson, R. T., Joel, S. P., Bakhshi, K., Clark, S. J., Langford, R. M., & Slevin, M. L. (2000). Randomized placebo-controlled trial of the activity of the morphine glucuronides. *Clin Pharmacol Ther, 68*(6), 667-676. doi:10.1067/mcp.2000.111934

Quillinan, N., Lau, E. K., Virk, M., von Zastrow, M., & Williams, J. T. (2011). Recovery from mu-opioid receptor desensitization after chronic treatment with morphine and methadone. *J Neurosci, 31*(12), 4434-4443. doi:10.1523/JNEUROSCI.4874-10.2011

Richner, M., Jager, S. B., Siupka, P., & Vaegter, C. B. (2017). Hydraulic Extrusion of the Spinal Cord and Isolation of Dorsal Root Ganglia in Rodents. *J Vis Exp*(119)doi:10.3791/55226

Roeckel, L. A., Utard, V., Reiss, D., Mouheiche, J., Maurin, H., Robe, A., et al. (2017). Morphine-induced hyperalgesia involves mu opioid receptors and the metabolite morphine-3-glucuronide. *Sci Rep, 7*(1), 10406. doi:10.1038/s41598-017-11120-4

Rush, G. F., Newton, J. F., & Hook, J. B. (1983). Sex differences in the excretion of glucuronide conjugates: the role of intrarenal glucuronidation. *J Pharmacol Exp Ther, 227*(3), 658-662.

Sandouk, P., Serrie, A., Scherrmann, J. M., Langlade, A., & Bourre, J. M. (1991). Presence of morphine metabolites in human cerebrospinal fluid after intracerebroventricular administration of morphine. *Eur J Drug Metab Pharmacokinet, Spec No 3*, 166-171.

Sarton, E., Olofsen, E., Romberg, R., den Hartigh, J., Kest, B., Nieuwenhuijs, D., et al. (2000). Sex differences in morphine analgesia: an experimental study in healthy volunteers. *Anesthesiology, 93*(5), 1245-1254; discussion 1246A. doi:10.1097/00000542-200011000-00018

Selley, D. E., Herbert, J. T., Morgan, D., Cook, C. D., Picker, M. J., & Sim-Selley, L. J. (2003). Effect of strain and sex on mu opioid receptor-mediated G-protein activation in rat brain. *Brain Res Bull, 60*(3), 201-208. doi:10.1016/s0361-9230(03)00014-5

Shelby, M. K., Cherrington, N. J., Vansell, N. R., & Klaassen, C. D. (2003). Tissue mRNA expression of the rat UDP-glucuronosyltransferase gene family. *Drug Metab Dispos, 31*(3), 326-333. doi:10.1124/dmd.31.3.326

Smith, G. D., & Smith, M. T. (1995). Morphine-3-glucuronide: evidence to support its putative role in the development of tolerance to the antinociceptive effects of morphine in the rat. *Pain, 62*(1), 51-60. doi:10.1016/0304-3959(94)00228-7

Smith, G. D., & Smith, M. T. (1998). The excitatory behavioral and antianalgesic pharmacology of normorphine-3-glucuronide after intracerebroventricular administration to rats. *J Pharmacol Exp Ther, 285*(3), 1157-1162.

Smith, M. T., Watt, J. A., & Cramond, T. (1990). Morphine-3-glucuronide--a potent antagonist of morphine analgesia. *Life Sci, 47*(6), 579-585.

Smith, M. T., Wright, A. W., Williams, B. E., Stuart, G., & Cramond, T. (1999). Cerebrospinal fluid and plasma concentrations of morphine, morphine-3-glucuronide, and morphine-6-glucuronide in patients before and after initiation of intracerebroventricular morphine for cancer pain management. *Anesth Analg, 88*(1), 109-116.

Soldin, O. P., Chung, S. H., & Mattison, D. R. (2011). Sex differences in drug disposition. *J Biomed Biotechnol, 2011*, 187103. doi:10.1155/2011/187103

Soldin, O. P., & Mattison, D. R. (2009). Sex differences in pharmacokinetics and pharmacodynamics. *Clin Pharmacokinet, 48*(3), 143-157. doi:10.2165/00003088-200948030-00001

South, S. M., Edwards, S. R., & Smith, M. T. (2009). Antinociception versus serum concentration relationships following acute administration of intravenous morphine in male and female Sprague-Dawley rats: differences between the tail flick and hot plate nociceptive tests. *Clin Exp Pharmacol Physiol, 36*(1), 20-28. doi:10.1111/j.1440-1681.2008.05019.x

South, S. M., Wright, A. W., Lau, M., Mather, L. E., & Smith, M. T. (2001). Sex-related differences in antinociception and tolerance development following chronic intravenous infusion of morphine in the rat: modulatory role of testosterone via morphine clearance. *J Pharmacol Exp Ther, 297*(1), 446-457.

Stain, F., Barjavel, M. J., Sandouk, P., Plotkine, M., Scherrmann, J. M., & Bhargava, H. N. (1995). Analgesic response and plasma and brain extracellular fluid pharmacokinetics of morphine and morphine-6-beta-D-glucuronide in the rat. *J Pharmacol Exp Ther, 274*(2), 852-857.

Stoffel, E. C., Ulibarri, C. M., & Craft, R. M. (2003). Gonadal steroid hormone modulation of nociception, morphine antinociception and reproductive indices in male and female rats. *Pain, 103*(3), 285-302. doi:10.1016/s0304-3959(02)00457-8

Stone, A. N., Mackenzie, P. I., Galetin, A., Houston, J. B., & Miners, J. O. (2003). Isoform selectivity and kinetics of morphine 3- and 6-glucuronidation by human udp-glucuronosyltransferases: evidence for atypical glucuronidation kinetics by UGT2B7. *Drug Metab Dispos, 31*(9), 1086-1089. doi:10.1124/dmd.31.9.1086

Strasser, S. I., Smid, S. A., Mashford, M. L., & Desmond, P. V. (1997). Sex hormones differentially regulate isoforms of UDP-glucuronosyltransferase. *Pharm Res, 14*(9), 1115-1121. doi:10.1023/a:1012130118186

Su, W., & Pasternak, G. W. (2013). The role of multidrug resistance-associated protein in the blood-brain barrier and opioid analgesia. *Synapse, 67*(9), 609-619. doi:10.1002/syn.21667

Swartjes, M., Mooren, R. A. G., Waxman, A. R., Arout, C., van de Wetering, K., den Hartigh, J., et al. (2012). Morphine Induces Hyperalgesia without Involvement of mu-Opioid Receptor or Morphine-3-glucuronide. *Molecular Medicine, 18*(9), 1320-1326. doi:10.2119/molmed.2012.00244

Trescot, A. M., Datta, S., Lee, M., & Hansen, H. (2008). Opioid pharmacology. *Pain Physician, 11*(2 Suppl), S133-153.

Wahlstrom, A., Winblad, B., Bixo, M., & Rane, A. (1988). Human brain metabolism of morphine and naloxone. *Pain, 35*(2), 121-127. doi:10.1016/0304-3959(88)90219-9

Weinsanto, I., Laux-Biehlmann, A., Mouheiche, J., Maduna, T., Delalande, F., Chavant, V., et al. (2018). Stable isotope-labelled morphine to study in vivo central and peripheral morphine glucuronidation and brain transport in tolerant mice. *Br J Pharmacol, 175*(19), 3844-3856. doi:10.1111/bph.14454

Williams, J. T., Ingram, S. L., Henderson, G., Chavkin, C., von Zastrow, M., Schulz, S., et al. (2013). Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol Rev, 65*(1), 223-254. doi:65/1/223 10.1124/pr.112.005942

Wobbrock, J. O., Findlater, L., Gergle, D., & Higgins, J. J. (2011). The aligned rank transform for nonparametric factorial analyses using only anova procedures. *Proceedings of the SIGCHI Conference on Human Factors in Computing Systems*, 143–146. doi:10.1145/1978942.1978963

Xie, R., Bouw, M. R., & Hammarlund-Udenaes, M. (2000). Modelling of the blood-brain barrier transport of morphine-3-glucuronide studied using microdialysis in the rat: involvement of probenecid-sensitive transport. *Br J Pharmacol, 131*(8), 1784-1792. doi:10.1038/sj.bjp.0703759

Xie, R., Hammarlund-Udenaes, M., de Boer, A. G., & de Lange, E. C. (1999). The role of P-glycoprotein in blood-brain barrier transport of morphine: transcortical microdialysis studies in mdr1a (-/-) and mdr1a (+/+) mice. *Br J Pharmacol, 128*(3), 563-568. doi:10.1038/sj.bjp.0702804

Yamada, H., Ishii, K., Ishii, Y., Ieiri, I., Nishio, S., Morioka, T., et al. (2003). Formation of highly analgesic morphine-6-glucuronide following physiologic concentration of morphine in human brain. *J Toxicol Sci, 28*(5), 395-401. doi:10.2131/jts.28.395

Zhang, Y., Huo, M., Zhou, J., & Xie, S. (2010). PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Comput Methods Programs Biomed, 99*(3), 306-314. doi:10.1016/j.cmpb.2010.01.007

TABLES AND FIGURES LEGENDS

TABLES

Table 1- Pharmacokinetic parameters obtained from the NCA for morphine and M3G in the blood of male and female control and tolerant mice following an injection of 10 mg/kg of morphine at day 10. Data are expressed as mean ± SEM, *n* are indicated in the table. Two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. Morphine volume of distribution at steady-state was analysed with the non-parametric approach ART ANOVA. Sex: #, $P<0.05$. Treatment: $, $P<0.05$. Interaction: £, $P<0.05$. Control males vs control females: a, $P<0.05$. Tolerant males vs tolerant females: b. Control females vs tolerant females: d, $P<0.05$.

Table 2- Levels of morphine and M3G found in the amygdala, the PAG, the LSC and the OB of male and female control and tolerant mice 30 min after an injection of 10 mg/kg of morphine at day 10. Data are expressed as mean ± SEM. *n* are indicated in the table. Two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. Morphine and M3G in the OB were analysed with the non-parametric approach ART ANOVA. Sex: #, $P<0.05$. Treatment: $, $P<0.05$. Control males vs control females: a, $P<0.05$. Tolerant males vs tolerant females: b, $P<0.05$. Control males vs tolerant males: c, $P<0.05$. Control females vs tolerant females: d, $P<0.05$.

Table S1. LC-MS/MS conditions. LC and MS/MS conditions for the purification, detection and quantification of morphine and M3G and their respective heavy-tagged counterparts. The flow rate was set at 90 µl/min on a ZORBAX SB-C18 column (150 x 1mm, 3.5µm).

Table S2. Statistical details for morphine antinociceptive effect and induction of tolerance (see Figure 1). Non-linear regression with a 4-parameters logistic equation was applied to define the ED_{50} of morphine and the 95% confidence intervals in both males and females. The two fits were compared using a nested-model comparison with the extra sum-of-the-squares F test.

For the tolerance experiment, the same analysis was applied to the data of each mouse. Then, the obtained parameters were averaged and compared with an unpaired t-test with Welch's correction. MPE, maximal possible effect.

Table S3. Statistical details for the pharmacokinetic parameters of morphine and M3G in the blood obtained from the NCA (see Figure 2). Two-way ANOVA was used to identify the differences between the pharmacokinetic parameters reported for each group. The non-parametric approach ART ANOVA (results highlighted in bold) was used for morphine volume of distribution at steady-state. C_{max}, maximal concentration reached over the time course; AUC, area under the curve; AUMC, area under the first moment curve; MRT, mean residence time; Cl/F, clearance over bioavailability; V_{dss}/F, volume of distribution at steady-state over bioavailability.

Table S4. Statistical details for the quantification of morphine and M3G in the brain and LSC (see Figure 3). Two-way ANOVA was used to identify the differences in morphine and M3G quantities between the groups. The non-parametric approach ART ANOVA (results highlighted in bold) was used for the M3G/Morphine ratio in the amgydala and morphine and M3G in the OB.

Table S5. Statistical details for morphine and M3G brain/blood ratio (see Figure 4). Two-way ANOVA was used to identify the differences in morphine and M3G brain/blood ratio between the groups. The non-parametric approach ART ANOVA (results highlighted in bold) was used for morphine and M3G brain/blood ratio in the PAG and for M3G brain/blood ratio in the LSC.

Table S6. Statistical details for M3G BBB permeability and central metabolism of morphine (see Figure 4). Linear regressions were applied and analysed through a nested-model comparison with the extra sum-of-squares F-test to compare the M3G BBB permeability between males and females, evaluate whether a significant morphine central metabolism is observed (*i.e.* comparison of the M3G brain/blood ratio fits obtained after an injection of morphine and M3G) and to compare this central metabolism between males and females (*i.e.* comparison of the M3G brain/blood ratio fits obtained after an injection of morphine in male and female mice).

FIGURES

Figure 1- Antinociceptive effect of morphine and development of morphine antinociceptive tolerance in male and female mice. (**a**) Dose-response curves for morphine antinociceptive effect in male and female mice. Antinociception is expressed as % of maximum possible effect (%MPE). 95% confidence intervals are represented as shaded area in blue for males and in red for females. Each group received one dose of morphine (1 mg/kg, 2,5 mg/kg, 5 mg/kg, 10 mg/kg or 20 mg/kg). n = 6 for all conditions except n = 5 for the injection of 20 mg/kg in males. (**b**) Protocol of induction of the antinociceptive tolerance to morphine. (**c**) Development of morphine antinociceptive tolerance throughout the chronic treatment (i.p. 10 mg/kg, from day 1 to 9). Antinociception is expressed as %MPE observed 30 min after morphine or saline injection for 9 successive days. Data are expressed as mean ±SEM; n are indicated in the figure. ED_{50} were extracted from each fitting, and fits were compared with the extra sum-of-the-square F test. Unpaired t-test with Welch's correction was used to compare the values for the day at which half of the MPE remains and the hill-slope coefficient values. *, $P<0.05$. Males are represented as blue circle dots and females as red square dots.

