

# Effects of alcohol and tramadol co-treatment on cognitive functions and neuro-inflammatory responses in the medial prefrontal cortex of juvenile male rats

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## Abstract

**Objectives:** Misuse and abuse of drugs are on the increase amongst juvenile individuals across the world. This study was carried out to evaluate the cognitive and subcellular neuropathologic events in the medial prefrontal cortex (mPFC) of juvenile male rats following exposure to alcohol and tramadol hydrochloride.

**Methods:** The rats were assigned into four groups; vehicle group, alcohol group, tramadol group, and the alcohol+tramadol combined group. Twenty-four hours after the administration of the last dose, 5 rats from each group were sacrificed. The mPFCs were excised and were stained with either cresyl violet or glia fibrillary acidic protein immunoreactivity. The remaining rats in each of the groups were subjected to cognitive behavioural tests.

**Results:** The administration of alcohol, tramadol and the co-administration of alcohol+tramadol triggers astrogliosis, glial scars and inflammatory responses relative to the vehicle-treated with well-preserved profile. The distribution of Nissl substances suggested that the neurons are either undergoing neurodegeneration or neuronal metabolism impairment. The behavioural tests showed that the administration of the respective substances impaired cognition in the treated rats compared to the vehicle-treated rats.

**Conclusion:** This study concluded that alcohol, tramadol, and alcohol+tramadol misuse can impair the functional integrity of the medial prefrontal cortex.

**Keywords:** brain; cognition; drug misuse; learning; memory; neural degeneration

Anatomy 2019;13(1):1–12 ©2019 Turkish Society of Anatomy and Clinical Anatomy (TSACA)

## Introduction

Tramadol is a synthetic centrally acting analgesic opioid which exerts its analgesic effect by blocking the re-uptake of norepinephrine and serotonin.<sup>[1-3]</sup> It is structurally related to codeine and morphine. Tramadol is a racemic mixture of two enantiomers, the (1R, 2R)-(+)- and (1S, 2S)-(-)-stereoisomers, which have differing affinities for  $\mu$ -receptors and monoamine re-uptake. (+)-Tramadol enantiomer preferentially blocks serotonin re-uptake and (-)-

tramadol is a potent suppressor of noradrenaline re-uptake.<sup>[1,4-6]</sup> It is used for the treatment of moderate to severe pain.<sup>[7-9]</sup> Observations from published reports have shown that tramadol can be effective in relieving the symptoms of anxiety, depression, and phobias,<sup>[9,10]</sup> and is used in the treatment of opiate withdrawal,<sup>[11]</sup> as well as premature ejaculation.<sup>[12]</sup>

Tramadol is assumed to be a safe drug devoid of the large number of severe adverse effects associated with many of the traditional opioids. However, tramadol might

be additive and could be a substantial contributor to fatal intoxication when consumed in excess with other drugs depressing the central nervous system.<sup>[13–16]</sup>

Alcohol use and abuse are factors associated with tremendous burden of diseases, injury, and medico-economical costs worldwide. Repeated and excessive alcohol consumption is associated with more than 60 diseases. According to published findings by Rehm et al.,<sup>[17]</sup> it was estimated that approximately 4% of the total mortality and between 4% and 5% of the disability-adjusted life-years (DALYs) recorded worldwide are attributable to alcohol. Furthermore, while the greater share of the alcohol-associated disease burden occurs in advanced countries, the medical burden inflicted per unit consumption of alcohol is highest in developing and poorer countries, thereby setting a major barrier for additional developments in these countries.<sup>[17]</sup>

In a demographic study by The World Health Organization, it was reported that about 10–16% of individuals who consume episodic alcohol excessively and repeatedly are aged 15 years or above are considered to be ‘problem drinkers’.<sup>[17,18]</sup> Many from these age group have a mild to moderate form of alcohol use disorder (AUD) and despite the negative consequences, are abnormally and excessively preoccupied with alcohol craving, seeking and consumption.<sup>[17,19]</sup>

Over time, there has been considerable progression in the pattern of alcohol consumption among teenagers.<sup>[20]</sup> A substantial body of evidence in clinical- and laboratory-based studies have described the vulnerability of the central nervous system to the deleterious effects of alcohol and that exposure to alcohol during ontogenesis can confer morphological and functional abnormality on the brain and other structures.<sup>[21–23]</sup> It has been reported that many of the neurotoxic abnormalities associated with adolescent or juvenile exposure to abused substances occur at the same time with biological modifications in the functional integrity of bio-chemistry of the nervous system, which significantly determine the excellent transmission of information from one brain area to another via neural circuits.<sup>[24,25]</sup>

The prefrontal cortex (PFC) is the association cortex of the frontal lobe.<sup>[26]</sup> It receives inputs from many cortical areas of the brain, and functions in executing affective, cognitive, and social behaviour and many other complex functions. The PFC constitutes the highest level of the cause of ontogeny. It has an extended ontogenesis, which permits the accession of higher cognitive and executive functions via experience, but makes it susceptible to factors that can lead to aberrant and defective operational

performance, manifested in neuropsychiatric disturbances.<sup>[27]</sup>

In the developing and advanced countries across the world, young people have the tendency of consuming alcohol in combination with other abused substances,<sup>[28]</sup> however, there is dearth of information on the effects of alcohol and its possible association with tramadol on the medial prefrontal cortex. Therefore, the main aim of this study was to examine the neuropathologic changes in the medial PFCs of juvenile male rats following exposure to alcohol and/or tramadol. Specifically, the objectives of the study were to determine the effects of treatments on the general cytoarchitecture, cell count of normal and degenerating neurons and astrocytes and expression of glial fibrillary acidic protein (GFAP) in the medial prefrontal cortex, and behavioural tests including MWM, passive avoidance, and novel object recognition.

