



Administration of the benzodiazepine midazolam increases tau phosphorylation in the mouse brain



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ABSTRACT

Preclinical studies have shown that anesthesia might accelerate the clinical progression of Alzheimer's disease (AD) and can have an impact on tau pathology, a hallmark of AD. Although benzodiazepines have been suggested to increase the risk of incident dementia, their impact on tau pathology *in vivo* is unknown. We thus examined the impact of midazolam, a benzodiazepine that is often administered perioperatively as an anxiolytic, on tau hyperphosphorylation in nontransgenic and in hTau mice, the latter a model of AD-like tau pathology. The acute administration of midazolam in C57BL/6 mice was associated with downregulation of protein phosphatase-1 and a significant and persistent increase in brain tau phosphorylation. In hTau mice, tau hyperphosphorylation was also observed; however, midazolam was neither associated with proaggregant changes nor spatial reference memory impairment. In C57BL/6 mice, chronic midazolam administration immediately increased hippocampal tau phosphorylation, and this effect was more pronounced in older mice. Interestingly, in young C57BL/6 mice, chronic midazolam administration induced hippocampal tau hyperphosphorylation, which persisted for 1 week. In hTau mice, chronic midazolam administration increased hippocampal tau phosphorylation and, although this was not associated with proaggregant changes, this correlated with a decreased capacity of tau to bind to preassembled microtubules. These findings suggest that midazolam can induce significant tau hyperphosphorylation *in vivo*, which persists well beyond recovery from its sedative effects. Moreover, it can disrupt one of tau's critical functions. Hence, future studies should focus on the impact of more prolonged or repeated benzodiazepine exposure on tau pathology and cognitive decline.

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1. Introduction

The two major neuropathological hallmarks of Alzheimer's disease (AD) are extracellular senile plaques comprised of aggregates of β -amyloid protein and intraneuronal neurofibrillary tangles (NFTs) composed of aggregated, hyperphosphorylated tau protein. Tau is a microtubule (MT)-associated protein, abundantly present in neuronal axons (Binder et al., 1985), whose main function is to bind to MTs and promote MT assembly and stabilization (Drechsel et al., 1992; Drubin et al., 1985, 1988; Weingarten et al., 1975). In AD and other tauopathies, tau can become aberrantly hyperphosphorylated, leading to its detachment from MTs,

subsequent aggregation, and ultimately the progression of NFT pathology (Brion et al., 1985; Grundke-Iqbal et al., 1986a,b; Kosik et al., 1986; Wood et al., 1986).

Previous studies have demonstrated that anesthetics can profoundly increase tau phosphorylation by inducing hypothermia-mediated inhibition of protein phosphatase 2A (PP2A) (Planel et al., 2007). However, more recent studies have shown that certain inhalational anesthetics and intravenous sedative agents, including sevoflurane (Le Freche et al., 2012), dexmedetomidine (Whittington et al., 2015), and propofol (Whittington et al., 2011) can directly increase tau phosphorylation even in the absence of hypothermia.

Benzodiazepines are commonly used anesthetic adjuncts that are administered clinically to produce sedative-hypnotic and anxiolytic effects. However, the clinical use of benzodiazepines has been associated with cognitive side effects (Acil et al., 2004; Buffett-Jerrott and Stewart, 2002; Ghoneim et al., 1984a,b;

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Santana Santos et al., 2005) and, more recently, an increased risk of incident dementia in the elderly (Billioti de Gage et al., 2012, 2014). Despite this, there is a dearth of information related to the direct impact of benzodiazepines on tau hyperphosphorylation in vivo. Given that benzodiazepines mediate their effects through the allosteric potentiation of GABAergic neurotransmission and that other sedatives that activate this receptor subtype have been shown to increase tau phosphorylation (Le Freche et al., 2012; Whittington et al., 2011), we hypothesized that this class of sedative agents induces tau phosphorylation, accelerates tau aggregation, and disrupts the capacity of tau to bind to MTs. Midazolam is a water-soluble benzodiazepine that is often administered perioperatively as an anxiolytic and on a prolonged basis for long-term sedation (Rhoney and Murry, 2003) in patients requiring mechanical ventilatory support. To test the aforementioned benzodiazepine-tau pathology hypothesis, we examined the impact of midazolam on tau phosphorylation, aggregation, and function in both non-transgenic and transgenic mice.

We observed that the acute administration of midazolam in nontransgenic mice, under normothermic conditions, resulted in PP1 downregulation and produced tau hyperphosphorylation in the brain that persisted up to 24 h in the cortex of nontransgenic mice. In hTau transgenic mice solely expressing nonmutant human tau, midazolam-induced tau hyperphosphorylation in a slightly different pattern than that observed in nontransgenic mice; however, this hyperphosphorylation was neither associated with a change in soluble tau levels in the cortex nor a downstream change in spatial reference memory. Chronic administration of midazolam-induced tau hyperphosphorylation in the brain of nontransgenic mice, and this effect increased as a function of age. Moreover, this midazolam-induced tau hyperphosphorylation was observed to persist for at least 1 week. Although chronic midazolam did not have pro-aggregant effects on tau in hTau mice, the capacity of tau to bind to MTs was diminished.

2. Materials and methods

2.1. Anesthetics and chemical reagents

Midazolam hydrochloride (Lipomed Inc, Cambridge, MA) was purchased from Lipomed Inc, (Cambridge, MA). All chemicals used in the preparation of mouse brain protein samples were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of the protease inhibitors (Cocktail set III, Calbiochem, EMD Biosciences Inc, La Jolla, CA) and the bicinchoninate protein assay reagents (Thermo Fisher Scientific Inc, Waltham, MA).

2.2. Antibodies

Tau phosphorylation levels were determined using antibodies directed at tau phosphorylated at the following epitopes: AT8 (pSer²⁰²/pThr²⁰⁵), CP13 (pSer²⁰²), PHF-1 (pSer³⁹⁶/pSer⁴⁰⁴), pSer²⁶², AT180 (pThr²³¹), AT270 (pThr¹⁸¹), 12E8 (pSer²⁶²/pSer³⁵⁶), and pSer¹⁹⁹. Total tau levels were measured with the monoclonal antibodies Tau46, TG5, or Tau A0024. These particular tau antibodies were selected, as the phosphoepitopes they recognize have been associated with pretangle formation (CP13), paired helical filament, and NFT formation (AT8, PHF-1) (Augustinack et al., 2002; Goedert, 1993; Goedert et al., 1994) as well as the MT binding domain (pSer²⁶², 12E8) (Buee et al., 2000). Hence, they were indeed suitable to provide a more comprehensive examination of the effects of midazolam on tau phosphorylation.

Furthermore, to dissect the mechanism underlying the tau phosphorylation changes following midazolam administration,

the expression and activation of several major tau kinases were examined using the following antibodies purchased from Cell Signaling Technology, Inc (Danvers, MA): CaMKII, phospho-CaMKII (Thr²⁸⁶), GSK-3 β , phospho-GSK-3 β (Ser⁹), SAPK/JNK, phospho-SAPK-JNK (Thr¹⁸³/Tyr¹⁸⁵), p44/42 MAPK (ERK 1/2), phospho-p44/42 MAPK (Thr²⁰²/Tyr²⁰⁴), CDK5, and P35/P25. The changes in the expression of the tau phosphatases were determined using antibodies directed at the catalytic subunit of PP2A-C (Sigma-Aldrich, St. Louis, Mo) and the demethylated PP2A-C subunit (Cell Signaling), PP1, PP2B, and PP5. Each immunoblot was normalized for gel loading with β -actin or β -tubulin depending on the molecular weight of the protein of interest. A detailed summary of all of the antibodies used in these studies is shown in Table 1.

2.3. Mice treatment midazolam administration protocols for tau phosphorylation studies

The Columbia University Animal Care and Use Committee approved the experimental protocol, and appropriate measures were taken to minimize pain and discomfort as per National Institutes of Health guidelines. Nontransgenic 8- to 10-week-old C57BL/6 male mice were purchased from a commercial vendor (Taconic, Germantown, NY). Three-month-old male hTau mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were aged accordingly in the Columbia University animal care facility. hTau mice, a model of AD-like tauopathy, express nonmutant human tau on a murine tau knock-out background and develop age-related increases in tau hyperphosphorylation and aggregation (Andorfer et al., 2003). All mice were housed at 22 °C in a temperature-controlled room and kept on a 12-h alternating light/dark cycle. Food and water were made available *ad libitum*, and all mice underwent an acclimatization period of at least 24 h before being used in the experiments.

In experiments examining whether midazolam had a dose response-related effect on tau phosphorylation, C57BL/6 mice were treated with either midazolam 10 mg/kg, 25 mg/kg, or an equivalent volume of 0.9% saline (control [Ctl]; 100 μ L per 10 g of body weight) via intraperitoneal (i.p.) injection. These doses of midazolam were based on a previous study demonstrating antinociception without any reported adverse effects (Chiba et al., 2009). In studies examining remote tau phosphorylation following acute midazolam administration, the mice were treated with either midazolam 25 mg/kg or 0.9% saline (100 μ L per 10 g body weight) i.p. and sacrificed 30 minutes, 6 h, or 24 h after treatment.

In the chronic midazolam administration studies, midazolam or 0.9% saline was administered subcutaneously using an ALZET 2001D osmotic pump (DURECT, Cupertino, CA), which was implanted in the interscapular region under 1%–2% isoflurane anesthesia (~5–10 minutes total anesthesia exposure) in 30% O₂. The pumps delivered the midazolam solution or saline at a rate of 8 μ L/h. The mice received preemptive analgesia with carprofen 5 mg/kg s.c. and, at the end of surgery, bupivacaine hydrochloride (2 mg/kg max) was applied to the surgical site by local subcutaneous infiltration. Midazolam was subcutaneously administered at a rate of 3 or 5 mg/kg/h.

On induction of sedation, normothermia was maintained in the midazolam-treated mice by transferring the mice to a water-jacketed incubator unit (Thermocare, Incline Village, NV), which was set to a target temperature of ~37 °C throughout the study. The Ctl mice were returned to their home cage at room temperature after receiving their treatment injections. Rectal temperature was monitored using an electronic thermometer (Thermalert TH-5, Physitemp, Clifton, NJ) to confirm normothermia.