Figure 2- Morphine and M3G kinetics in the blood. (**a**) Protocol of induction of morphine antinociceptive tolerance across days 1 to 10 (D1-D10, 10 mg/kg morphine or saline i.p.). At day 10, blood was collected at the tail vein at different time points during 180 min. (**b**) Blood levels of morphine in control male and female mice after a single injection of morphine at day 10. (**c**) Blood levels of M3G in control mice. (**d**) M3G/morphine metabolic ratios in the blood of control mice. (**e**) Blood levels of morphine in male and female tolerant mice after injection of morphine at day 10. (**f**) Blood levels of M3G in tolerant mice. (**g**) M3G/morphine metabolic ratios in the blood of tolerant mice. (**h**) Overall quantities (area

under the curve; AUC) of morphine expressed in nmol.min/ml. (**i**) AUC expressed in µmol.min/ml of M3G; (**j**) Ratio M3G/morphine of the corresponding AUC. Data are expressed as means ± SEM, *n* are indicated in the figure. Two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. Tukey's multiple comparisons results are reported as *, $P<0.05$. Males are represented as blue circle dots and females as red square dots.

Figure 3- Levels of morphine and M3G in the different brain areas and LSC of male and female control and tolerant mice. (**a**) Protocol of induction of morphine antinociceptive tolerance across days 1 to 10 (D1-D10, 10 mg/kg of morphine or saline i.p.). At day 10, brain areas and lumbar spinal cord were collected 30 min after the injection of morphine and, morphine and M3G were quantified by LC-MS/MS. M3G/morphine ratios found in (**b**) the amygdala, (**c**) PAG, (**d**) LSC and (**e**) OB. Data are expressed as means ± SEM, *n* are indicated in the figure. Two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. The M3G/Morphine ratio in the amygdala was analysed with the non-parametric approach ART ANOVA. Tukey's multiple comparisons results are reported as *, $P<0.05$. Males are represented as blue circle dots and females as red square dots.

Figure 4- Brain/blood ratio of morphine and M3G in the different brain areas and lumbar spinal cord of male and female control and tolerant mice. Brain/blood ratio of (**a**) morphine and (**b**) M3G in the amygdala following an i.p injection of morphine. (**c**) M3G brain/blood ratio obtained in the amygdala as a function of M3G blood concentration after i.p. injections of increasing concentrations of M3G. $n = 4$ for the injection of 10mg/kg of M3G in males and females, $n = 5$ for the other conditions. Brain/blood ratio of (**d**) morphine and (**e**)

M3G in the PAG following an i.p injection of morphine. (**f**) M3G brain/blood ratio obtained in the PAG as a function of M3G blood concentration after i.p. injections of increasing concentrations of M3G. *n* = 3 for the injection of 10mg/kg of M3G in females, *n* = 5 for the other conditions. Brain/blood ratio of (**g**) morphine and (**h**) M3G in the LSC following an i.p injection of morphine. (**i**) M3G brain/blood ratio obtained in the LSC as a function of M3G blood concentration after i.p. injections of increasing concentrations of M3G. *n* = 4 for the injection of 10mg/kg of M3G in males, *n* = 5 for the other conditions. Brain/blood ratio of (**j**) morphine and (**k**) M3G in the OB following an i.p injection of morphine. (**l**) M3G brain/blood ratio obtained in the OB as a function of M3G blood concentration after i.p. injections of increasing concentrations of M3G. *n* = 4 for the injection of 40mg/kg of M3G in males, *n* = 5 for the other conditions. Ratio values are displayed as x 10^3 for morphine and x 10^4 for M3G. M3G injected doses are depicted as (▼) 10 mg/kg, (♦) 20 mg/kg and (▲) 40 mg/kg in blue for males and red for females. The blue and red lines represent linear curve fitting of the BBB permeability for M3G in males and females, respectively. 95% Confidence intervals are represented as dotted-line with the appropriate color. Data are expressed as means ± SEM, *n* are indicated in the figure. Two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. The morphine and M3G brain/blood ratio in the PAG and the M3G brain/blood ratio in the LSC were analysed with the non-parametric approach ART ANOVA. Tukey's multiple comparisons results are reported as *, $P<0.05$.

Figure S1- Pharmacokinetic parameters for morphine and M3G obtained from the NCA. Values of parameters obtained for (**a**) Morphine C_{max}, (**b**) morphine AUMC, (**c**) morphine MRT, (**d**) morphine half-life, (**e**) morphine clearance, (**f**) morphine V_{dss}, (**g**) M3G C_{max}, (**h**) M3G AUMC and (**i**) M3G MRT. Data are expressed as means ± SEM, *n* are indicated

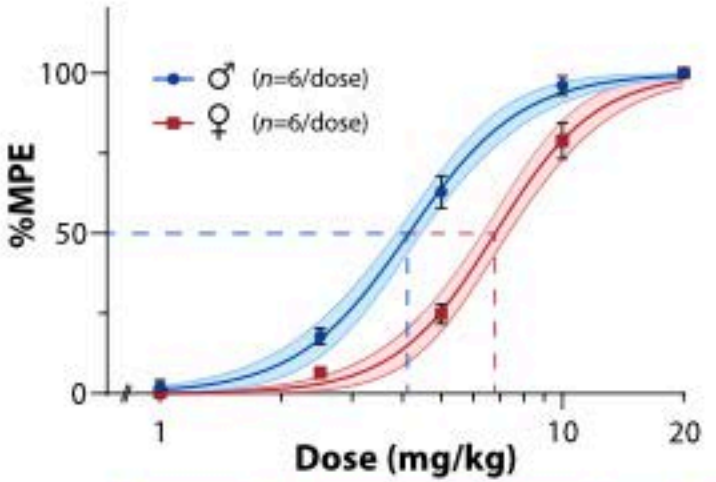
in the figure. Two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. Morphine volume of distribution at steady-state was analysed with the non-parametric approach ART ANOVA. Tukey's multiple comparisons results are reported as *, $P<0.05$. Males are represented as blue circle dots and females as red square dots.

Figure S2- Quantities of morphine and M3G in the different brain areas and lumbar spinal cord of male and female control and tolerant mice. Levels of (**a**) morphine and (**b**) M3G in the amygdala. Levels of (**c**) morphine and (**d**) M3G in the PAG. Levels of (**e**) morphine and (**f**) M3G in the LSC. Levels of (**g**) morphine and (**h**) M3G in the OB. Data are expressed as means ± SEM, *n* are indicated in the figure. Two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. Morphine and M3G in the OB were analysed with the non-parametric approach ART ANOVA. Tukey's multiple comparisons results are reported as *, $P<0.05$. Males are represented as blue circle dots and females as red square dots.

Figure S3- Quantities of M3G found in the different brain areas and lumbar spinal cord of male and female naïve mice following i.p. injection of increasing concentration of M3G. Levels of M3G found in (**a**) the amygdala following i.p. injection of 10, 20 and 40 mg/kg of M3G. $n = 4$ for the injection of 10mg/kg of M3G in males and females, $n = 5$ for the other conditions. Levels of M3G found in (**b**) the PAG following i.p. injection of 10, 20 and 40 mg/kg of M3G. $n = 3$ for the injection of 10mg/kg of M3G in females, $n = 5$ for the other conditions. Levels of M3G found in (**c**) the LSC following i.p. injection of 10, 20 and 40 mg/kg of M3G. $n = 4$ for the injection of 10mg/kg of M3G in males, $n = 5$ for the other conditions. Levels of M3G found in (**d**) the OB following i.p. injection of 10, 20 and 40 mg/kg

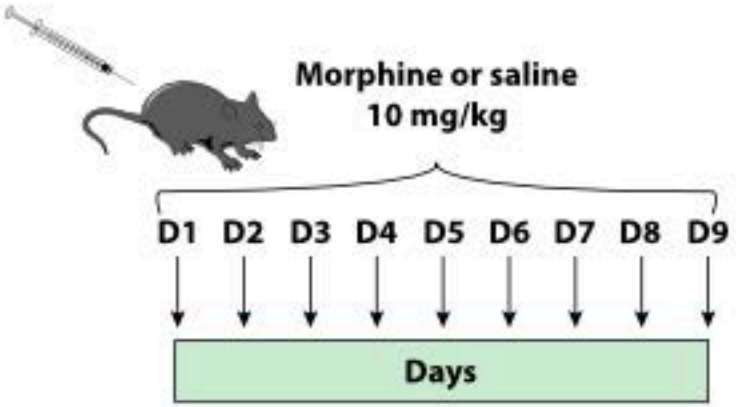
of M3G. $n = 4$ for the injection of 40mg/kg of M3G in males, $n = 5$ for the other conditions. The blue and red lines represent linear regressions of the M3G quantities found in the brain area and LSC as a function of increasing concentration of M3G injected in males and females, respectively. Data are expressed as means ± SEM. Males are represented as circle dots and females as square dots.

a

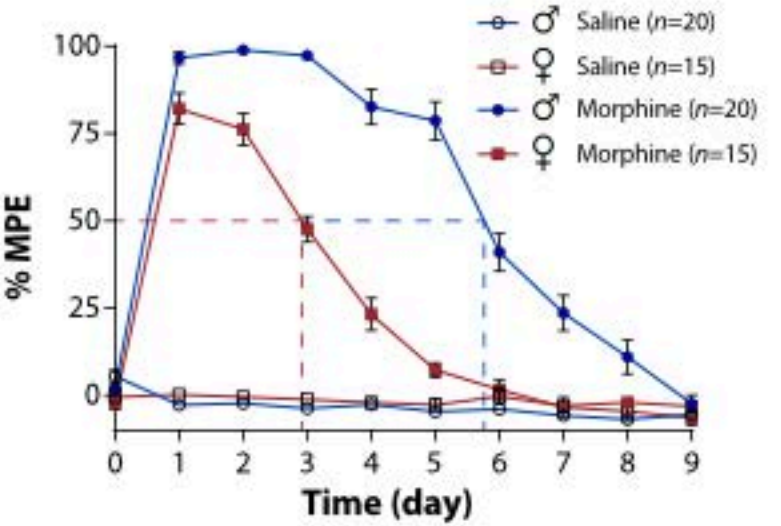


	Males	Females
ED_{50}	**4.15 mg/kg** [CI95% : 3.87 - 4.45]	**6.81 mg/kg*** [CI95%: 6.33 - 7.33]

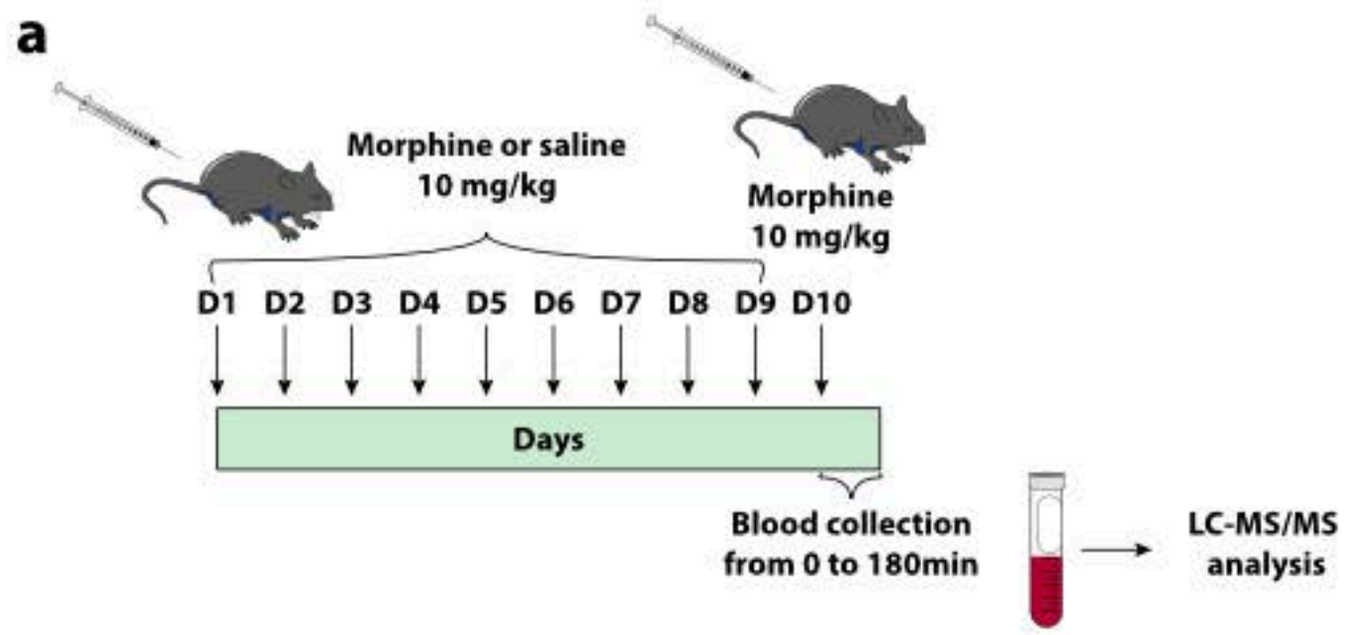
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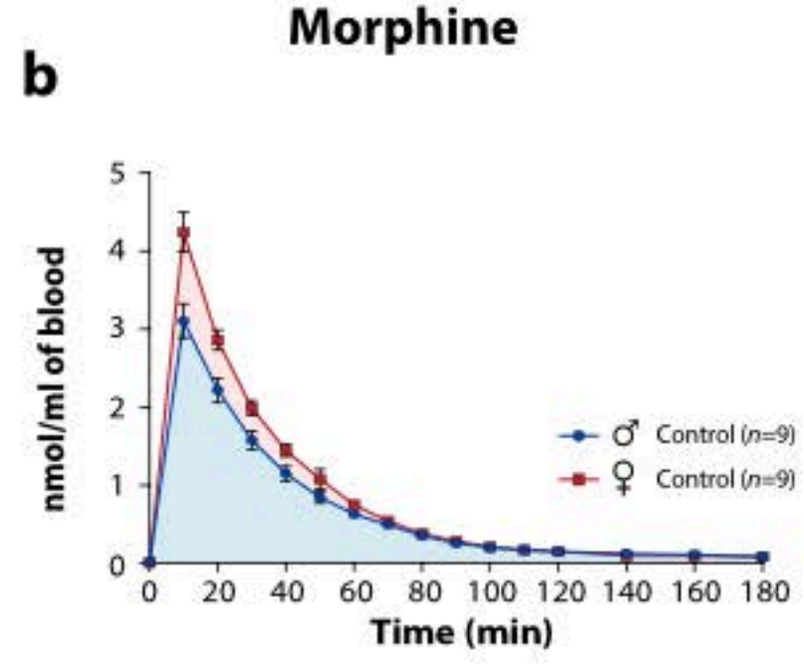


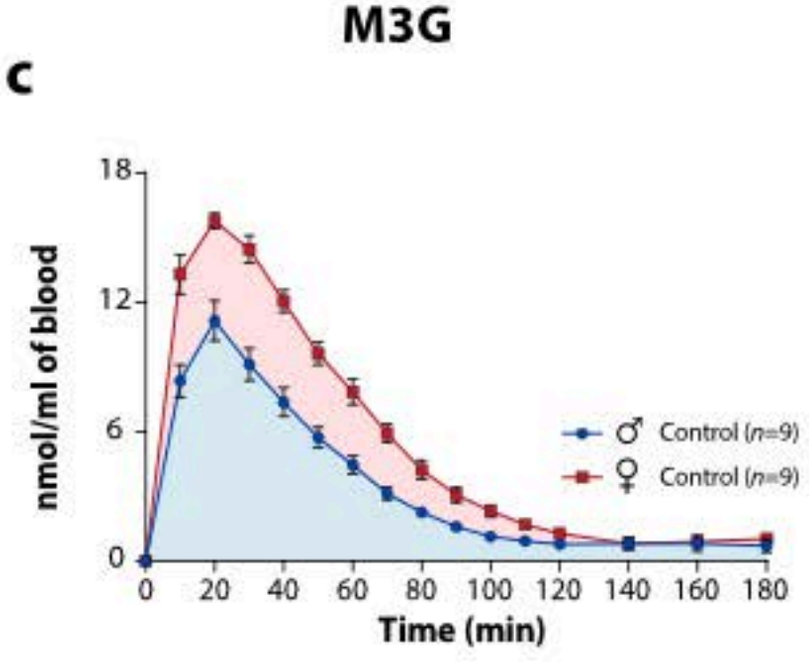
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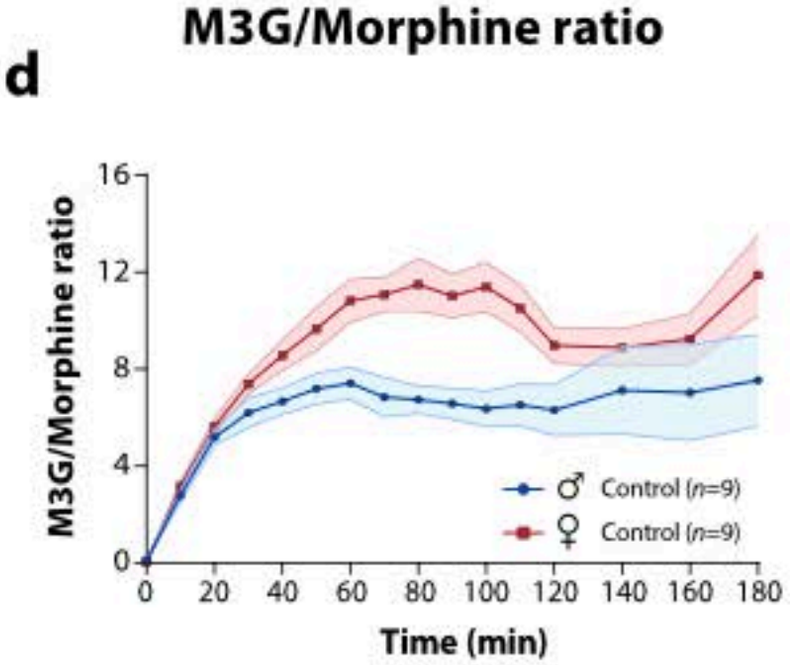


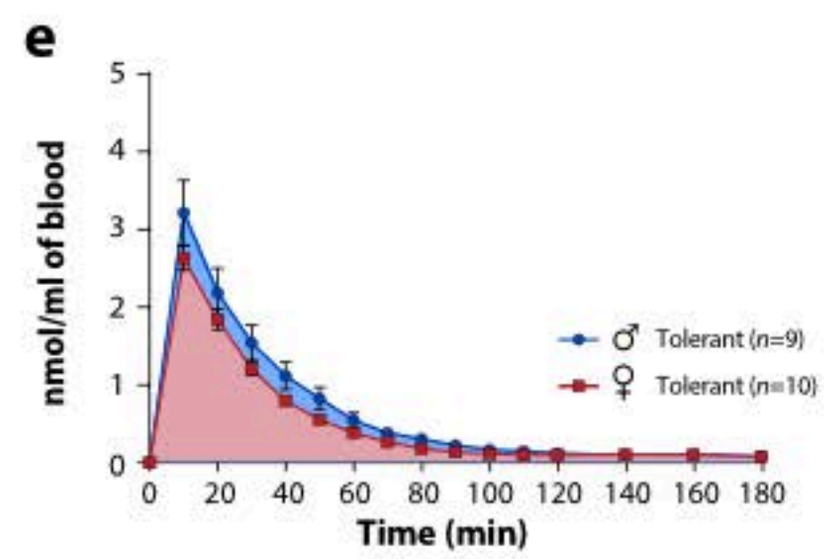
	Males	Females
Day at which 50% MPE remained	**Day 5.88±0.17**	**Day 2.89±0.10***
Hill-slope coefficient	**-0.79±0.14**	**-0.57±0.07**

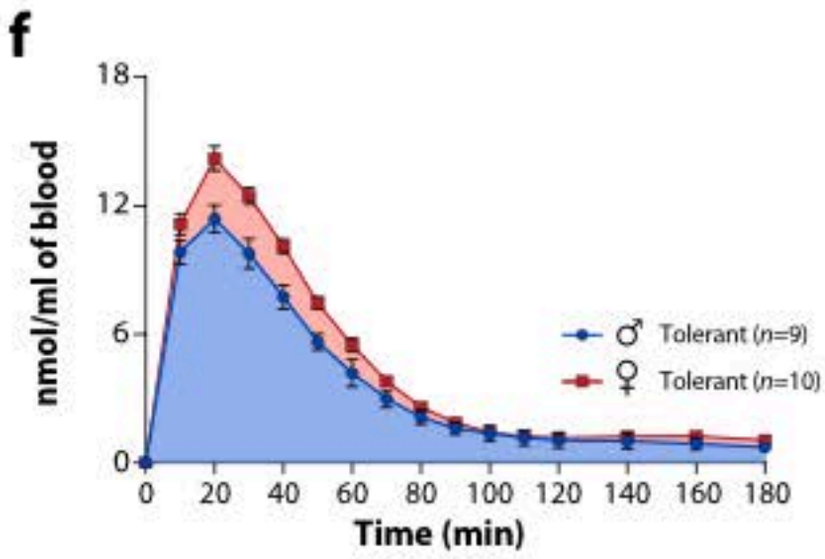

a
Morphine or saline
10 mg/kg
Morphine
10 mg/kg
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10
Days
Blood collection
from 0 to 180min
LC-MS/MS
analysis

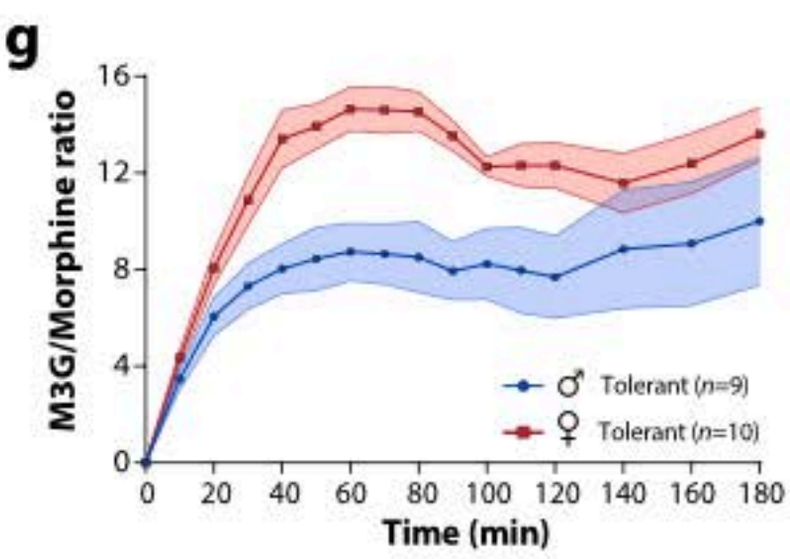

Morphine
b
nmol/ml of blood
Time (min)
♂ Control (n=9)
♀ Control (n=9)

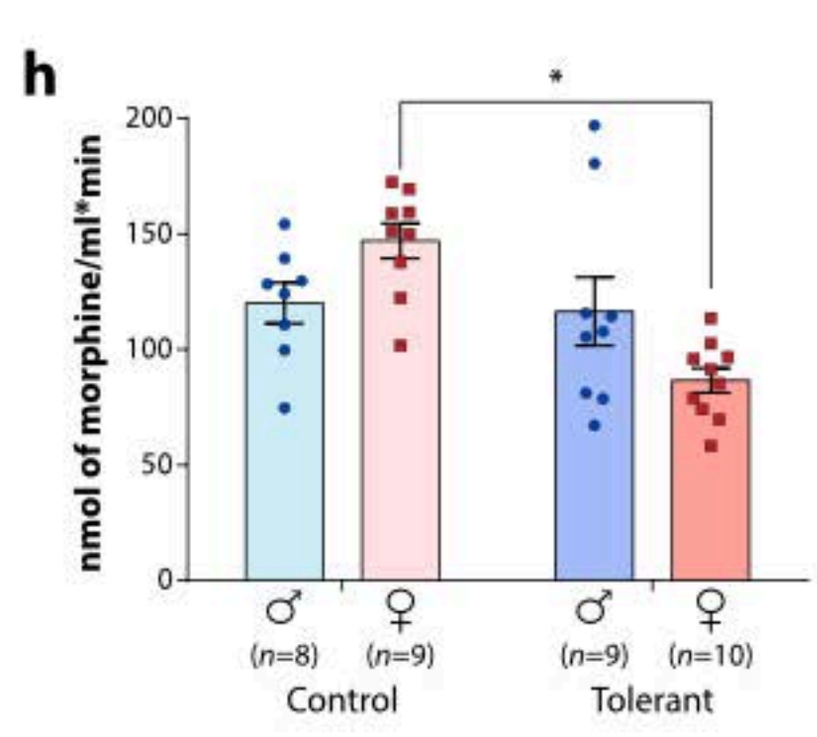

M3G
c
nmol/ml of blood
Time (min)
♂ Control (n=9)
♀ Control (n=9)

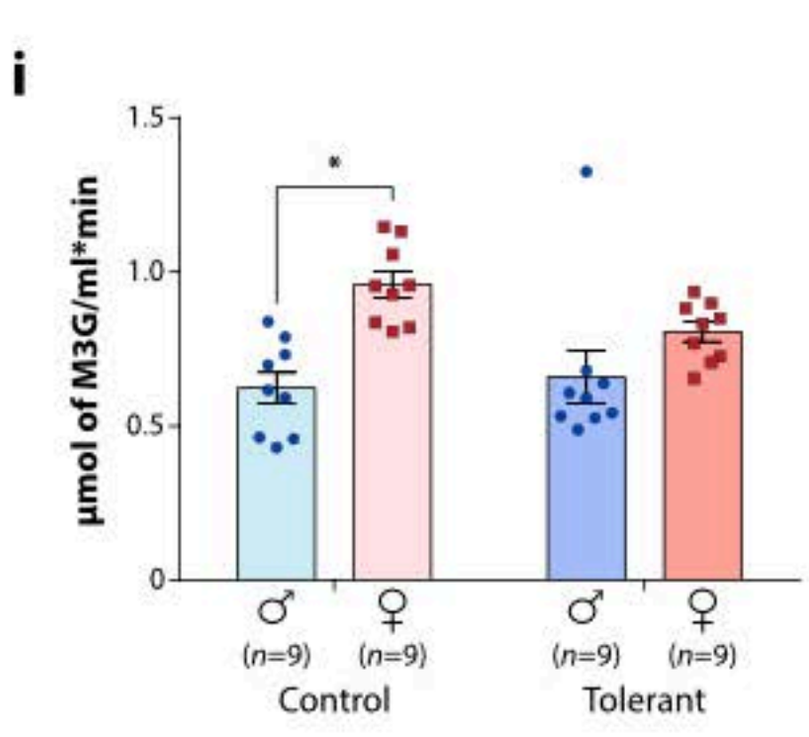

M3G/Morphine ratio
d
M3G/Morphine ratio
Time (min)
♂ Control (n=9)
♀ Control (n=9)