Table 1
List of antibodies used

| Antibody | Phospho-epitope | Type | Source | Manufacturer | Location | Catalog # | WB dilution |
|------------------------------|-------------------|------------|--------|--|----------------------------|-----------|-------------|
| Tau | | | | | | | |
| Tau46 | Total Tau | Monoclonal | Mouse | Cell Signaling | Danvers, MA | 4019 | 1:2500 |
| Anti-Human Tau A0024 | Total Tau 243–441 | Polyclonal | Rabbit | DakoCytomation | Carpinteria, CA | A002401-2 | 1:10,000 |
| TG5 | Total Tau 220–240 | Monoclonal | Mouse | Peter Davies | Bronx, NY | N/A | 1:1000 |
| AT8 | pSer202/pThr205 | Monoclonal | Mouse | Thermo Scientific | Waltham, MA | MN1020 | 1:10,000 |
| CP13 | pSer202 | Monoclonal | Mouse | Peter Davies | Bronx, NY | N/A | 1:1000 |
| PHF-1 | pSer396/pSer404 | Monoclonal | Mouse | Peter Davies | Bronx, NY | N/A | 1:1000 |
| AT180 | pThr231 | Monoclonal | Mouse | Thermo Scientific | Waltham, MA | MN1040 | 1:1000 |
| PS422 | pSer422 | Polyclonal | Rabbit | Life Technologies | Carlsbad, CA | 44-764G | 1:1000 |
| PS262 | pSer262 | Polyclonal | Rabbit | Life Technologies | Carlsbad, CA | 44-750G | 1:1000 |
| PS199 | pSer199 | Polyclonal | Rabbit | Life Technologies | Carlsbad, CA | 44-734G | 1:1000 |
| AT270 | pThr181 | Monoclonal | Mouse | Thermo Scientific | Waltham, MA | MN1050 | 1:1000 |
| 12 E8 | pSer262/pSer356 | Monoclonal | Mouse | Peter Seubert, Elan Pharmaceuticals | South San Francisco, CA | N/A | 1:1000 |
| Kinases | | | | | | | |
| GSK-3 β | | Monoclonal | Rabbit | Cell Signaling | Danvers, MA | 9315 | 1:1000 |
| Phospho-GSK-3 β (Ser9) | Ser9 | Polyclonal | Rabbit | Cell Signaling | Danvers, MA | 9336 | 1:1000 |
| SAPK/JNK | | Monoclonal | Rabbit | Cell Signaling | Danvers, MA | 9258 | 1:1000 |
| Phospho-SAPK/JNK | Thr183/Tyr185 | Monoclonal | Rabbit | Cell Signaling | Danvers, MA | 4671 | 1:1000 |
| p44/42 MAPK | | Monoclonal | Mouse | Cell Signaling | Danvers, MA | 9107 | 1:2000 |
| Phospho-p44/42 MAPK | Thr202/Tyr204 | Monoclonal | Mouse | Cell Signaling | Danvers, MA | 9106 | 1:2000 |
| CaMKII | | Polyclonal | Rabbit | Cell Signaling | Danvers, MA | 3362 | 1:1000 |
| Phospho-CaMKII | Thr286 | Polyclonal | Rabbit | Cell Signaling | Danvers, MA | 3361 | 1:1000 |
| p35/25 | | Monoclonal | Rabbit | Cell Signaling | Danvers, MA | 2680 | 1:1000 |
| Phosphatases | | | | | | | |
| PP2A-C (catalytic subunit) | | Monoclonal | Mouse | Sigma-Aldrich | St. Louis, MO | A5316 | 1:10,000 |
| Demethylated PP2A-C | | Monoclonal | Mouse | Cell Signaling | Danvers, MA | 4466 | 1:1000 |
| Gel loading Controls | | | | | | | |
| β -actin | | Monoclonal | Mouse | Sigma-Aldrich | St. Louis, MO | A5316 | 1:10,000 |
| β 3-tubulin | | Monoclonal | Mouse | Cell Signaling | Danvers, MA | 4466 | 1:1000 |
| Other | | | | | | | |
| α -tubulin | | Monoclonal | Mouse | Sigma-Aldrich | St. Louis, MO | T6074 | 1:10,000 |

2.4. Preparation of mouse brain protein samples

All mice were killed by cervical dislocation followed by immediate decapitation at 30 minutes, 6 h, 24 h, or 1 week following midazolam or saline treatment. The brains were immediately harvested, and both the hippocampi and the cerebral cortex were dissected in ice-cold Tris-EDTA buffer (100 mM Tris-HCl, 1 mM EDTA, pH 7.4). These brain tissues were then immediately flash frozen in liquid nitrogen and stored at -80°C until they were used in the analyses. Tissue homogenates were prepared as previously described (Planel et al., 2008). Briefly, hippocampal or cortical tissues were homogenized in 5X vol/w radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Nonidet P-40, phosphatase inhibitors (Cocktails 1 and 2, 1:100 dilution), and protease inhibitors (Cocktail set III, 1:200 dilution). All homogenates were incubated on ice for 30 minutes, sonicated for 30 s in pulse mode, and then centrifuged at $11,000 \times g$ for 10 minutes at 4°C . Total protein content was determined in the supernatant using a bicinchoninic acid protein assay (Smith et al., 1985).

2.5. SDS-PAGE and Western blot analysis

The protein expressions of phosphorylated tau (p-tau) and total tau were determined using SDS-polyacrylamide gel electrophoresis coupled with Western blot analysis, as we have previously described (Whittington et al., 2011). For monoclonal tau antibodies, we used a goat anti-mouse light chain-specific secondary antibody to avoid nonspecific signals (Petry et al., 2014). Immunoreactive band signal intensity was visualized by enhanced chemiluminescence (ECL Plus, GE Healthcare Life Sciences, Piscataway,

NJ) using an ImageQuant LAS 4000 imaging system (GE Healthcare Life Sciences, Piscataway, NJ). ImageQuant TL 8.1 software (GE Healthcare Life Sciences, Piscataway, NJ) was used to perform densitometric quantification of the immunoreactive bands.

2.6. Tau solubility analyses

Assessment of tau solubility was performed using a modification of the protocol of Greenberg and Davis (Greenberg and Davies, 1990) as we have previously described (Planel et al., 2009; Whittington et al., 2015). Briefly, the cortices were homogenized with a mechanical homogenizer in 5:1 volume/weight RIPA buffer containing 50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% Na-deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mM Na_3VO_4 ; 1 mM NaF; 1:100 dilution of a protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO, USA). The samples were next centrifuged at $20,000 \times g$ for 20 minutes at 4°C . One aliquot of the supernatant was used to analyze the total tau fraction. A second aliquot of the supernatant was used to obtain the heat-stable, soluble, aggregate-free fraction. This was achieved by boiling the aliquot for 5 minutes, followed by subsequent centrifugation at $20,000 \times g$ for 20 minutes at 4°C to remove the protein aggregates. The remaining supernatants were adjusted to the same protein content with RIPA buffer to a final concentration of 1% sarkosyl (*N*-lauroyl sarcosinate). The supernatants were subsequently incubated for 30 minutes at room temperature with constant shaking, followed by centrifugation at $100,000 \times g$ for 1 hour at 20°C . The pellets containing the sarkosyl-insoluble aggregated tau were resuspended in an appropriate volume of Laemmli buffer and analyzed using Western blotting. Aliquots of the supernatant were analyzed by Western blotting of the sarkosyl-soluble tau fraction.

2.7. Barnes maze testing

Barnes maze testing was used to assess short-term spatial reference memory following acute midazolam administration as we have previously described (Whittington et al., 2015). Briefly, the Barnes maze apparatus (Stoelting, Inc, Wood Dale, IL) consisted of a circular metal platform that was 91 cm in diameter, with 20 holes around the perimeter of the platform. Each hole was 5 cm in diameter, and the platform was elevated 90 cm from the ground. All of the holes, with the exception of one, were blocked with a piece of metal as a means to prevent the mouse from entering and escaping the platform. The unblocked hole, referred to as the target hole, had a chamber through which the mouse was able to escape from the platform. Spatial cues were placed around the maze and incandescent bright light (580 lux) as well as white noise (85 dB), generated by the ANY-maze software (Stoelting, Inc, Wood Dale, IL), were used as reinforcers. The movements of the mice were continuously tracked and recorded using the ANY-maze video tracking software for subsequent “offline” analysis.

The Barnes maze testing consisted of four phases. During an initial adaptation phase (day 0), the mice were acclimated to the testing platform, target area, and the escape box. This was followed by the spatial acquisition phase (days 1–4), where the mice were trained (3 trials a day for 4 days) to locate the target escape hole within 180 seconds. At the end of the 4th day of the spatial acquisition phase, the hTau mice that reached asymptotic performance were randomized to receive either midazolam 25 mg/kg or 0.9% saline i.p.

On the 5th day, 18–20 hours after receiving the midazolam or saline injection, the mice underwent probe test I, which assessed short-term memory as previously described (Whittington et al., 2015). During probe test I, the mice were placed in the center of the Barnes maze; however, the target escape hole was now blocked. Probe test I lasted 90 seconds, and the latency to reach the target hole, total and primary errors (number of errors committed before the first attempted entry into the target hole), total and primary (distance traveled before reaching the target hole for the first time), distance traveled as well as the mean speed (m/s) were measured. Following the completion of probe test I, the mice were sacrificed via cervical dislocation, and the hippocampal tissues were subsequently analyzed for total tau and p-tau levels.

2.8. Microtubule binding assay

We performed a MT binding assay to measure the binding of tau to preassembled, taxol-stabilized MTs to determine whether midazolam disrupted this critical function of tau. The assay was performed as we have described previously (Planel et al., 2008) based on a modification of a method originally described by Maas et al. (Maas et al., 2000). Briefly, brain hemispheres were homogenized in 5X weight/volume of BRB80 (Brinkley Reassembly Buffer; 80 mM PIPES/KOH pH 6.8, 1 mM EGTA, 1 mM MgCl₂) with protease and phosphatase inhibitors. The homogenates were then incubated on ice for 15 minutes and centrifuged at 20,000 × g for 20 minutes at 4 °C. The resulting supernatants were then centrifuged again at 100,000 × g for 1 hour at 4 °C. These high-speed supernatant fractions were then adjusted to 1 mM GTP, 10 μM taxol, and incubated with taxol-stabilized MTs (30 μM, Cytoskeleton, Inc, Denver, CO) in a final volume of 50 μL for 10 minutes at 37 °C. The mixtures were then centrifuged through 100 μL of 30% (w/v) sucrose cushions in BRB80 containing 1 mM GTP and 10 μM taxol, at 100,000 × g for 30 minutes at room temperature. The supernatant, which contained the MT-free fraction, was collected and diluted with a modified O⁺ buffer (O’Farrell, 1975) (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 5% 2-mercaptoethanol; 2.3% SDS; 1 mM EGTA; 1 mM

EDTA; 1 mM PMSF; 1 mM Na₃VO₄; 1 mM NaF; 10 U/mL Protease Inhibitor Cocktail P8340, Sigma-Aldrich). The pellet, which contained the MT-bound fraction, was then resuspended in the O⁺ buffer. The two fractions were then analyzed by Western blot using total tau and α-tubulin antibodies for quantification.

2.9. Statistical analysis

Group comparisons of immunoblot relative band intensities were performed using either a one- or two-way analysis of variance with Tukey’s *post hoc* test applied when appropriate or by an unpaired *t*-test. Statistical calculations were performed using Prism 5 software (GraphPad Software, Inc, San Diego, CA), and all biochemical data are reported as mean ± SD with a value of *p* < 0.05 considered statistically significant.

Analysis of the Barnes Maze acquisition testing variables was performed by means of a two-way repeated-measures analysis of variance with Newman-Keuls Multiple Comparison *post hoc* test used when appropriate. An unpaired *t*-test was used to compare group analysis of probe test I variables. Prism 5 software was also used to complete all of the Barnes Maze statistical analyses, with data reported as mean ± SEM and again *p* < 0.05 deemed statistically significant.

3. Results

3.1. Acute midazolam administration increases tau phosphorylation under normothermic conditions in the mouse hippocampus

Using 8- to 10-week-old C57BL/6 male mice, we initially examined the impact of two intraperitoneally administered sedative doses of midazolam, 10 mg/kg and 25 mg/kg, versus saline (Ctl) on hippocampal tau phosphorylation under normothermic conditions. These doses were selected as they both produced mild gait impairment and decreased locomotion but did not affect the loss of righting reflex; moreover, these motor effects resolved within 1 h of its administration. Thirty min after the administration of midazolam 10 mg/kg and 25 mg/kg (*n* = 5 for each dose), significant increases (expressed as % of Ctl) were observed at the AT8 (pSer202/pThr205; 247 ± 70 and 225 ± 89% of Ctl, respectively) and CP13 (pSer202; 215 ± 65 and 223 ± 81% of Ctl, respectively) phosphoepitopes (Fig. 1A and B), when compared to the saline-treated mice (Ctl, *n* = 4). Both doses of midazolam increased tau phosphorylation to a similar degree; however, neither midazolam dose had an effect on p-tau levels at PHF-1 (pSer396/pSer404) or total tau levels (Fig. 1C and D). Rectal temperatures at the end of the experiment were similar in all of the study groups: Ctl 37.4 ± 0.3, midazolam 10 mg/kg 37.3 ± 0.3, and midazolam 25 mg/kg 37.4 ± 0.2 °C.

We next examined whether tau phosphorylation persists in the mouse hippocampus following its acute administration. Given that we recently reported that acute administration of dexmedetomidine, a sedative-analgesic agent, produces tau hyperphosphorylation that lasts up to 6 h (Whittington et al., 2015), we first examined hippocampal tau phosphorylation 6 h after the administration of midazolam 25 mg/kg. In these studies, the C57BL/6 mice received either midazolam 25 mg/kg (*n* = 5) or saline (Ctl, *n* = 4) i.p. and were sacrificed 6 h after treatment. Six hours after midazolam 25 mg/kg, tau hippocampal hyperphosphorylation was still present at the AT8 and CP13 phosphoepitopes (Fig. 1E and F). Interestingly, the PHF-1 epitope was also increased (Fig. 1G), although it was not at 30 min (Fig. 1G), indicating a different kinetic phosphorylation profile than that observed with the other epitopes. There were no observed changes in total tau levels at 6 h (Fig. 1H).

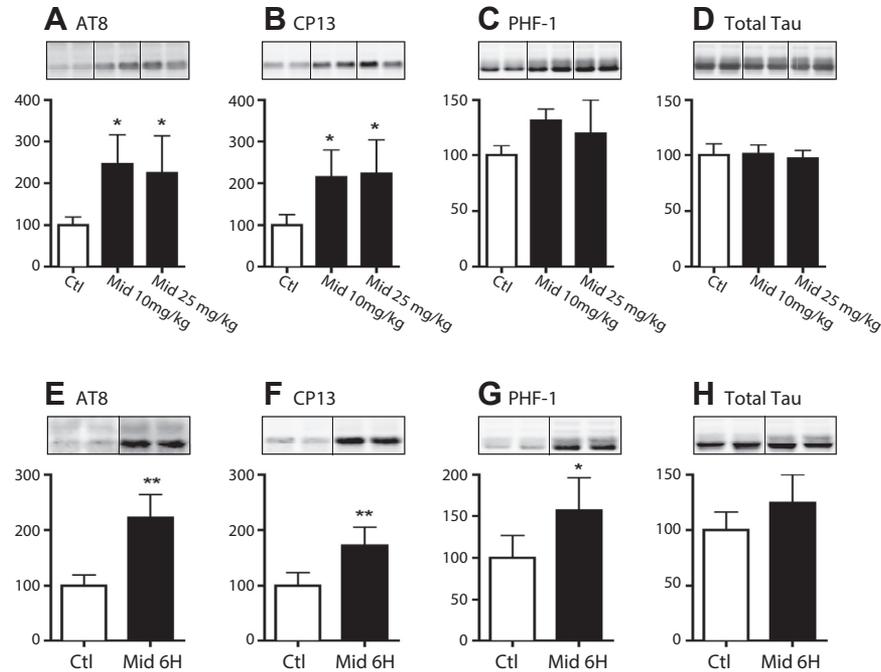


Fig. 1. Acute administration of midazolam increases hippocampal tau phosphorylation in C57BL/6 mice. Hippocampal tau phosphorylation (% Ctl) at the AT8 (pSer²⁰²/pThr²⁰⁵), CP13 (pSer²⁰²), and PHF-1 (pSer³⁹⁶/Ser⁴⁰⁴) phosphoepitopes (A–C) in 8- to 10-week-old C57BL/6 treated with midazolam (Mid) 10 mg/kg (n = 5) or 25 mg/kg (n = 5) i.p. Control (Ctl) mice (n = 4) were treated with 0.9% saline (100 μ l per 10 g of body weight). All mice were sacrificed 30 minutes after treatment. No statistically significant difference in rectal temperatures ($^{\circ}$ C) was observed at the time of tissue harvest: Ctl 37.4 ± 0.3 $^{\circ}$ C, Mid 10 mg/kg 37.3 ± 0.3 $^{\circ}$ C, and Mid 25 mg/kg 37.4 ± 0.2 $^{\circ}$ C. Hippocampal tau phosphorylation (% Ctl) at the AT8, CP13, and PHF-1 phosphoepitopes (E–G) in 8- to 10-week-old C57BL/6 mice treated with midazolam 25 mg/kg (MID 6 h, n = 5) or 0.9% saline (Ctl, n = 4) i.p. and sacrificed 6 h later. Normothermia was maintained throughout the study and rectal temperatures were similar at the time of hippocampal tissue harvest: Ctl 36.4 ± 0.3 $^{\circ}$ C and MID 6h 36.9 ± 0.2 $^{\circ}$ C. Phosphorylated tau was normalized to total tau (D and H) after controlling for gel loading with β -actin. Relative immunoreactive band intensities are expressed as a percent of Ctl and are displayed for each phosphoepitope and total tau. For each condition, 2 representative immunoblot bands are displayed. Data are expressed as mean \pm SD and were analyzed using ANOVA with Tukey's post hoc test. * and ** denote $p < 0.05$ and $p < 0.01$ versus Ctl, respectively. Abbreviation: ANOVA, analysis of variance.

Overall, our results indicate that a single injection of midazolam can lead to rapid (30 minutes) and prolonged (6 hours) tau hyperphosphorylation in the hippocampus.

3.2. Midazolam induces cortical tau phosphorylation for up to 24 hours after its acute administration

As robust tau phosphorylation in the hippocampus was still evident 6 h after midazolam administration, which was long after its sedative effects had dissipated, we next examined tau phosphorylation in the cortex of C57BL/6 mice that received midazolam 25 mg/kg i.p. and were sacrificed 30 minutes or 24 h later (n = 6 for each treatment group). For these studies, tau phosphorylation was measured using a wider panel of phosphoepitope antibodies to more fully assess the degree of phosphorylation across several domains of tau, including the MT binding domain (i.e., pSer262 with 12E8).

At 30 minutes, increases in tau phosphorylation were again observed at AT8 and CP13 (Fig. 2A and B), with no change of phosphorylation at PHF-1 or total tau, confirming our results in the hippocampus (Fig. 1C). In addition, significant increases in tau phosphorylation were observed at 30 minutes at AT180 (pThr231; Fig. 2E) and 24 h at pSer199 (Fig. 2G). No change in tau phosphorylation was observed at the 12E8 (pSer262/pSer356) or AT270 (pThr181) phosphoepitopes at either time point.

Overall, these data indicate that the acute administration of midazolam, under normothermic conditions, quickly increases tau phosphorylation in the hippocampus and cortex following its administration in nontransgenic mice. However, the tau hyperphosphorylation response observed following acute midazolam administration is complex, with some epitopes responding

immediately, such as AT8 and CP13 (30 min), while other epitopes have a more delayed onset, such as PHF-1 (6 h) and pS199 (24 h).