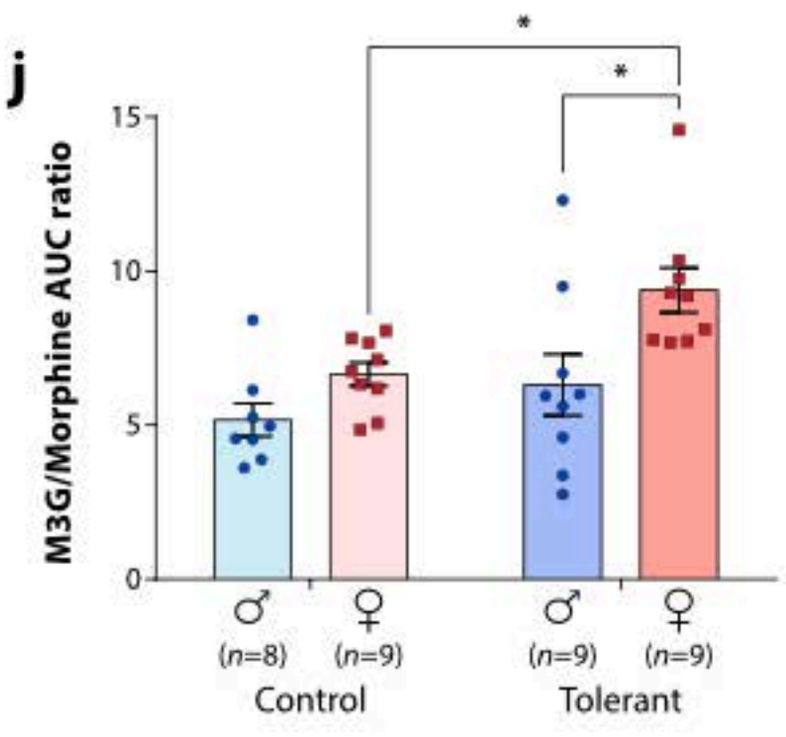

e
nmol/ml of blood
Time (min)
♂ Tolerant (n=9)
♀ Tolerant (n=10)

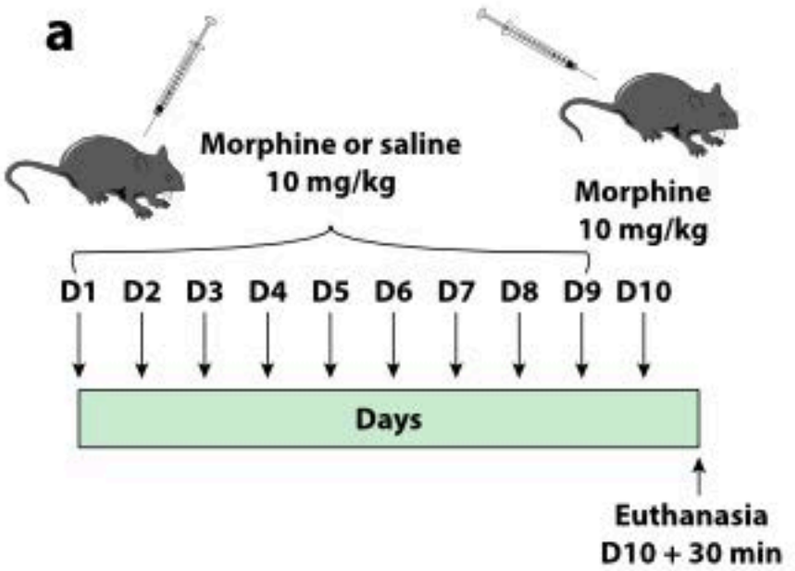

f
nmol/ml of blood
Time (min)
♂ Tolerant (n=9)
♀ Tolerant (n=10)


g
M3G/Morphine ratio
Time (min)
♂ Tolerant (n=9)
♀ Tolerant (n=10)

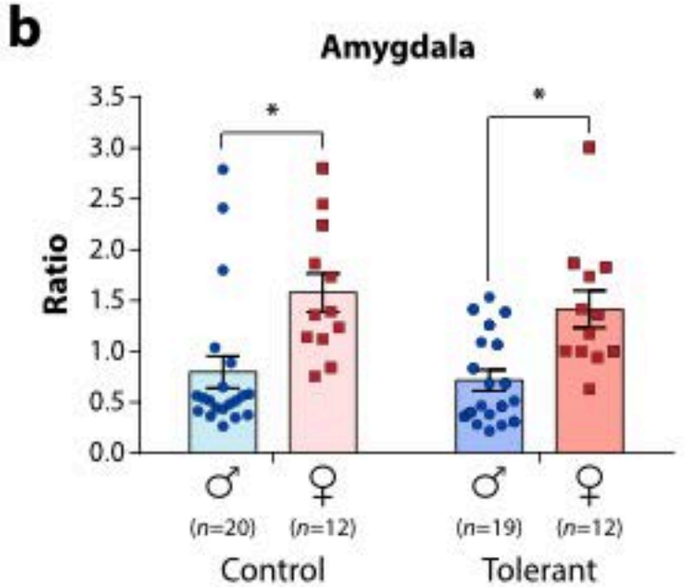

h
nmol of morphine/ml*min
*
(n=8) (n=9) (n=9) (n=10)
Control
Tolerant

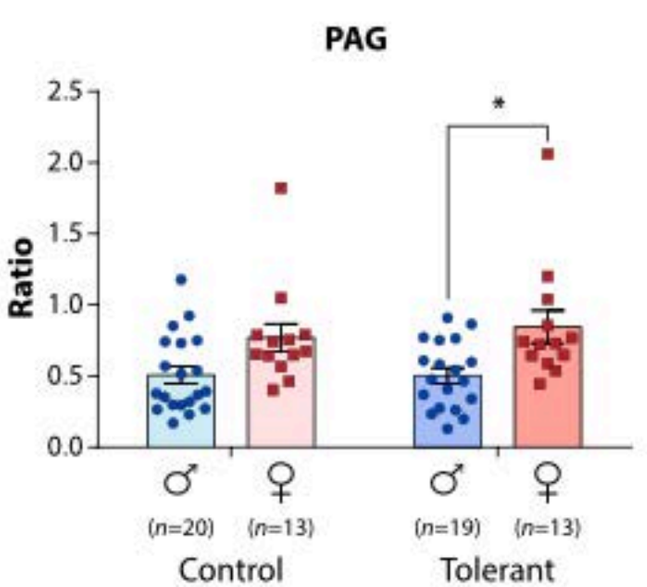

i
µmol of M3G/ml*min
*
(n=9) (n=9) (n=9) (n=9)
Control
Tolerant

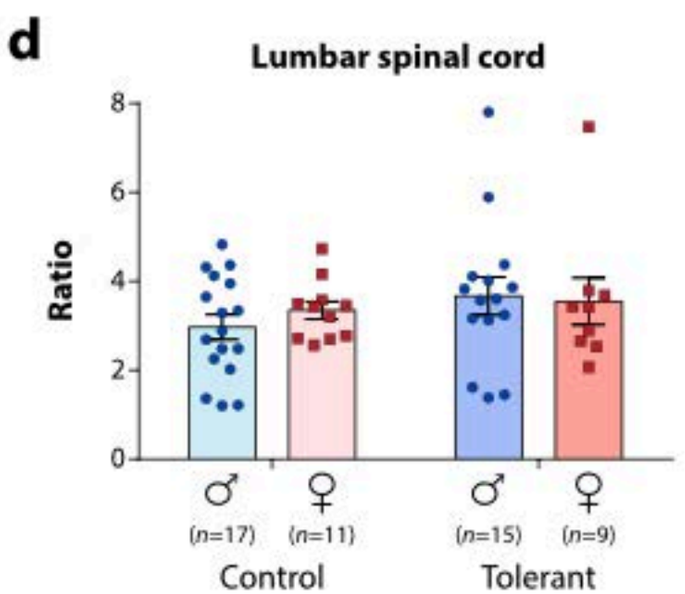

j
M3G/Morphine AUC ratio
* *
(n=8) (n=9) (n=9) (n=9)
Control
Tolerant

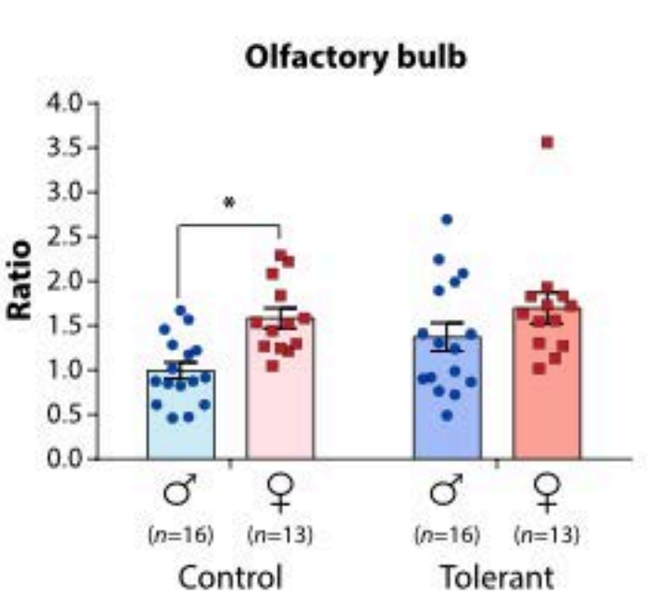


M3G/Morphine ratio









i.p. injection of morphine

i.p injection of M3G

Morphine brain/blood ratio

M3G brain/blood ratio

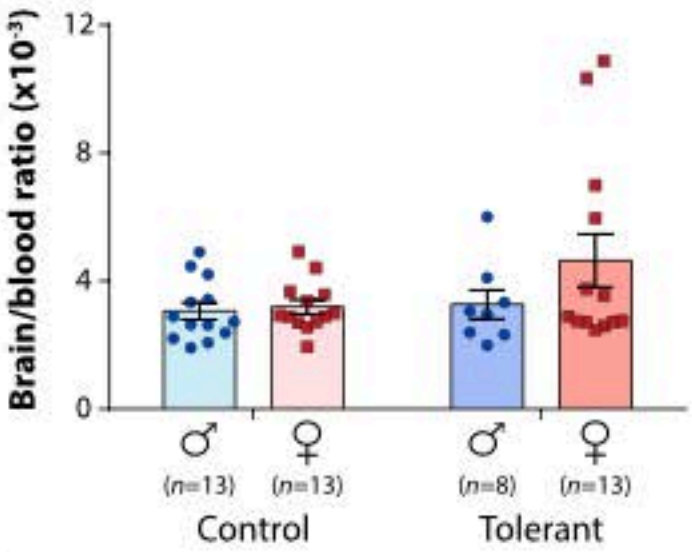
M3G brain/blood ratio

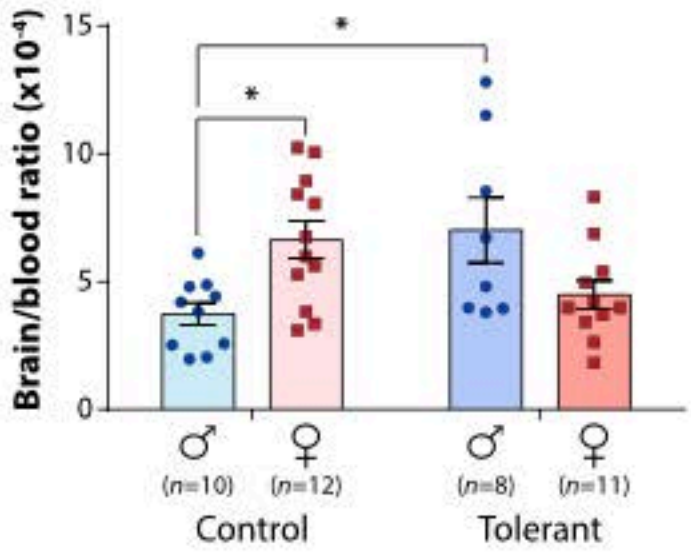
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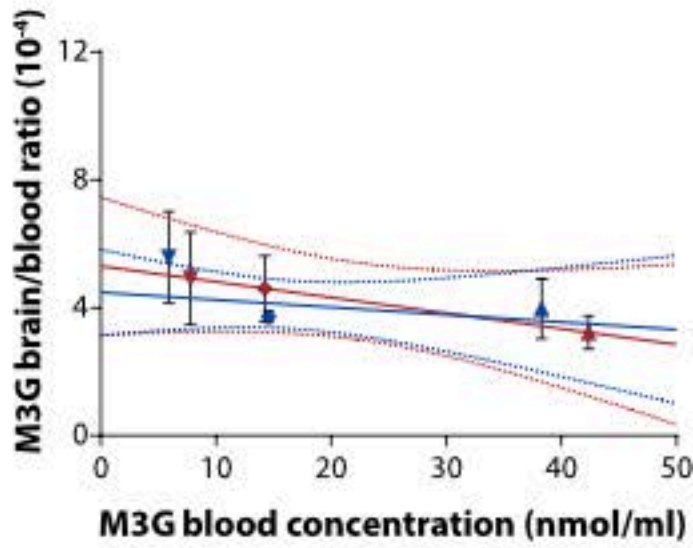
b