3.3. Mechanisms of tau hyperphosphorylation after midazolam administration

The tau phosphorylation state is dependent on a balance between the activity of several major tau protein kinases such as glycogen synthase kinase-3 β (GSK-3 β), stress-activated protein kinase (SAPK)/Jun-amino terminal kinase (JNK), extracellular signal-regulated kinase (ERK or MAPK1), calmodulin-dependent kinase II (CaMKII), cyclin-dependent kinase 5 (CDK5), and its neuron-specific activator P35. Tau phosphorylation also depends on the activity of several PPs, including PP1, PP2B, and PP2A, the latter being the main tau phosphatase in the brain (Ferrer et al., 2001, 2002, 2005; Gong et al., 2000; Planel et al., 2002, 2007; Tian and Wang, 2002). Therefore, we subsequently dissected the underlying tau phosphorylation mechanism by specifically determining the activation pattern of GSK-3 β , ERK 1/2, SAPK/JNK, CAMKII, CDK5, and examining the protein expression of PP2A-C (catalytic subunit) and demethylated-PP2A-C in the same cortical samples obtained from the C57BL/6 mice at 30 minutes and 24 h following acute midazolam or saline treatment.

At 30 minutes, a significant increase in phospho-GSK-3 β (Ser⁹) levels (Fig. 3C) and a significant decrease in phospho-ERK 1/2 (Fig. 3G) levels were observed, when normalized to their respective total kinase levels (Fig. 3D and H), in the midazolam-treated mice. This was consistent with inactivation of these enzymes and, consequently, could not explain the tau hyperphosphorylation observed at this time point. Moreover, no significant kinase

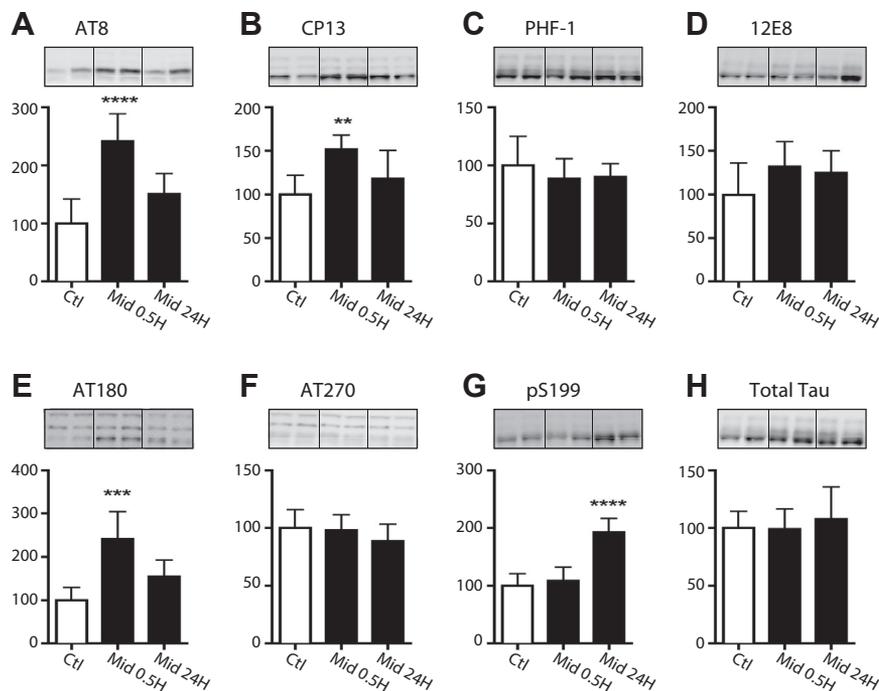


Fig. 2. Cortical tau hyperphosphorylation following acute midazolam administration persists up to 24 h in C57BL/6 mice. Cortical tau phosphorylation (% control [Ctl]) at the AT8 (A), CP13 (B), PHF-1 (C), 12E8 (D), AT180 (E), AT270 (F), and pS199 (G) phosphoepitopes in 8- to 10-week-old C57BL/6 mice treated with midazolam (Mid) 25 mg/kg i.p. and sacrificed 30 minutes (0.5 h, $n = 6$) or 24 h ($n = 6$) later. Control mice ($n = 6$) were treated with 0.9% saline i.p. and sacrificed 30 minutes later. Normothermia was maintained throughout and rectal temperatures at the end of the study were 37.4 ± 0.2 °C (Ctl), 37.0 ± 0.3 °C (Mid 0.5 h), and 37.0 ± 0.4 °C (Mid 24 h). Phosphorylated tau levels were normalized to total tau (H) after controlling for gel loading with β -actin. Data are expressed as mean \pm SD and **, ***, **** denote $p < 0.01$, $p < 0.001$, and $p < 0.0001$ versus Ctl, respectively, using ANOVA with Tukey's post hoc test. Two representative immunoblot bands are displayed for each condition. Abbreviation: ANOVA, analysis of variance.

expression changes consistent with hyperphosphorylation at 30 minutes were observed in phospho-CaMKII and phospho-SAPK/JNK (Fig. 3A and E) when normalized to their respective total kinase level (Fig. 3B and F) and in levels of CDK5 (Fig. 3I) and its activator P35 (Fig. 3J). Furthermore, at 24 h, no changes in cortical kinase activation were observed that could explain the persistence of phosphorylation at pSer199.

Given the absence of kinase activation at these time points, levels of relevant tau PPs were also examined at 30 minutes and 24 h in these same cortical tissues. At 30 minutes, midazolam-induced a decrease in demethylated PP2A-C levels (Fig. 4A), which again was inconsistent with increased tau phosphorylation, as it reflects increased activity. Overall, there were no changes in the protein expression of the catalytic subunit of PP2A (Fig. 4B), PP2B (Fig. 4D), and PP5 (Fig. 4E). Interestingly, a decrease in PP1 (Fig. 4C) levels was observed at 30 minutes, suggesting that perhaps this enzyme may play a role in midazolam-induced tau phosphorylation. At 24 h, there was still an equivalent decrease in PP1; however, it did not reach statistical significance.

Taken together, our data do not allow the identification of a definite mechanism. Nevertheless, they suggest that PP1 down-regulation following midazolam administration might contribute to the observed hyperphosphorylation of tau.

3.4. Acute midazolam administration increases hippocampal tau phosphorylation levels 6 h after its administration in hTau mice without altering cortical tau solubility

To determine whether the tau hyperphosphorylation response following midazolam administration is impacted by pre-existing tau pathology, the tau phosphorylation pattern was also examined in 3-month-old male hTau mice, a transgenic strain solely

expressing nonmutant human tau that develops neurofibrillary pathology similar to that observed in AD (Andorfer et al., 2003). These mice were studied at 3 months of age, as Andorfer et al. previously demonstrated that, by 3 months of age, accumulation of CP13 reactive tau occurs in the hippocampal cell bodies of this transgenic strain (Andorfer et al., 2003).

The hTau mice received either midazolam 25 mg/kg ($n = 6$) or 0.9% saline (Ctl, $n = 6$) i.p. and were sacrificed 6 h after administration (Fig. 5). Midazolam produced a significant increase in hippocampal tau phosphorylation at 6 h solely at CP13, $153 \pm 20\%$ of Ctl (Fig. 5B); furthermore, unlike the C57BL/6 mice, no persistence of tau hyperphosphorylation was observed at AT8 (Fig. 5A).

We also examined the impact of midazolam on cortical tau solubility 6 h after its administration. Cortical sarkosyl-soluble tau levels were very variable, and no significant changes were detected at 6 h (data not shown), with no change in unfractionated total tau (Fig. 5E) sarkosyl-insoluble (Fig. 5F), as well as the heat-stabilized tau fraction (Fig. 5G). Rectal temperatures were similar in all groups at the end of this study: 36.8 ± 0.9 and 36.8 ± 0.7 °C.

In summary, acute midazolam administration in hTau mice was associated with persistent phosphorylation at the CP13 phosphoepitope; however, this phosphorylation was not associated with any increase in cortical sarkosyl-insoluble tau levels. This suggests that despite the presence of persistent hyperphosphorylation at 6 h in the hTau mice, midazolam was not associated with any tau-related proaggregant changes in this transgenic strain.

3.5. Acute midazolam administration does not impact short-term spatial reference memory in hTau mice

Barnes Maze testing was performed in hTau mice to assess short-term spatial reference memory following midazolam 25 mg/

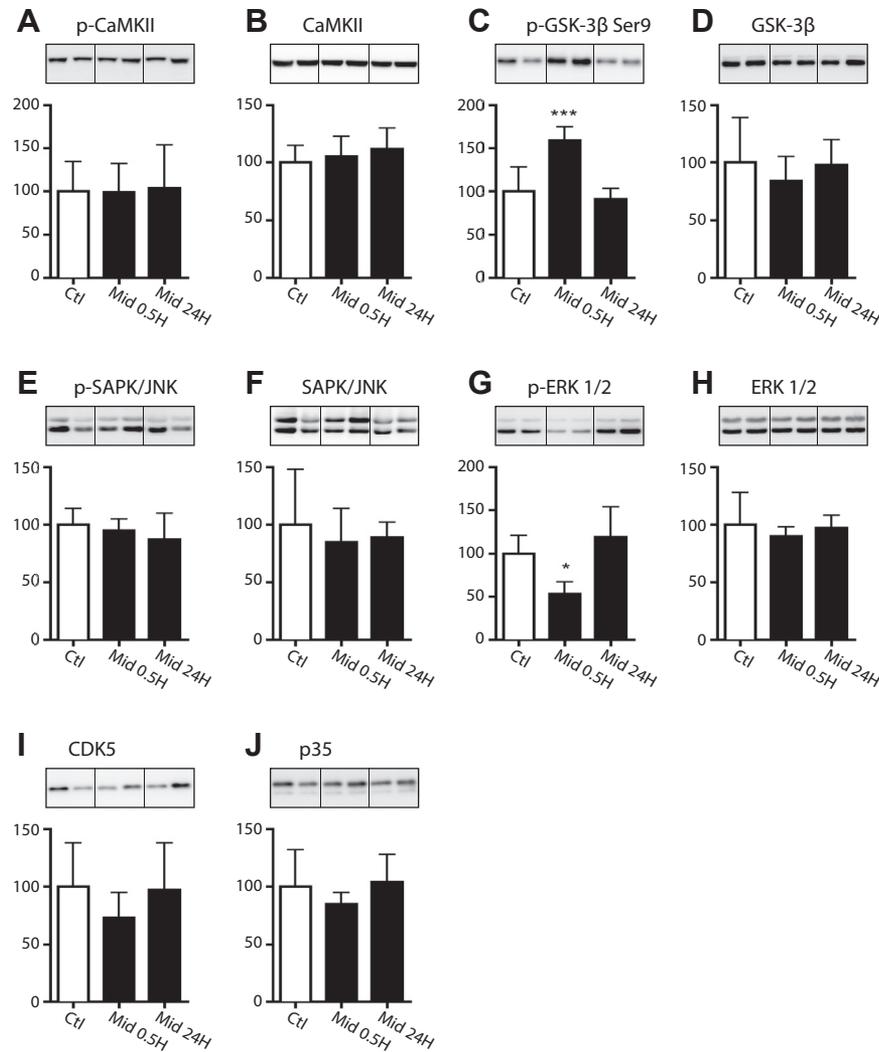


Fig. 3. The effect of acute midazolam administration on tau kinases in the mouse cortex at 0.5 h and 24 h. Cortical proteins from the 8- to 10-week-old C57BL/6 mice that received either saline (Ctl) or midazolam 25 mg/kg i.p. and were sacrificed 30 minutes (Mid 0.5) or 24 h (Mid 24) were separated by SDS-PAGE and protein levels of kinases were determined using antibodies directed at the following proteins: (A) phospho-CaMKII, (B) total CaMKII, (C) phospho-GSK-3β Serine (Ser) 9, (D) total GSK-3β, (E) phospho-SAPK/JNK, (F) total SAPK/JNK, (G) phospho-ERK 1/2 (phospho-p44/42 MAPK), (H) total ERK 1/2 (p44/42 MAPK), (I) CDK5, and (J) P35 (activator of CDK5). Relative immunoreactive band intensities are expressed as a percent of saline control (Ctl) after appropriately normalizing for gel loading with β -actin or β -tubulin based on the molecular weight of the protein of interest. Phosphorylated proteins were also normalized to each respective nonphosphorylated total protein. For each condition, 2 representative immunoblot bands are displayed with Ctl (n = 6), 0.5 h (n = 6), and 24 h (n = 6). Data are expressed as mean \pm SD. *, *** denote $p < 0.05$, $p < 0.001$, versus Ctl, respectively; ANOVA with Tukey's post hoc test. Abbreviations: ANOVA, analysis of variance; CaMKII, calmodulin-dependent kinase II; GSK-3 β , glycogen synthase kinase-3 β ; ERK, extracellular signal-regulated kinase.

kg (n = 8) or 0.9% saline (10 μ L/kg; n = 8) administration. Data from the acquisition phase (days 1–4) demonstrate that both mice groups learned rapidly and reach asymptotic performance by day 4 (Fig. 6). By day 4, there were no significant between-group differences in total distance, total latency, and errors (Fig. 6A–C). On day 5, 18–20 hours after the administration of midazolam or saline, a probe test (Fig. 6D–I) assessing spatial reference memory was performed and revealed that midazolam had no significant effect on primary and total distance (Fig. 6D and E), primary and total number of errors (Fig. 6G and H), as well as primary latency (Fig. 6I). The mean speed was similar in both groups demonstrating that midazolam produced no motor impairment at that time point (Fig. 6F).

Thus, our results reveal that administration of midazolam, at a dose previously demonstrated to induce tau hyperphosphorylation, is not associated with short-term memory impairment in young hTau mice.

3.6. Chronic midazolam administration increases hippocampal tau phosphorylation in an age-dependent manner in nontransgenic mice

As midazolam is often administered as a prolonged infusion for long-term sedative purposes, we next examined the impact of chronic midazolam administration on hippocampal tau phosphorylation in 8- to 10-week-old and 11-month-old C57BL/6 mice. We initially measured hippocampal tau phosphorylation in 8- to 10-week-old C57BL/6 mice (n = 6 per group) following a 24 h infusion of midazolam 3 mg/kg/h or 0.9% saline. These mice were sacrificed immediately upon termination of the infusion. Hippocampal tau phosphorylation solely increased at the CP13 phosphoepitope ($150 \pm 16\%$ of Ctl; Fig. 7B), demonstrating that the CP13 phosphoepitope is again susceptible to midazolam-induced tau hyperphosphorylation. No significant increases in total tau and tau phosphorylation at AT8 and PHF-1, AT270, pSer262, AT180, pSer199

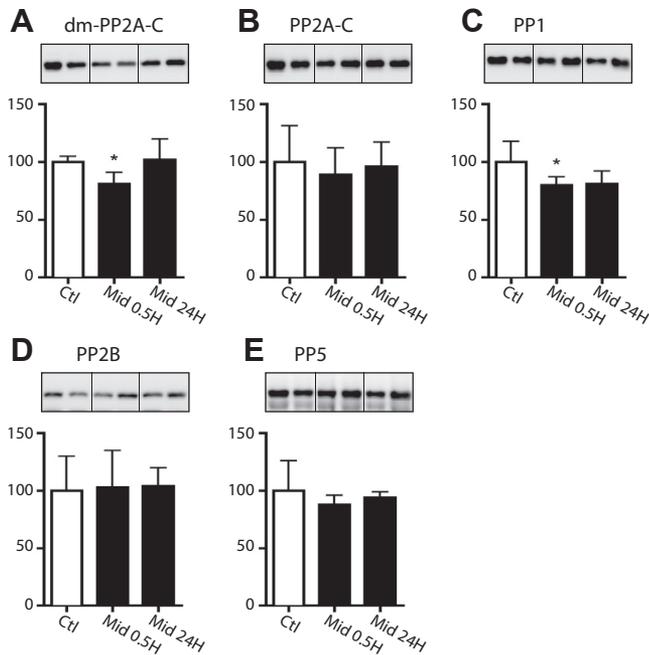


Fig. 4. The effect of acute midazolam administration on phosphatase levels in the mouse cortex at 0.5 h and 24 h. Cortical proteins from the 8- to 10-week-old C57BL/6 mice that received either saline (Ctl) or midazolam 25 mg/kg i.p. and were sacrificed 30 minutes (Mid 0.5) or 24 h (Mid 24) were separated by SDS-PAGE, and protein levels of several protein phosphatases were determined using antibodies directed at (A) the demethylated protein phosphatase catalytic subunit (dm-PP2A-C), (B) the PP2A catalytic subunit (PP2A-C), (C) protein phosphatase-1 (PP1), (D) protein phosphatase 2B (PP2B), and (E) protein phosphatase 5 (PP5). Relative immunoreactive band intensities are expressed as a percent of saline control (Ctl) after appropriately normalizing for gel loading with β -actin or β -tubulin. For each condition, 2 representative immunoblot bands are displayed with Ctl (n = 6), 0.5 h (n = 6), and 24 h (n = 6). Data are expressed as mean \pm SD. * denotes $p < 0.05$ versus Ctl using ANOVA with Tukey's *post hoc* test. Abbreviation: ANOVA, analysis of variance.

phosphoepitopes were observed (Fig. 7A, C–H). In the young mice, there was no significant difference in rectal temperatures between the Ctl and midazolam-treated groups: 36.5 ± 0.2 and 36.8 ± 0.8 °C, respectively. In contrast, when the 11-month-old C57BL/6 mice (n = 5) per group received similar midazolam or saline treatment, significant increases in hippocampal tau phosphorylation were observed in CP13, PHF-1, and AT180 in the midazolam group (Fig. 7J–L), while no significant changes were still not observed at AT270, pSer199, pSer262, or total tau (Fig. 7M–P). Again, in these aged mice, there was no significant difference in rectal temperatures between the Ctl and midazolam-treated groups: 36.9 ± 0.4 and 36.5 ± 0.04 °C, respectively.

Hence, these data suggest the susceptibility to tau phosphorylation after chronic midazolam administration increases as a function of age in this nontransgenic strain.

3.7. Chronic midazolam administration induces hippocampal tau hyperphosphorylation that persist for up to 1 week in young nontransgenic mice

The persistence of hippocampal tau phosphorylation was also examined 1 week after the start of a 24-h infusion of midazolam 3 mg/kg/h (n = 5) or 0.9% saline (n = 6) in 8- to 10-week-old C57BL/6 mice. Interestingly, 1 week after a chronic infusion of midazolam, increased hippocampal p-tau levels at AT8 and CP13 (152 ± 29 and $211 \pm 69\%$ of Ctl, respectively; Fig. 8A and B) were observed, with no similar change detected in phosphorylation at PHF-1 or total tau levels (Fig. 8C and D) at this same time point.

Therefore, these studies indicate that chronic midazolam administration, in young nontransgenic mice, results in persistent hippocampal tau phosphorylation for up to 1 week, which is a period long after the sedative effects of midazolam have dissipated.

3.8. The impact of chronic midazolam administration on tau phosphorylation, solubility, and MT binding

As we observed an increase in tau phosphorylation in the nontransgenic mice after chronic midazolam administration, the impact of this type of exposure on tau phosphorylation, solubility, and function were subsequently investigated in 7-month-old hTau mice.

3.9. Tau phosphorylation and solubility studies

7-month-old hTau mice received either midazolam 5 mg/kg/h or an equal volume of 0.9% saline (n = 6 per group) for 24 h. On termination of the 24-h infusion, hippocampal tau phosphorylation was increased at AT8 and CP13 phosphoepitopes in the midazolam-treated group with no change in phosphorylation at pSer262 or total tau levels (Fig. 9A–D).

Using tissues from this same experiment, we then determined whether this midazolam-induced increase in tau phosphorylation was associated with any changes in tau solubility in cortical homogenates. Interestingly, there were no significant changes in the sarkosyl-insoluble tau, sarkosyl-soluble tau, heat-stabilized tau, and total tau levels (Fig. 9E–H) observed between the midazolam- and saline-treated groups.

Thus, although this chronic infusion of midazolam produced gait impairment and significant hippocampal tau phosphorylation, these changes did not result in changes in tau aggregation.