c

Amygdala





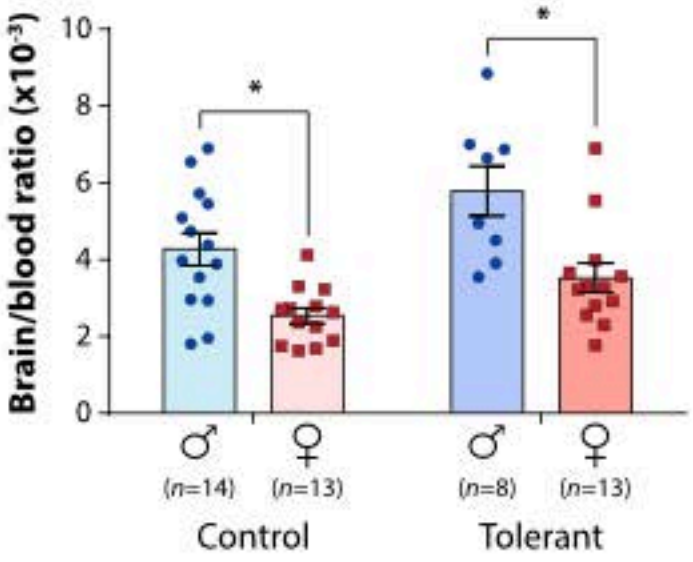


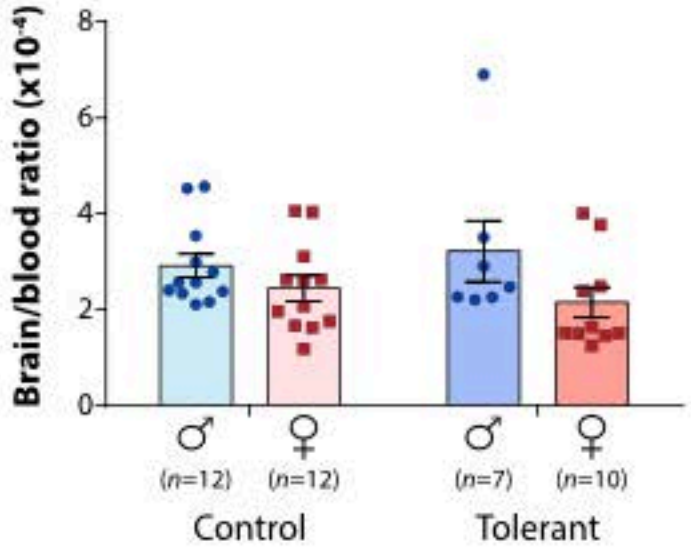
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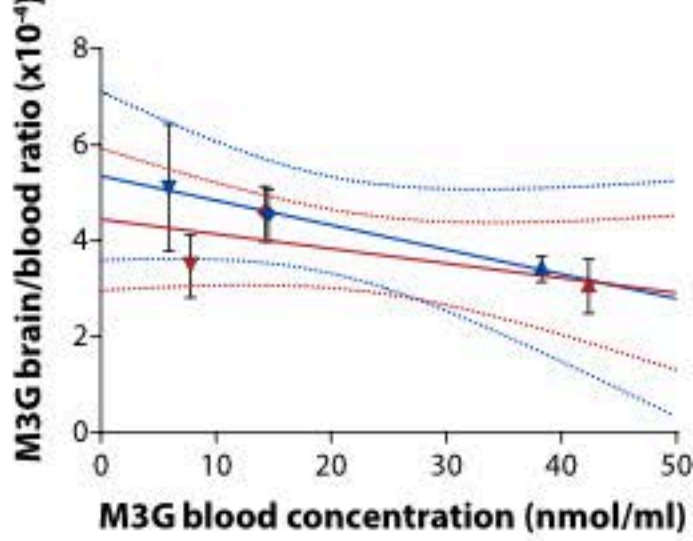
e