3.10. Tau microtubule binding studies

We then examined whether chronic midazolam administration, affected the capacity of tau to bind to preassembled, taxol-stabilized MTs (MTs) using tissues from the midazolam and saline-treated hTau mice. Compared to Ctl mice, we observed a significant decrease in tau levels in the MT-bound fraction, for equivalent amounts of α -tubulin, in the midazolam-treated mice (Fig. 10). Although a slight increase in tau levels in the MT-free fraction was observed in the midazolam-treated mice versus Ctl, this was not statistically significant.

Nevertheless, these data indicate that the chronic administration of midazolam has the ability to impact the ability of tau to bind to preformed MTs in a transgenic mouse model expressing nonmutant human tau.

4. Discussion

Although it has been well established that anesthetics can induce profound tau hyperphosphorylation by producing anesthesia-induced hypothermia (Planel et al., 2007, 2008, 2009), our current studies further support the notion that certain anesthetics can still increase tau phosphorylation in the absence of hypothermia. The present findings specifically demonstrate that midazolam, a commonly used benzodiazepine, directly increases tau phosphorylation in nontransgenic and a transgenic AD mouse model expressing nonmutant human tau. To our knowledge, it is the first study reporting tau hyperphosphorylation in vivo following acute and chronic administration of a benzodiazepine.

We initially observed that even a single dose of midazolam, in nontransgenic mice, was associated with increased cortical tau phosphorylation at AT8, CP13, and AT180 at 30 minutes, and

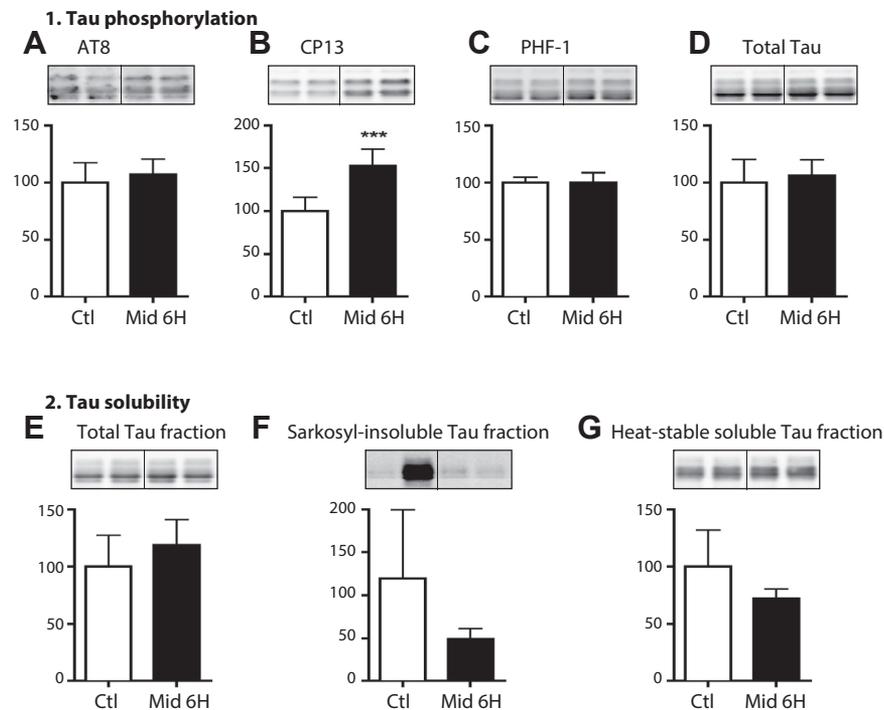


Fig. 5. Hippocampal tau phosphorylation is present at the CP13 phosphoepitopes 6 h after acute midazolam administration in hTau mice but is not associated with an increase in insoluble tau levels. Hippocampal tau phosphorylation (% Ctl) at the AT8, CP13, and PHF-1 phosphoepitopes (A–C) in 3-month-old hTau mice treated with midazolam 25 mg/kg ($n = 6$) or saline (Ctl, $n = 6$) i.p. and sacrificed 6 h later. Normothermia was maintained throughout the study and rectal temperatures were similar at the time of hippocampal tissue harvest: Ctl 36.8 ± 0.9 °C and Midazolam 36.8 ± 0.7 °C. Phosphorylated tau levels were normalized to total tau (D), after controlling for gel loading with β -actin. Levels of tau in the total (E), sarkosyl-insoluble (F), and heat-stabilized fractions (G) were measured using immunoblotting and total tau antibody. Relative immunoreactive band intensities are expressed as a percent of Ctl and are displayed for each phosphoepitope and total tau. For each condition, 2 representative immunoblot bands are displayed. Data are expressed as mean \pm SD and *** $p < 0.001$ versus Ctl using an unpaired t -test.

pSer199 for up to 24 h. Interestingly, compared to the 2-h and 6-h durations of tau phosphorylation that we previously reported after an acute dose of propofol (Whittington et al., 2011) and dexmedetomidine (Whittington et al., 2015), respectively, the duration of tau phosphorylation at pS199 after midazolam administration lasted up to 24 h. As those previous studies involving the acute administration of sedatives used even more profound levels of sedation than that observed with midazolam, our present findings suggest that midazolam possesses a much greater capacity to directly increase the duration of tau phosphorylation than these two commonly used intravenous hypnotic agents, having an effect long after its sedative effect has disappeared.

As a benzodiazepine, midazolam produces many of its effects by allosterically binding to the benzodiazepine receptor on GABA_A receptors, which in turn potentiates the action of GABA on this receptor subtype (Ehlert et al., 1983; Sigel and Baur, 1988). Previous studies have demonstrated that activation of GABA_A receptor using the benzodiazepine desalkylflurazepam (an active metabolite of flurazepam), increased AT8 tau phosphorylation in vitro but had no effect on TG3 (pSer231) or PHF13 (pSer396) (Nykanen et al., 2012). Interestingly, Nykanen et al. also observed persistent tau phosphorylation in vitro at AT8 after treatment following a washout period; in contrast, we solely observed persistent tau hyperphosphorylation at pS199 in vivo. Nevertheless, our findings are consistent with those of Nykanen et al. as the AT8 (pSer202/pThr205) and pSer199 phosphoepitopes are located in the same proline-rich region of tau (Augustinack et al., 2002; Buee et al., 2000), suggesting a propensity for benzodiazepines to induce persistent phosphorylation in this area.

Our studies examining tau kinases and phosphatases did not allow for the isolation of the mechanism underlying tau

hyperphosphorylation but show that midazolam administration led to the downregulation of PP1. Interestingly, Shibasaki et al. have shown that in vivo administration of zolpidem, an imidazopyridine that binds and activates GABA_A receptors at the same location than benzodiazepines, results in the downregulation of PP1 (Shibasaki et al., 2013), suggesting that benzodiazepines might have the same effect and supporting our results. We did not find a downregulation of PP2A, but Nykanen et al. observed that GABA_A receptor-mediated increases in tau phosphorylation in vitro were associated with decreases in PP2A binding to tau and not a decrease in global PP2A level or activity per se (Nykanen et al., 2012). Overall, and in light of the literature, our results suggest that tau hyperphosphorylation after midazolam treatment might be due in part to PP1 downregulation.

Importantly, acute midazolam-induced tau hyperphosphorylation in 3-month-old hTau mice was not associated with increases in cortical insoluble tau levels or with impaired short-term spatial reference memory. This is in stark contrast to what we recently observed in hTau mice of the same age with dexmedetomidine, where increases in insoluble tau and impaired spatial memory were observed following its acute administration (Whittington et al., 2015). The differences in tau aggregation might be due to different epitopes being hyperphosphorylated. For example, acute dexmedetomidine administration immediately induced tau hyperphosphorylation at the PHF-1 site (Whittington et al., 2015), whereas midazolam did not. In contrast to our results, midazolam administered before the probe test impaired retrieval of spatial memory in rats tested with the Morris water maze (Timic et al., 2013). However, the rats were tested 20 minutes after midazolam administration, although the probe test was performed 18–20 hours after in our mice. However, other studies

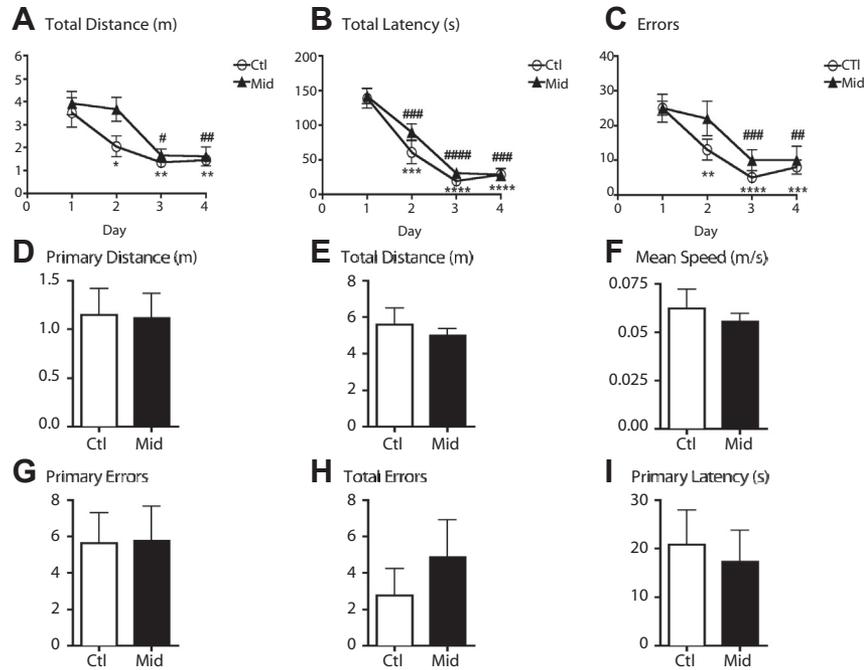


Fig. 6. Acute Administration of a midazolam dose known to induce tau hyperphosphorylation does not result in the impairment of spatial memory in hTau mice. Acquisition phase testing data (mean \pm SEM) in 6-month-old hTau mice demonstrate that asymptotic performance is readily achieved in the Barnes maze with 4 days of training (A–C), before treatment. By day 4, total distance (m), total latency (s), and total errors in the saline and midazolam groups ($n = 8$ per group) were significantly decreased from their respective day 1 performance for each variable, denoting good learning performance. *, **, ***, and **** denote $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ versus day 1 (saline), respectively, and #, ##, ###, and #### denote $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ versus day 1 (midazolam) using repeated-measures analysis of variance (ANOVA) with Newman-Keuls post hoc test. Probe test data (mean \pm SEM) were obtained one day after the end of the acquisition training phase (day 5). Primary and total distance (m), primary and total errors, primary latency (s) and mean speed (m/s) were obtained 18–20 hours after treatment with midazolam 25 mg/kg or saline i.p. Midazolam-treated mice demonstrated no deterioration of spatial memory retention as measured by distance (D and E), errors (G and H), and latency (I). Moreover, there were no differences in terms of mean speed (F) suggesting the absence of midazolam-induced motor impairment.

confirm our results because the administration of midazolam at different stages of learning did not affect acquisition, consolidation, or recall of spatial memory (Valentim et al., 2013a,b). Overall, our results suggest that acute midazolam administration does not affect tau aggregation or spatial memory in our experimental design.