f

PAG





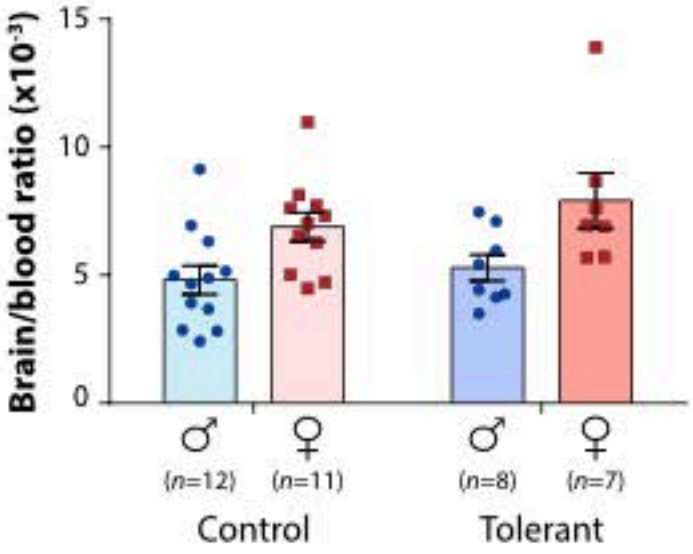


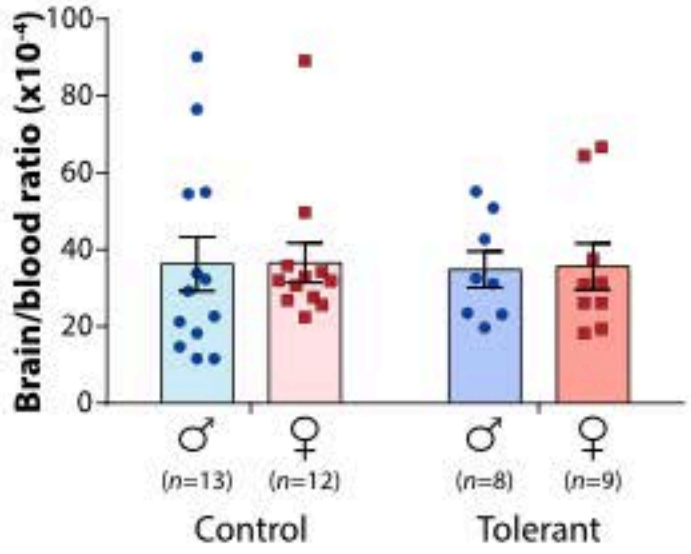
g

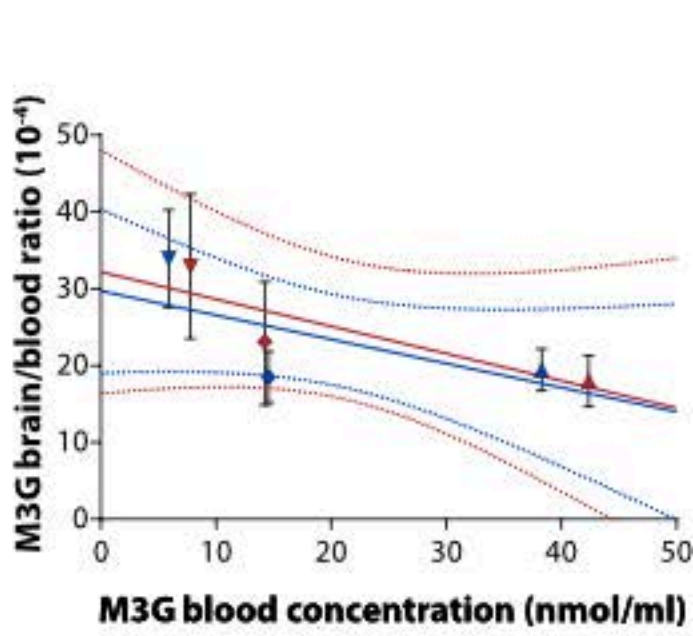
h

i

Lumbar spinal cord





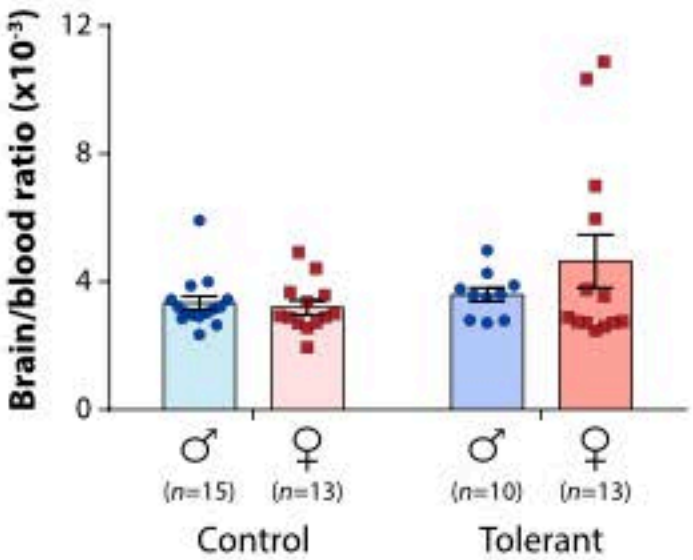


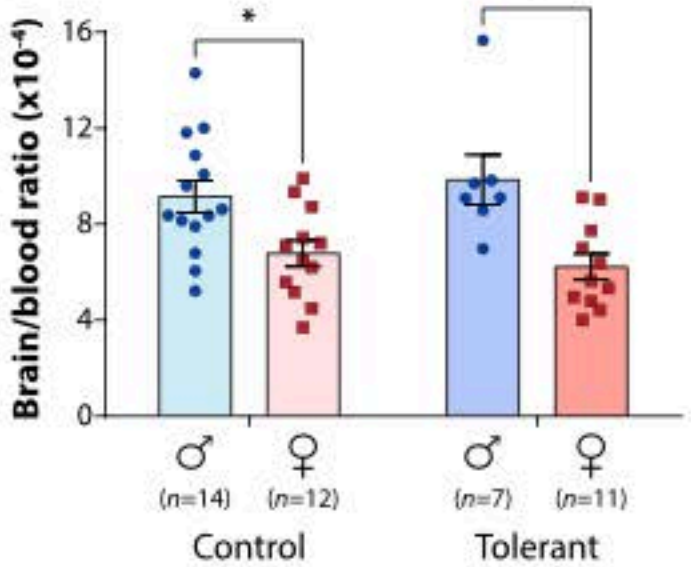
j

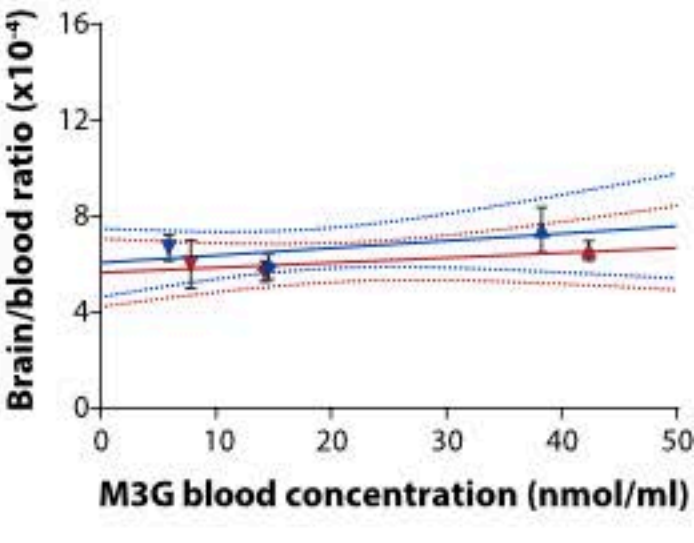
k

l

Olfactory bulb

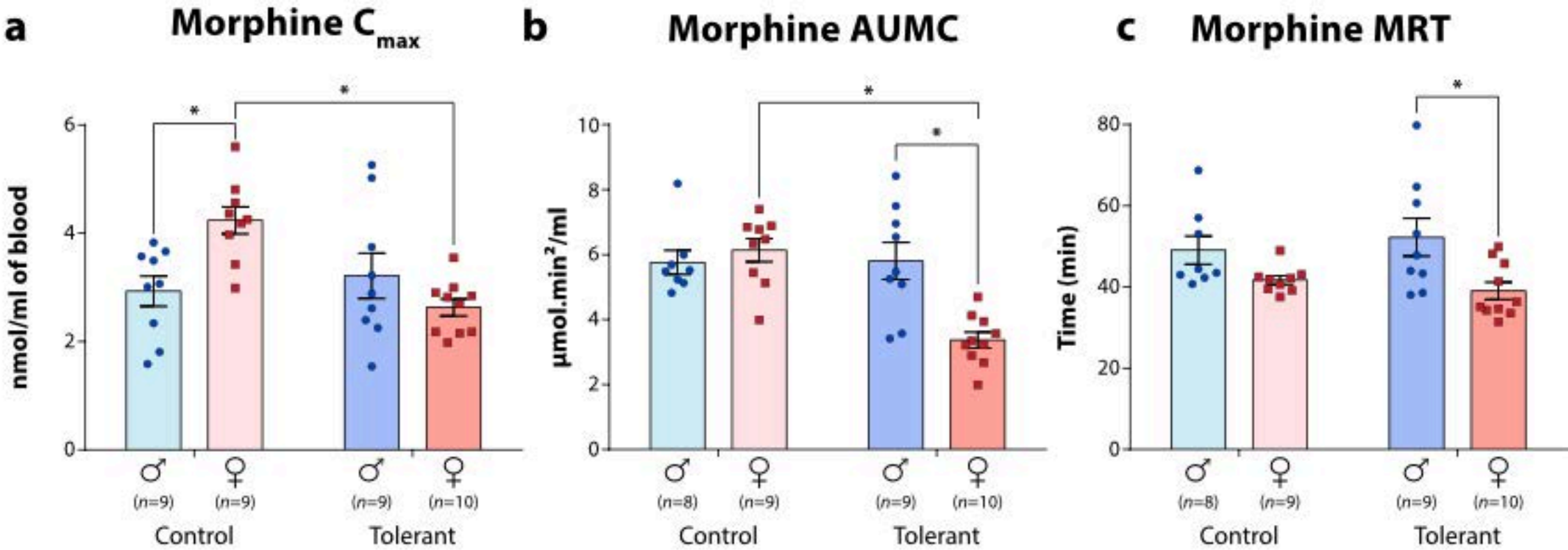


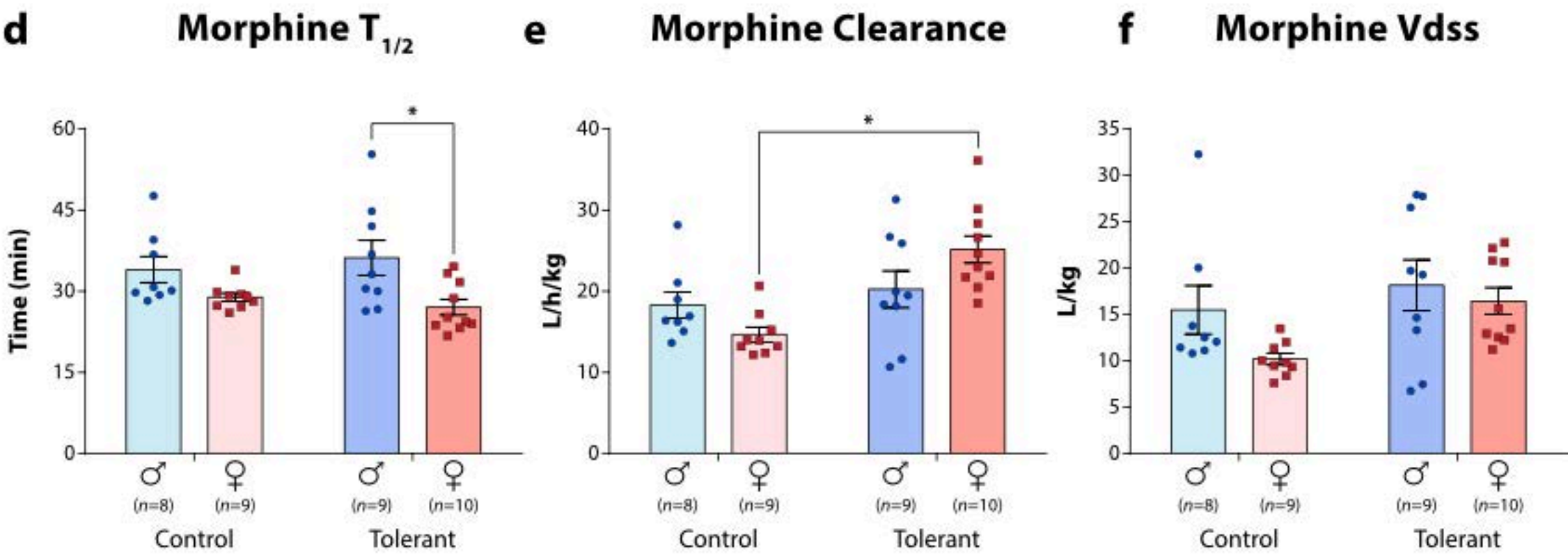


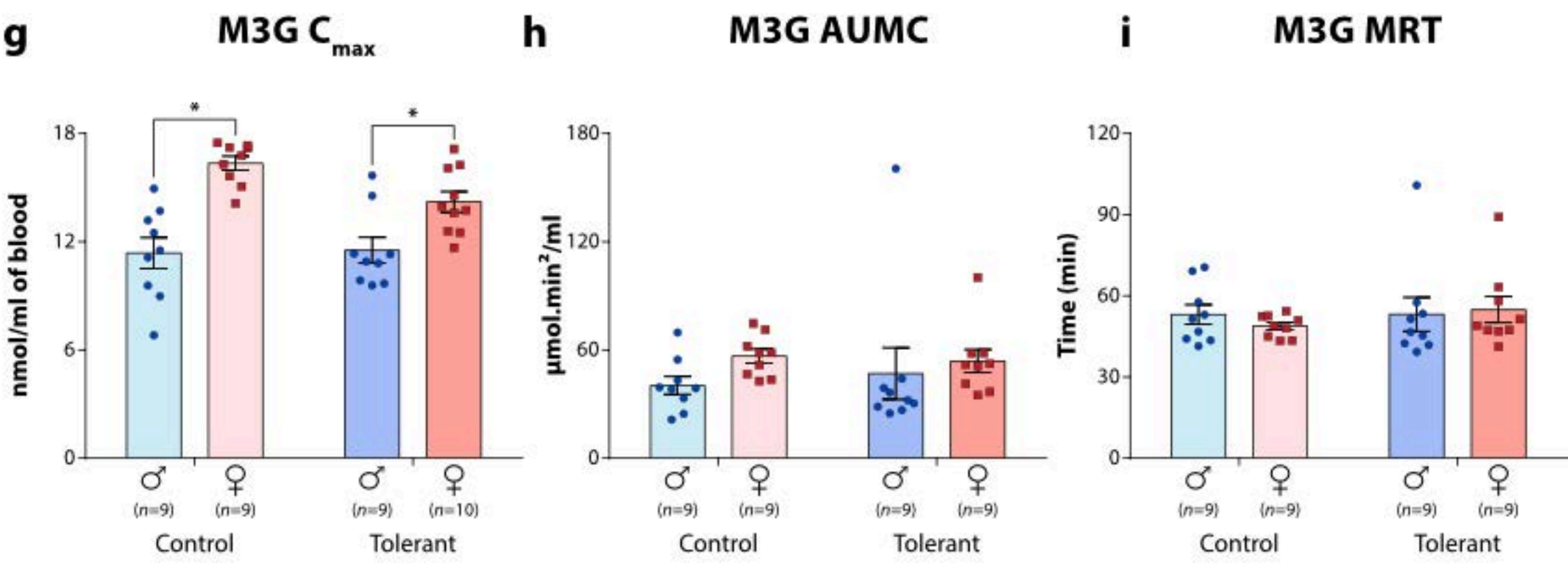


	Morphine			
	CT males	CT females	Tolerant males	Tolerant females
C_{max} (nmol/ml)$^{\$, £}$	**2.93 ± 0. 28** (*n*=9)	**4.24 ± 0.25^{a}** (*n*=9)	**3.22 ± 0.42** (*n*=9)	**2.63 ± 0.16^{d}** (*n*=10)
$AUC_{0\text{-}inf}$ (nmol.min/ml)$^{\$, £}$	**120.12 ± 8.7** (*n*=8)	**146.99 ± 7.7** (*n*=9)	**116.41 ± 14.9** (*n*=9)	**86.66 ± 52.7^{d}** (*n*=10)
$AUMC_{0\text{-}inf}$ c (µmol.min²/ml)$^{\#, \$, £}$	**5.76 ± 0.37** (*n*=8)	**6.14 ± 0.36** (*n*=9)	**5.80 ± 0.57** (*n*=9)	**3.37 ± 0.24$^{b, d}$** (*n*=10)
MRT (min)$^{\#}$	**49.10 ± 3.46** (*n*=8)	**41.72 ± 1.09** (*n*=9)	**52.21 ± 4.63** (*n*=9)	**39.06 ± 2.12^{b}** (*n*=10)
$T_{1/2}$ (min)$^{\#}$	**34.03 ± 2.40** (*n*=8)	**28.91 ± 0.75** (*n*=9)	**36.19 ± 3.21** (*n*=9)	**27.07 ± 1.47^{b}** (*n*=10)
Cl/F (L/h/kg)$^{\$, £}$	**18.31 ± 1.62** (*n*=8)	**14.68 ± 0.91** (*n*=9)	**20.25 ± 2.27** (*n*=9)	**25.16 ± 1.67^{d}** (*n*=10)
Vdss/F (L/kg)$^{\$}$	**15.51 ± 2.61** (*n*=8)	**10.18 ± 0.61** (*n*=9)	**18.16 ± 2.74** (*n*=9)	**16.44 ± 1.46** (*n*=10)
	M3G			
	CT males	CT females	Tolerant males	Tolerant females
C_{max} (nmol/ml)$^{\#}$	**11.38 ± 0.85** (*n*=9)	**16.35 ± 0.39^{a}** (*n*=9)	**11.52 ± 0.72** (*n*=9)	**14.21 ± 0.57^{b}** (*n*=10)
$AUC_{0\text{-}inf}$ (nmol.min/ml)$^{\#}$	**624.4 ± 50.3** (*n*=9)	**959.7 ± 42.9^{a}** (*n*=9)	**659.1 ± 85.8** (*n*=9)	**805.8 ± 32.2** (*n*=9)
AUMC0-inf (µmol.min²/ml)	**33.62 ± 4.1** (*n*=9)	**47.22 ± 3.2** (*n*=9)	**39.14 ± 12.0** (*n*=9)	**44.90 ± 5.4** (*n*=9)
MRT (min)	**53.12 ± 3.6** (*n*=9)	**48.85 ± 1.4** (*n*=9)	**53.22 ± 6.3** (*n*=9)	**54.88 ± 4.8** (*n*=9)

	Morphine (pmol/mg of protein)			
	Males CT	Females CT	Males tolerant	Females tolerant
Amygdala[#, $]	**9.70 ± 1.01** (*n*=20)	**7.94 ± 0.41** (*n*=12)	**7.65 ± 0.63** (*n*=19)	**5.10 ± 0.51** (*n*=12)
PAG[#]	**10.26 ± 0.75** (*n*=20)	**6.76 ± 0.54**[a] (*n*=13)	**9.43 ± 0.68** (*n*=20)	**5.28 ± 0.80**[b] (*n*=13)
LSC[#]	**12.68 ± 1.31** (*n*=17)	**18.20 ± 1.17** (*n*=11)	**11.51 ± 1.54** (*n*=15)	**16.71 ± 4.06** (*n*=9)
OB[#, $]	**13.12 ± 2.28** (*n*=16)	**8.26 ± 0.37** (*n*=13)	**8.55 ± 0.50** (*n*=16)	**6.48 ± 1.04**[b, d] (*n*=13)
	M3G (pmol/mg of protein)			
	Males CT	Females CT	Males tolerant	Females tolerant
Amygdala [#, $]	**6.56 ± 0.94** (*n*=20)	**12.23 ± 1.41**[a] (*n*=12)	**4.88 ± 0.62** (*n*=19)	**7.41 ± 1.37**[d] (*n*=12)
PAG	**4.89 ± 0.65** (*n*=20)	**5.29 ± 0.84** (*n*=13)	**4.34 ± 0.48** (*n*=19)	**4.64 ± 1.15** (*n*=13)
LSC[#]	**34.87 ± 5.63** (*n*=17)	**59.71 ± 3.49**[a] (*n*=11)	**36.76 ± 4.09** (*n*=15)	**49.59 ± 9.29** (*n*=9)
OB[#]	**15.64 ± 1.87** (*n*=16)	**13.04 ± 1.07** (*n*=13)	**13.67 ± 0.80** (*n*=16)	**9.59 ± 1.23** (*n*=13)


a
Morphine C_{max}
nmol/ml of blood
b
Morphine AUMC
µmol.min²/ml
c
Morphine MRT
Time (min)
(n=8) (n=9) (n=9) (n=10)
Control
Tolerant

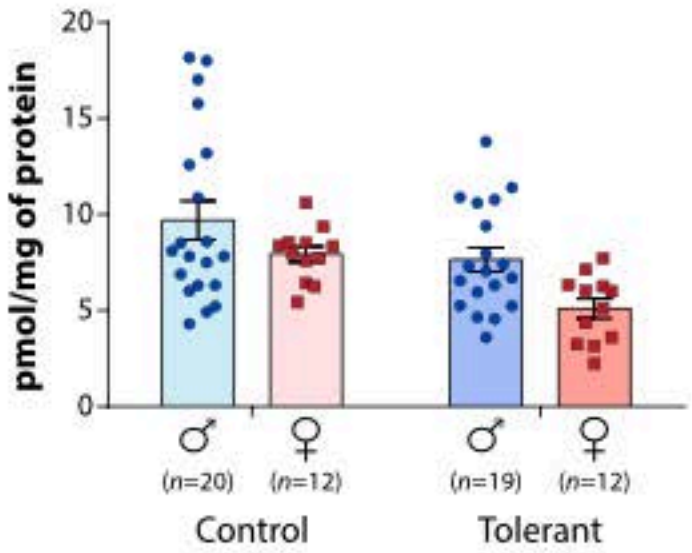

d
Morphine $T_{1/2}$
Time (min)
e
Morphine Clearance
L/h/kg
f
Morphine Vdss
L/kg
(n=8) (n=9) (n=9) (n=10)
Control
Tolerant

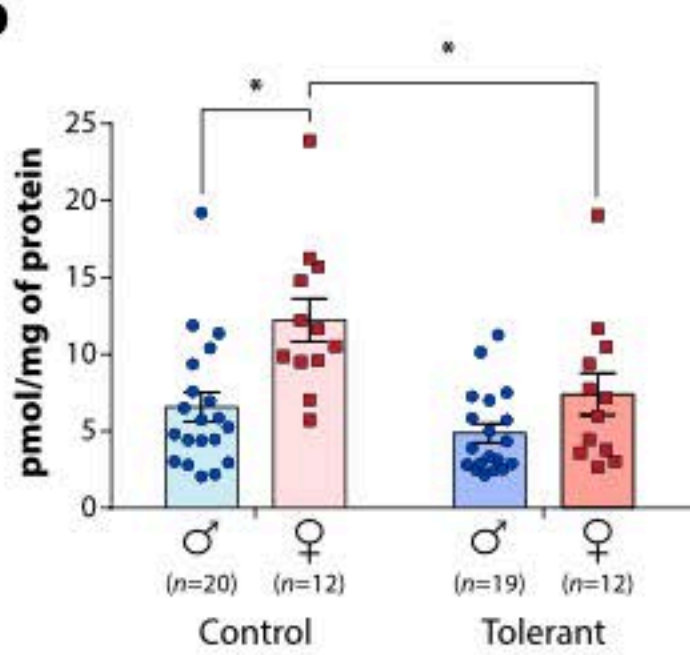

g
M3G C_{max}
nmol/ml of blood
h
M3G AUMC
µmol.min²/ml
i
M3G MRT
Time (min)
(n=9) (n=9) (n=9) (n=10)
Control
Tolerant