Given this persistence of tau phosphorylation after its acute administration, we also examined hippocampal tau phosphorylation in nontransgenic mice following a 24-hour midazolam infusion, an experimental paradigm with clinical relevance as this benzodiazepine is still used for prolonged sedation in intensive care unit settings (Spence et al., 2018; Zhang et al., 2017). Again, despite achieving a modest level of sedation, characterized primarily by mild ataxia, hippocampal tau phosphorylation was observed immediately on cessation of the 24-hour midazolam infusion. Moreover, this effect was augmented by age suggesting that, after its chronic administration, the aged brain is more susceptible to midazolam-induced increases in hippocampal tau phosphorylation.

Surprisingly, we also observed pronounced hippocampal tau hyperphosphorylation in young nontransgenic mice, one week after the administration of a 24-hour midazolam infusion. The degree of tau phosphorylation at one week was somewhat different compared with the 24-hour time point, as it became more pronounced at AT8, while disappearing at PHF-1. This suggests that in the setting of chronic midazolam administration, hippocampal tau phosphorylation not only persists for up to 1 week but also implies that the pattern of epitopes affected shifts over time. Hence, future studies are indicated to determine whether this phosphorylation persists at time points even more remote than 1 week following midazolam exposure. This persistence of tau phosphorylation following chronic administration of an anesthetic is not a unique

finding, as Le Freche et al. observed a similar phenomenon following the chronic administration of the inhalational anesthetic sevoflurane (Le Freche et al., 2012). However, this persistence of midazolam-induced tau phosphorylation is the first time this has been described with an intravenous sedative that has been clinically associated with cognitive decline.

One of the key functions of tau is to bind and stabilize MTs. Tau hyperphosphorylation has been associated with the dissociation of tau from MTs resulting in disruption of the MT network and the impairment of axonal transport (Feinstein and Wilson, 2005; Mandelkow et al., 2003; Mi and Johnson, 2006). In hTau mice, we observed that the chronic administration of midazolam resulted in a decrease capacity of tau to bind to preassembled, taxol-stabilized MTs. This suggests that one of the major functions of tau, which is also critical to normal axonal transport (Mandelkow et al., 2003), is disrupted by midazolam. As fast axonal transport has been consistently demonstrated to be disrupted in several animal models of AD (Buxbaum et al., 1998; Morfini et al., 2002; Smith et al., 2007), any anesthetic that can further cause MT dysregulation could theoretically accelerate this neurodegenerative disorder.

Interestingly, just as was the case after acute midazolam administration, we did not observe any increases in insoluble tau levels in the cortex of 6-month-old hTau mice after a chronic infusion, despite the aforementioned disruption of tau function. Although this is somewhat surprising, it could be related to the fact that the tau aggregation analyses were performed immediately on cessation of midazolam infusion. Thus, although the tau hyperphosphorylation was associated with an increase in tau levels in the free (unbound) fraction and a concurrent decrease in the bound fraction, it is conceivable that not enough time elapsed for the

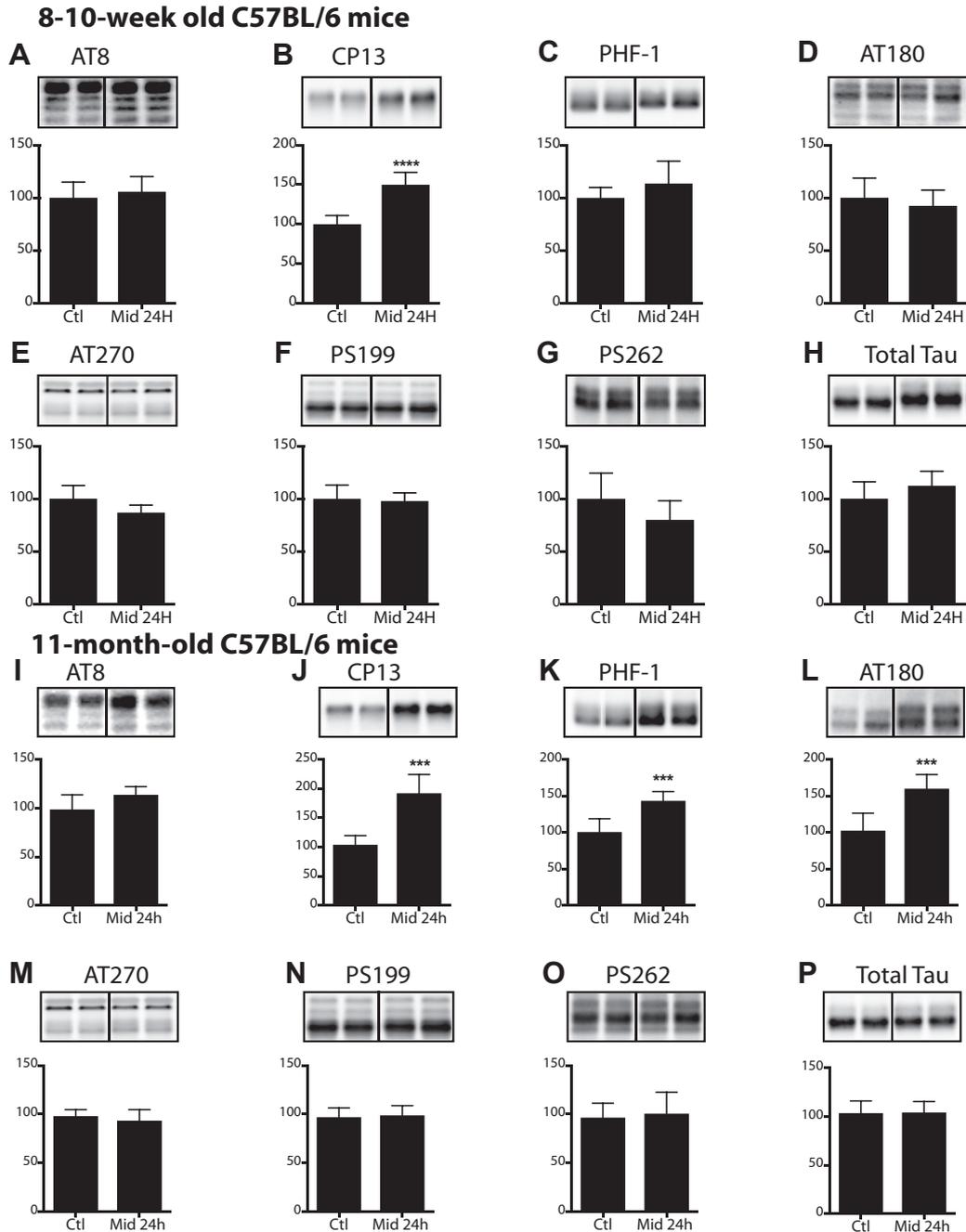


Fig. 7. Hippocampal tau phosphorylation after chronic midazolam administration increases as a function of age in C57BL/6 mice. Hippocampal tau phosphorylation (% Ctl) at the AT8, CP13, PHF-1, AT180, AT270, pSer199, pSer262 phosphoepitopes in 8- to 10-week-old (A–G) or 11-month-old (I–O) C57BL/6 mice treated with a subcutaneous infusion of midazolam 3 mg/kg/h ($n = 6$ young; $n = 5$ old) or 0.9% saline (Ctl) ($n = 6$ young; $n = 5$ old) i.p. and sacrificed 24 h after the start of the infusion. Normothermia was maintained throughout the administration of midazolam or saline, and rectal temperatures were similar at the time of hippocampal tissue harvest: young Ctl 36.5 ± 0.2 °C and midazolam 36.8 ± 0.8 °C and old Ctl 36.9 ± 0.4 °C and midazolam 36.5 ± 0.04 °C. Phosphorylated tau levels were normalized to each groups respective level of total tau (H, P), after controlling for gel loading with β -actin. Relative immunoreactive band intensities are expressed as a percent of Ctl and are displayed for each phosphoepitope and total tau. For each condition, 2 representative immunoblot bands are displayed. Data are expressed as mean \pm SD and ***, **** denote $p < 0.001$ and $p < 0.0001$, respectively, versus Ctl using an unpaired t -test.

unbound tau to more fully aggregate. Moreover, hTau mice express human, nonmutant tau, which would be expected to aggregate slower than other mice expressing the proaggregant P301L mutation (Lewis et al., 2000). In addition, the hTau used in our studies were still relatively young, which could have contributed to the absence of an increase in insoluble tau levels.

Recent studies have demonstrated that both intravenous (Run et al., 2009; Whittington et al., 2011, 2015) and inhalational

anesthetics (Le Freche et al., 2012; Tao et al., 2014) can induce tau hyperphosphorylation in the absence of hypothermia; however, clinically most of these classes of anesthetics have not been unequivocally associated with cognitive impairment in humans. In contrast, benzodiazepines are commonly used sedative and anxiolytic agents that have been clinically associated with an increased risk of short-term cognitive impairment (Chun, 2005; Curran, 1986; Ghoneim and Mewaldt, 1990; Hirshman et al., 2003; Lister, 1985).