Morphine

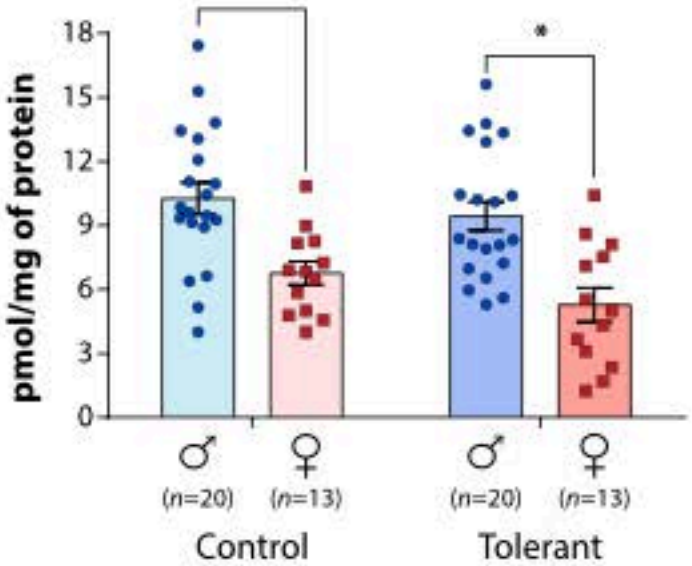
M3G

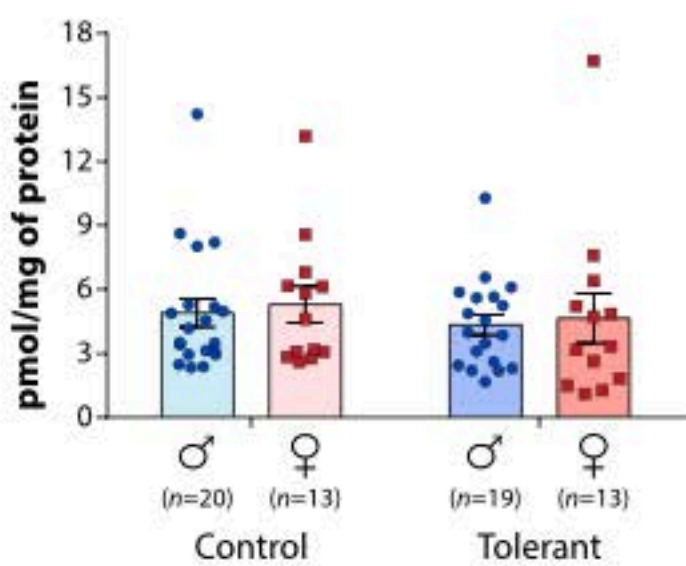
Amygdala



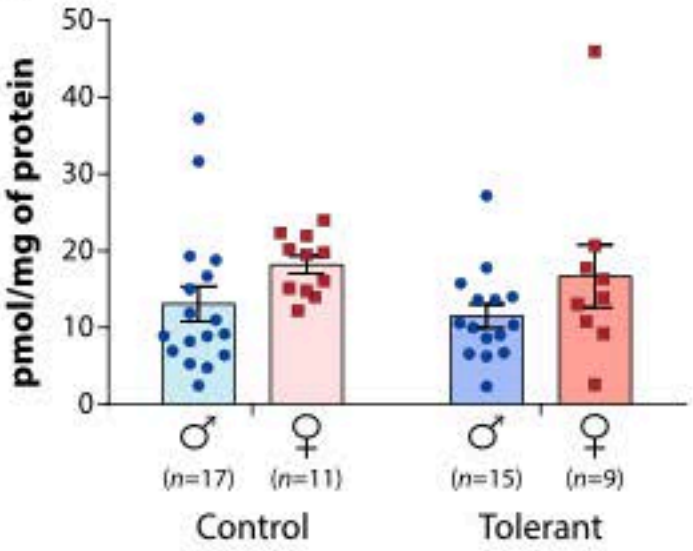


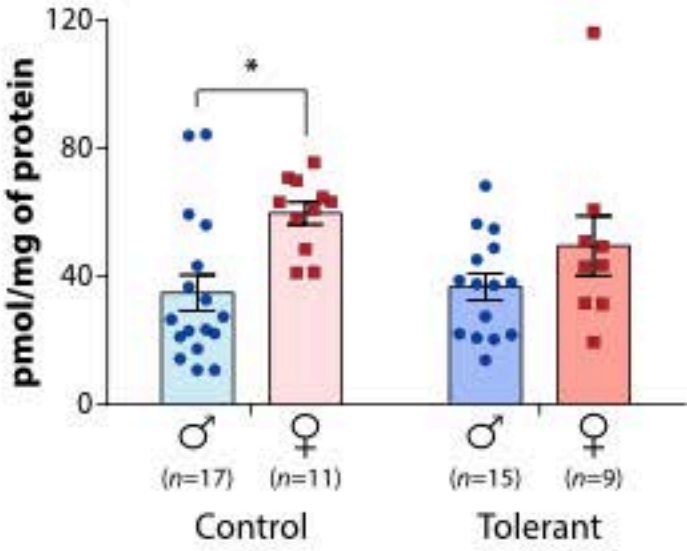
PAG



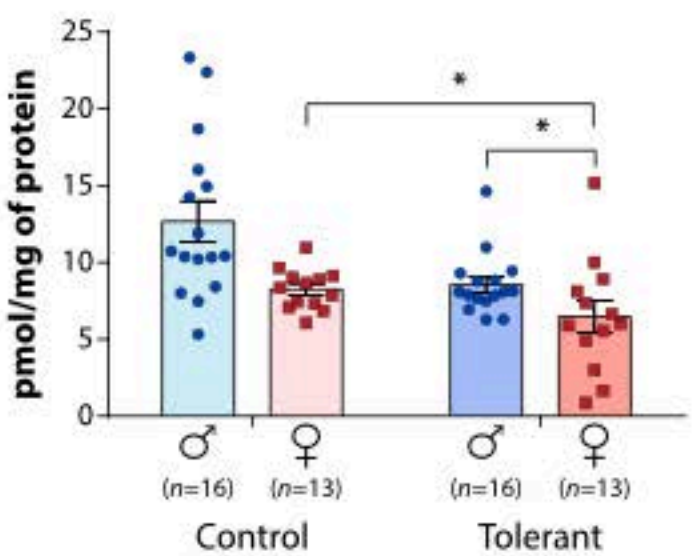


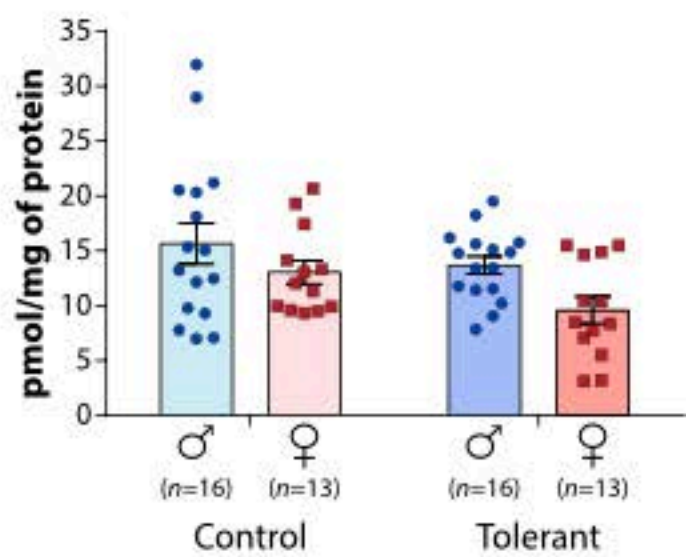
Lumbar spinal cord





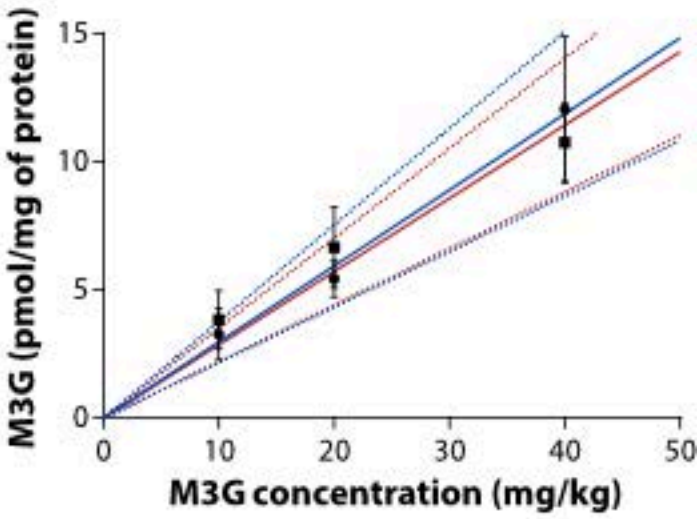
Olfactory bulb





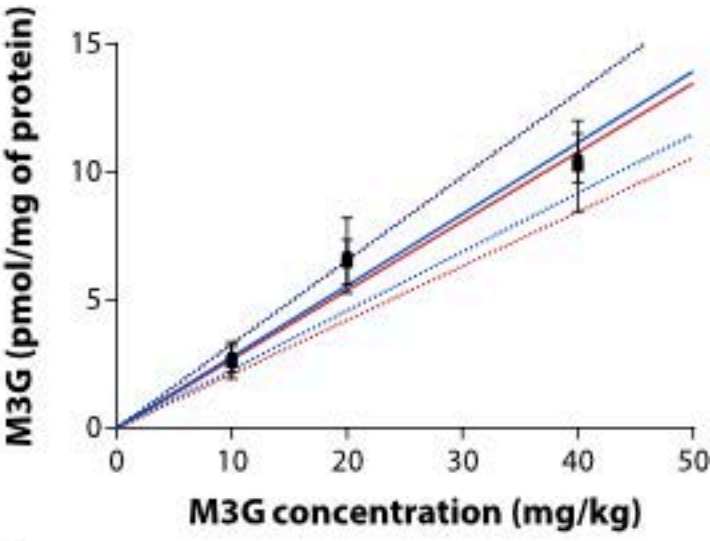
a

Amygdala



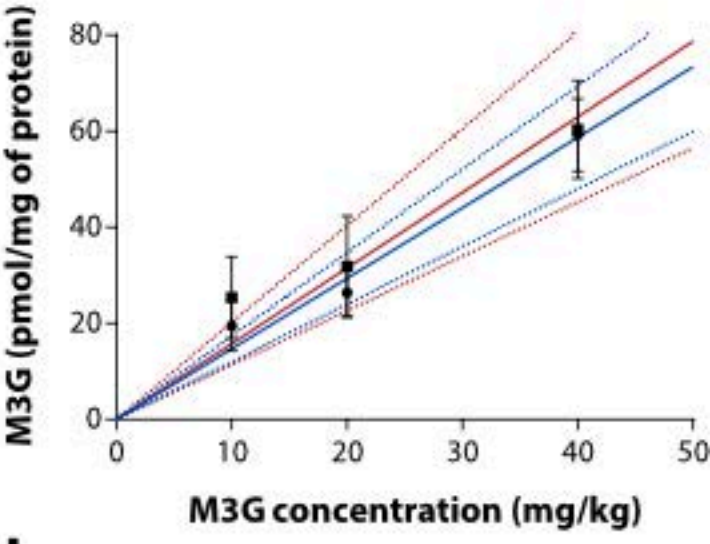
b

PAG



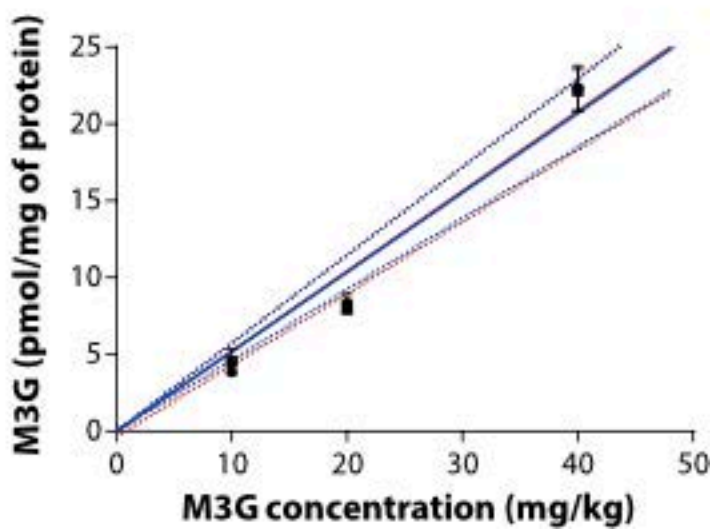
c

Lumbar spinal cord



d

Olfactory bulb



Supporting Information

Central metabolism as a potential origin of sex differences in morphine antinociception but not in the induction of antinociceptive tolerance in mice

Abbreviated title: Morphine central metabolism is potentially driving the sex differences observed in morphine antinociception.

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TABLES

Table S1. LC-MS/MS conditions. LC and MS/MS conditions for the purification, detection and quantification of morphine and M3G and their respective heavy-tagged counterparts. The flow rate was set at 90 µl/min on a ZORBAX SB-C18 column (150 x 1mm, 3.5µm).

Table S2. Statistical details for morphine antinociceptive effect and induction of tolerance (see Figure 1). Non-linear regression with a 4-parameters logistic equation was applied to define the ED_{50} of morphine and the 95% confidence intervals in both males and females. The two fits were compared using a nested-model comparison with the extra sum-of-the-squares F test.