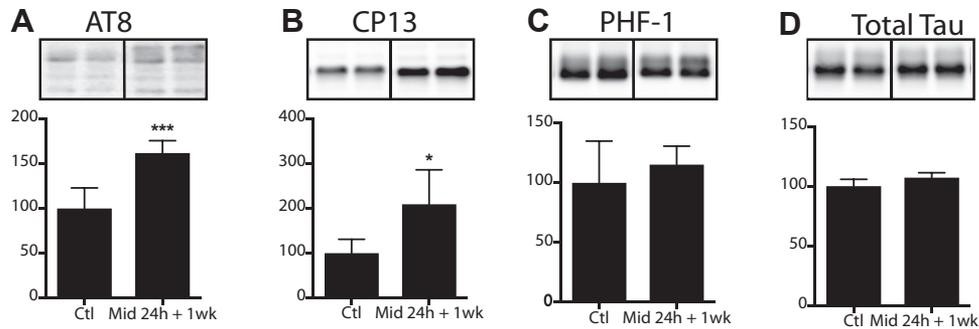


Fig. 8. Hippocampal tau phosphorylation following chronic midazolam administration persists as a function of age in young C57BL/6 mice. Hippocampal tau phosphorylation (% Ctl) at the AT8, CP13, PHF-1 phosphoepitopes (A–C) in 8- to 10-week-old (A–C) C57BL/6 mice treated with a subcutaneous infusion of midazolam 3 mg/kg/h ($n = 5$) or 0.9% saline (Ctl; $n = 6$) and sacrificed 1 week after the start of the 24 h infusion. Normothermia was maintained throughout the administration of midazolam or saline. Phosphorylated tau levels were normalized to total tau (D), after controlling for gel loading with β -actin. Relative immunoreactive band intensities are expressed as a percent of Ctl and are displayed for each phosphoepitope and total tau. For each condition, 2 representative immunoblot bands are displayed. Data are expressed as mean \pm SD and *, *** denote $p < 0.05$ and $p < 0.001$, respectively, versus Ctl using an unpaired t -test.

Moreover, using a prospective population-based study design with long-term follow-up, Billioti de Gage et al. recently demonstrated that ever use of benzodiazepines was associated with a 50% increase in dementia risk within 15 years after the start of this pharmacological class of drugs (Billioti de Gage et al., 2012), findings that, although controversial, have been further supported by a follow-up study (Billioti de Gage et al., 2014). The present study demonstrates that acute and chronic midazolam exposure increases tau phosphorylation, which theoretically could contribute to development or exacerbation of dementia. However, this transient exposure appears

to be insufficient to propagate higher degrees of pathology typically associated with tau hyperphosphorylation such as aggregation and the development of memory deficits.

As with many preclinical anesthesia neurotoxicity studies, it would be certainly premature to extrapolate these findings to clinical practice at this juncture. First of all, midazolam is extremely valuable as a sedative and anxiolytic agent in the immediate perioperative period as well as in the patient population requiring postoperative sedation. Nevertheless, these preclinical findings further strengthen the notion that clinical studies should

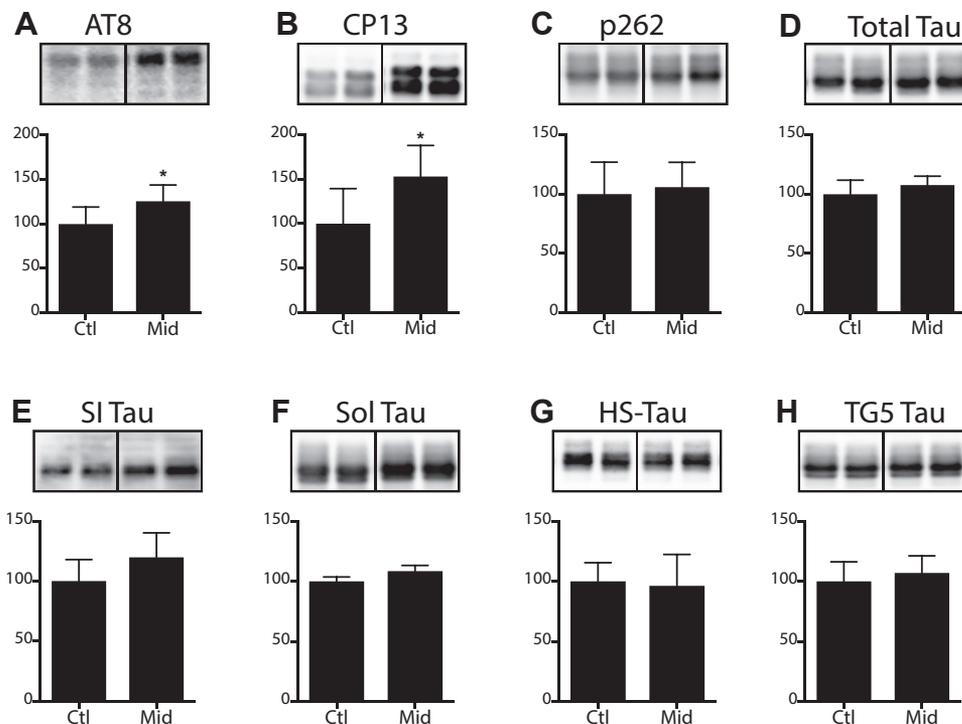


Fig. 9. Chronic midazolam administration increases hippocampal tau phosphorylation in hTau mice without altering tau solubility. Hippocampal tau phosphorylation (% Ctl) at the AT8, CP13, and PHF-1 phosphoepitopes (A–C) in 7-month-old hTau mice treated midazolam 5 mg/kg/h or 0.9% saline ($n = 6$ per group) and sacrificed 24 h after the start of the chronic infusion. Normothermia was maintained throughout the study and rectal temperatures were similar at the time of hippocampal tissue harvest: Ctl 37.3 ± 1.0 °C and midazolam 36.9 ± 0.3 °C. Phosphorylated tau levels were normalized to total tau (D), after controlling for gel loading with β -actin. Levels of tau in the sarkosyl-insoluble (SI; E), sarkosyl-soluble (Sol; F), and heat-stabilized (HS; G) and total (TG5) fractions (H) in cortical homogenates were measured using immunoblotting and total tau (TG5) antibody. Relative immunoreactive band intensities are expressed as a percent of Ctl and are displayed for each phosphoepitope and total tau. For each condition, 2 representative immunoblot bands are displayed. Data are expressed as mean \pm SD and * denotes $p < 0.05$ versus Ctl using an unpaired t -test.

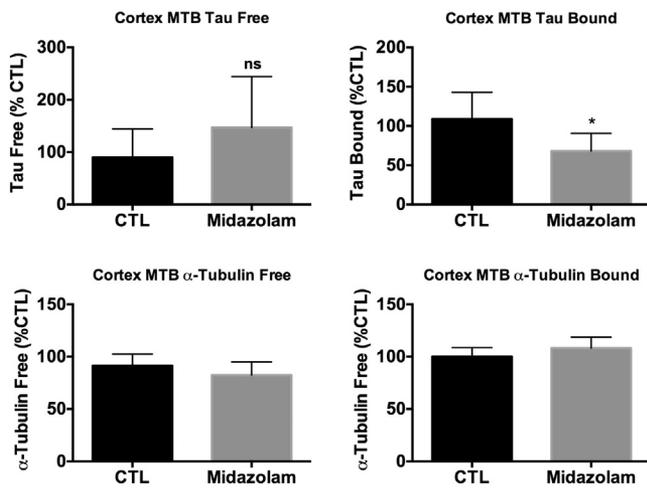


Fig. 10. Chronic midazolam administration decreases the capacity of tau to bind to taxol-stabilized preformed microtubules (MTs) in hTau mice. Neocortical brain proteins from 7-month-old hTau mice treated midazolam 5 mg/kg/h or 0.9% saline ($n = 6$ per group) for 24 h were extracted and incubated with taxol-stabilized MTs. Total tau and α -tubulin levels from the MT-free and -bound fractions were evaluated by immunoblot analysis. Levels of total tau and α -tubulin in MT-free (A and C, respectively) and -bound fractions (B and D, respectively) after midazolam treatment were compared to saline (Ctl) after controlling for gel loading with β -actin. Relative immunoreactive band intensities are expressed as a percent of Ctl and, for each condition, 2 representative immunoblot bands are displayed. Data are expressed as mean \pm SD and * denotes $p < 0.05$ versus Ctl using an unpaired t -test.

ultimately examine the impact of more prolonged benzodiazepine exposure on tau pathology in humans, especially given that this sedative class has been associated with a higher incidence of delirium in humans when compared with alpha-2 agonists (Riker et al., 2009) and an increased risk of incident dementia (Billioti de Gage et al., 2012).

In summary, acute midazolam administration in nontransgenic and transgenic mice results in hippocampal and cortical tau hyperphosphorylation that persists beyond recovery from its immediate sedative effects. This hippocampal tau phosphorylation pattern, after acute midazolam administration, differs greatly between C57BL/6 mice and hTau mice. Furthermore, acute midazolam-induced hyperphosphorylation does not impact tau aggregation or spatial memory in hTau mice, suggesting that acute exposure is unlikely to accelerate neurofibrillary pathology. Chronic midazolam administration is associated with the persistence of tau phosphorylation and a decrease in the capacity of tau to bind to exogenous, preassembled MTs. Hence, future studies should be directed at further establishing the pathological impact of a more prolonged or repeated exposure to this benzodiazepine.

Midazolam is not the most commonly used benzodiazepines. The most prescribed are alprazolam, diazepam, clonazepam, and lorazepam, but their effects on pathological markers of dementia are not yet understood. It is particularly a concern because a report has found that around 5.2% of U.S. adults used benzodiazepines, with this percentage climbing to 8.7% in the elderly (Olsson et al., 2015). Thus, future in vivo studies should be performed with other clinically used benzodiazepines to determine their comparative effects on tau pathology. The identification of such mechanisms may be important for ultimately determining how benzodiazepine use increases the risk of incident dementia.

Disclosure

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