For the tolerance experiment, the same analysis was applied to the data of each mouse. Then, the obtained parameters were averaged and compared with an unpaired t-test with Welch's correction. MPE, maximal possible effect.

Table S3. Statistical details for the pharmacokinetic parameters of morphine and M3G in the blood obtained from the NCA (see Figure 2). Two-way ANOVA was used to assess the differences between the pharmacokinetic parameters reported for each group. The non-parametric approach ART ANOVA (results highlighted in bold) was used for morphine volume of distribution at steady-state. C_{max}, maximal concentration reached over the time course; AUC, area under the curve; AUMC, area under the first moment curve; MRT, mean residence time; Cl/F, clearance over bioavailability; V_{dss}/F, volume of distribution at steady-state over bioavailability.

Table S4. Statistical details for the quantification of morphine and M3G in the brain and LSC (see Figure 3). Two-way ANOVA was used to assess the differences in morphine and M3G quantities between the groups. The non-parametric approach ART ANOVA (results highlighted in bold) was used for the M3G/Morphine ratio in the amgydala and morphine and M3G in the OB.

Table S5. Statistical details for morphine and M3G brain/blood ratio (see Figure 4). Two-way ANOVA was used to assess the differences in morphine and M3G brain/blood ratio between the groups. The non-parametric approach ART ANOVA (results highlighted in bold) was used for morphine and M3G brain/blood ratio in the PAG and for M3G brain/blood ratio in the LSC.

Table S6. Statistical details for M3G BBB permeability and central metabolism of morphine (see Figure 4). Linear regressions were applied and analysed through a nested-model comparison with the extra sum-of-squares F-test to compare the M3G BBB permeability between males and females, evaluate whether a significant morphine central metabolism is observed (*i.e.* comparison of the M3G brain/blood ratio fits obtained after an injection of morphine and M3G) and to compare this central metabolism between males and females (*i.e.* comparison of the M3G brain/blood ratio fits obtained after an injection of morphine in male and female mice).

FIGURES

Figure S1- Pharmacokinetic parameters for morphine and M3G obtained from the NCA. Values of parameters obtained for (**a**) Morphine C_{max}, (**b**) morphine AUMC, (**c**) morphine MRT, (**d**) morphine half-life, (**e**) morphine clearance, (**f**) morphine V_{dss}, (**g**) M3G C_{max}, (**h**) M3G AUMC and (**i**) M3G MRT. Data are expressed as means ± SEM, *n* are indicated in the figure. Two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. Morphine volume of distribution at steady-state was analysed with the non-parametric approach ART ANOVA. Tukey's multiple comparisons results are reported as *, $P<0.05$. Males are represented as blue circle dots and females as red square dots.

Figure S2- Quantities of morphine and M3G in the different brain areas and lumbar spinal cord of male and female control and tolerant mice. Levels of (**a**) morphine and (**b**) M3G in the amygdala. Levels of (**c**) morphine and (**d**) M3G in the PAG. Levels of (**e**) morphine and (**f**) M3G in the LSC. Levels of (**g**) morphine and (**h**) M3G in the OB. Data are expressed as means ± SEM, *n* are indicated in the figure. Two-way ANOVA was applied and followed by Tukey's multiple comparisons test only if F was significant and there was no variance homogeneity. Morphine and M3G in the OB were analysed with the non-parametric approach ART ANOVA. Tukey's multiple comparisons results are reported as *, $P<0.05$. Males are represented as blue circle dots and females as red square dots.

Figure S3- Quantities of M3G found in the different brain areas and lumbar spinal cord of male and female naïve mice following i.p. injection of increasing concentration of M3G. Levels of M3G found in (**a**) the amygdala following i.p. injection of 10, 20 and 40 mg/kg of M3G. $n = 4$ for the injection of 10mg/kg of M3G in males and females, $n = 5$ for the other conditions. Levels of M3G found in (**b**) the PAG following i.p. injection of 10, 20 and 40 mg/kg of M3G. $n = 3$ for the injection of 10mg/kg of M3G in females, $n = 5$ for the other conditions. Levels of M3G found in (**c**) the LSC following i.p. injection of 10, 20 and 40 mg/kg of M3G. $n = 4$ for the injection of 10mg/kg of M3G in males, $n = 5$ for the other conditions. Levels of M3G found in (**d**) the OB following i.p. injection of 10, 20 and 40 mg/kg of M3G. $n = 4$ for the injection of 40mg/kg of M3G in males, $n = 5$ for the other conditions. The blue and red lines represent linear regressions of the M3G quantities found in the brain area and LSC as a function of increasing concentration of M3G injected in males and females, respectively. Data are expressed as means ± SEM. Males are represented as circle dots and females as square dots.

Table S1.

	Mobile phase		
	ACN	**H_2O**	**Formic acid**
Mobile phase A	1%	98.9%	0.1%
Mobile phase B	99.9%	0	0.1%

HPLC gradient						
Time (min)	0	2.5	3.5	4.5	5.0	8
% B mobile phase	0	0	98	98	0	0

MS parameters	
Mode	positive
Spray voltage	3,500 V
Nebulizer gas	Nitrogen
Desolvation (nitrogen) sheath gas	18 Arb
Aux gas	7 Arb
Ion transfer tube temperature	297°C
Vaporizer temperature	131°C

Q1 and Q3 resolutions	0.7 FWHM
Collision gas (CID, argon) pressure	2 mTorr

MS ionization, selection, fragmentation and identification parameters

Compound	Polarity	Precursor (m/z)	Product (m/z)	Ion product type	Collision Energy (V)	RF Lens (V)
Morphine	Positive	285.98	201.11	Quantification	26.23	183
Morphine	Positive	285.98	165.36	Qualification	40.89	183
Morphine	Positive	285.98	181.06	Qualification	36.24	183
D3-morphine	Positive	289.1	201.06	Quantification	26.48	178
D3-morphine	Positive	289.1	153.13	Qualification	43.16	178
D3-morphine	Positive	289.1	165.04	Qualification	39.02	178
M3G	Positive	462.19	286.11	Quantification	30.02	276
D3-M3G	Positive	465.19	289.17	Quantification	29.92	242

Table S2

	Behavioural experiments		
	Dose-response curve experiment	**Tolerance experiment**	
Parameters	**ED_{50}**	**Time at which half %MPE**	**Hill-slope coefficient**
Test	Extra-sum of the Squares F test	Unpaired t-test with Welch's correction	Unpaired t-test with Welch's correction
F, t, df	F (2, 55) = 50.96	t=15.40 df=27.33	t=1.419 df=25.31
p-value	< 0.05*	< 0.05*	0.1680

Table S3.

	Peripheral metabolism		
	Cmax		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	F (1, 33) = 10.99 p < 0.05*	F (1, 33) = 3.07 p > 0.05	/
Treatment	F (1, 33) = 5.37 p < 0.05*	F (1, 33) = 2.33 p > 0.05	/
Sex	F (1, 33) = 1.60 p > 0.05	F (1, 33) = 34.42 p < 0.05*	/
	AUC		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	F (1, 32) = 8.54 p < 0.05*	F (1, 32) = 2.79 p > 0.05	F (1, 31) = 1.26 p > 0.05
Treatment	F (1, 32) = 10.92 p < 0.05*	F (1, 32) = 1.11 p > 0.05	F (1, 31) = 7.44 p < 0.05*
Sex	F (1, 32) = 0.022 p > 0.05	F (1, 32) = 18.20 p < 0.05*	F (1, 31) = 10.31 p < 0.05*
	AUMC		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	F (1, 32) = 12.44 p < 0.05*	F (1, 32) = 0.31 p > 0.05	/
Treatment	F (1, 32) = 11.71 p < 0.05*	F (1, 32) = 0.052 p > 0.05	/
Sex	F (1, 32) = 6.59 p < 0.05*	F (1, 32) = 1.88 p > 0.05	/
	MRT		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	F (1, 32) = 0.88 p > 0.05	F (1, 32) = 0.45 p > 0.05	/
Treatment	F (1, 32) = 0.0055 p > 0.05	F (1, 32) = 0.49 p > 0.05	/
Sex	F (1, 32) = 11.16 p < 0.05*	F (1, 32) = 0.088 p > 0.05	/
	$T_{1/2}$		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	F (1, 32) = 0.88 p > 0.05	/	/
Treatment	F (1, 32) = 0.0055 p > 0.05	/	/
Sex	F (1, 32) = 11.16 p < 0.05*	/	/

	Cl/F		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	F (1, 32) = 6.31 p < 0.05*	/	/
Treatment	F (1, 32) = 13.34 p < 0.05*	/	/
Sex	F (1, 32) = 0.14 p > 0.05	/	/
	Vdss/F		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	**F (1, 31) = 0.12 p > 0.05**	/	/
Treatment	**F (1, 32) = 7.275 p < 0.05***	/	/
Sex	**F (1, 32) = 2.185 p > 0.05**	/	/

Table S4.

	CNS regions quantification		
	Amygdala		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	F (1, 59) = 0.2349 p > 0.05	F (1, 59) = 2.210 p > 0.05	**F (1, 59) = 0.82** **p > 0.05**
Treatment	F (1, 59) = 9.01 p < 0.05*	F (1, 59) = 9.49 p < 0.05*	**F (1, 59) = 0.41** **p > 0.05**
Sex	F (1, 59) = 7.04 p < 0.05*	F (1, 59) = 15.12 p < 0.05*	**F (1, 59) = 29.84** **p < 0.05***
	PAG		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	F (1, 62) = 0.19 p > 0.05	F (1, 61) = 0.0049 p > 0.05	F (1, 61) = 0.26 p > 0.05
Treatment	F (1, 62) = 2.46 p > 0.05	F (1, 61) = 0.62 p > 0.05	F (1, 61) = 0.20 p > 0.05
Sex	F (1, 62) = 26.86 p < 0.05*	F (1, 61) = 0.20 p > 0.05	F (1, 61) = 14.4 p < 0.05*
	LSC		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	F (1, 48) = 0.0005 p > 0.05	F (1, 48) = 1.08 p > 0.05	F (1, 48) = 0.44 p > 0.05
Treatment	F (1, 48) = 0.43 p > 0.05	F (1, 48) = 0.50 p > 0.05	F (1, 48) = 1.44 p > 0.05
Sex	F (1, 48) = 4.76 p < 0.05*	F (1, 48) = 10.57 p < 0.05*	F (1, 48) = 0.12 p > 0.05
	OB		
	Morphine	**M3G**	**M3G/Morphine**
Interaction	**F (1, 54) = 0.54** **p > 0.05**	**F (1, 54) = 0.91** **p > 0.05**	F (1, 54) = 0.9002 p > 0.05
Treatment	**F (1, 54) = 16,64** **p < 0.05***	**F (1, 54) = 3.13** **p > 0.05**	F (1, 54) = 3.155 p > 0.05
Sex	**F (1, 54) = 20.69** **p < 0.05***	**F (1, 54) = 5.85** **p < 0.05***	F (1, 54) = 10.97 p < 0.05*

Table S5.

	Brain/blood ratio	
	Amygdala	
	Morphine	**M3G**
Interaction	F (1, 43) = 1.35 p > 0.05	F (1, 37) = 12.88 p < 0.05*
Treatment	F (1, 43) = 2.43 p > 0.05	F (1, 37) = 0.56 p > 0.05
Sex	F (1, 43) = 2.010 p > 0.05	F (1, 37) = 0.059 p > 0.05
	PAG	
	Morphine	**M3G**
Interaction	**F (1, 44) = 0.40 p > 0.05**	**F (1, 37) = 0.98 p > 0.05**
Treatment	**F (1, 44) = 3.795 p > 0.05**	**F (1, 37) = 1.63 p > 0.05**
Sex	**F (1, 44) = 18.74 p < 0.05***	**F (1, 37) = 5.27 p < 0.05***
	LSC	
	Morphine	**M3G**
Interaction	F (1, 34) = 0.1681 p > 0.05	**F (1, 38) = 0.34 p > 0.05**
Treatment	F (1, 34) = 1.25 p > 0.05	**F (1, 38) = 0.01 p > 0.05**
Sex	F (1, 34) = 12.27 p < 0.05*	**F (1, 38) = 0.06 p > 0.05**
	OB	
	Morphine	**M3G**
Interaction	F (1, 47) = 1.62 p > 0.05	F (1, 40) = 0.82 p > 0.05
Treatment	F (1, 47) = 3.31 p > 0.05	F (1, 40) = 0.010 p > 0.05
Sex	F (1, 47) = 0.97 p > 0.05	F (1, 40) = 18.80 p < 0.05*

Table S6.

	BBB and central metabolism of morphine		
	BBB permeability	**Morphine central metabolism : Injection M3G vs Morphine**	**Sex differences in morphine central metabolism**
	Amygdala		
Males	/	F (1, 22) = 1.61 p > 0.05	/
Females	/	F (1, 24) = 6.74 p < 0.05*	/
M vs F	F (2, 24) = 0.046 p > 0.05	/	F (1, 20) = 12.84 p < 0.05*
	PAG		
Males	/	F (1, 25) = 11.53 p < 0.05*	/
Females	/	F (1, 23) = 10.25 p < 0.05*	/
M vs F	F (2, 24) = 0.41 p > 0.05	/	F (1, 22) = 0.052 p > 0.05
	LSC		
Males	/	F (1, 25) = 1.88 p > 0.05	/
Females	/	F (1, 25) = 3.02 p > 0.05	/
M vs F	F (2, 25) = 0.059 p > 0.05	/	F (1, 23) = 0.12 p > 0.05
	OB		
Males	/	F (2, 24) = 6.27 p < 0.05*	/
Females	/	F (2, 23) = 2.79 p > 0.05	/
M vs F	F (2, 25) = 0.66 p > 0.05	/	F (1, 24) = 8.52 p < 0.05*