## Materials and Methods

Forty juvenile male Wistar rats (postnatal day (PND) 28, body weight: 78–98 g) were used in this study. The rats were randomly divided into four groups. The rats were housed under standard conditions (n=10 per group; 12h light/dark cycle; 24±1°C room temperature; 53±12% relative humidity; rodent feed and clean drinking water *ad libitum*). All the experimental procedures documented in this experiment were performed in strict compliance with the ethical guidelines for the use of animals in laboratory research outlined by the Health Research Ethics Committee (HREC), College of Health Sciences, Osun State University (Osogbo, Nigeria) which is in conformity with the approved NIH Guidelines for the Care and Use of Laboratory Animals.<sup>[29]</sup>

The rats were randomly assigned into one of the following four groups; vehicle, alcohol-treated, tramadol hydrochloride-treated, and alcohol+tramadol hydrochloride co-treated. The rats in the vehicle group (n=10) were subcutaneously (s.c.) injected with double distilled water two times daily; alcohol-treated group (n=10) 1 ml of 15% v/v ethanol twice daily; tramadol-treated group (n=10) with 1 ml of 60 mg/kg/bw tramadol hydrochloride twice daily, and the alcohol-tramadol hydrochloride group (n=10) were with a combination of 1.0 ml of 15% v/v ethanol and 1 ml of 60 mg/kg/bw of tramadol hydrochloride two times daily. The dose of the drugs used in this study was adopted from our previous pilot study. The duration of treatments was 30 days. 24 h after the administrations of the last respective doses, 5 rats from each of the experimental groups were deeply anaesthetized and transcardially perfused for histochemical or immunohisto-

chemical staining procedures while the remaining five rats were exposed to MWM, passive avoidance, and novel object recognition behavioural tests. The summary of the experimental procedures is presented in **Table 1**.

Twenty-four hours after the administration of the last subcutaneous injection, 20 rats (n=5 from each of the experimental groups) were subjected to MWM, passive avoidance, and novel object recognition behavioural tests. All behavioural procedures were recorded with ANY-maze video tracking system (Stoelting; <http://www.anymaze.co.uk>). This test plays pivotal role in the validation of rodent models for cognitive evaluation and results are expressed by escape latencies to find the hidden platform in milky water. The modified method of Piermartiri<sup>[30]</sup> was used in this study. Briefly, the experimental apparatus consisted of white metallic circular white pool (diameter of 120 cm and a depth of 50 cm) containing non-toxic milky water (temperature of 25±1°C and height of 35 cm). An opaque Plexiglas escape platform (10×10 cm) was submerged 1.2 cm below the water surface at a constant position in the middle of the North-West (NW) quadrant. To reduce stress effects, the rats in each of the experimental groups were habituated to the MWM 24 hours prior to training sessions. The pool was placed in a test room containing various prominent visual cues. The animals were allowed to a spatial reference memory version of the water maze as previously described.<sup>[30]</sup> To reduce stress effects, the rats in each of the experimental groups were habituated to the MWM 24 hours prior to training sessions. The acquisition training sessions was performed on PND 59 and consisted of 10 consecutive trials, during which the animals were left in the pool facing the wall and allowed to swim freely to the escape platform. If an animal did not find the escape platform within a period of 60 s, it was gently guided to it. The animal was allowed to remain on the platform for 10 s after locating the escape platform, and it was then removed from the tank for 5 min before being placed at the next starting point in the pool. This procedure was repeated 10 times, with the starting points varying in a quasi-randomized manner. The test session was carried out on PND 60 after the training session. The test session consisted of a single probe trial where the escape platform was removed from the tank and each animal was allowed to swim for 60 s in the maze. The ANY-maze (Stoelting; [www.anymaze.co.uk](http://www.anymaze.co.uk) digital tracking system) was used in videotaping and recording the trials and probe tests. The tracks from trials and probe tests were statistically analyzed.

On PND 61, the rats in the respective experimental groups were trained on a one-trial step-through passive

**Table 1**  
Synopsis of experimental protocols (n=40 rats).

Days	Procedure
0 – 27	Animal breeding (40 F1 generation)
28 – 58	Experimental treatments/drug exposure
59	Animal sacrifice (n=5 from each group) Morris water maze training (n=5 from each group)
60	Morris water maze testing (n=5 from each group)
61	Passive avoidance training (n=5 from each group)
62	Passive avoidance testing (n=5 from each group)
63	Habituation to the novel object recognition chamber (n=5 from each group)
64	Novel object recognition testing (n=5 from each group)

avoidance test. The passive avoidance chamber was compartmentalized into two (one illuminated and one dark; a door separated the illuminated section from the dark section) equal sections, fitted with a white plastic laminate grid floor. During the training trial, each rat was placed in the illuminated section; as soon as the rat entered the dark section, the door was automatically shut, and the rat received an inescapable foot shock (0.25 mA, 1 s).<sup>[31]</sup> In the testing trial, given on PND 62, the rat was once again placed in the illuminated section and the duration the rats entered the dark section again was measured and the step-through latency maximum testing limit duration was 180 seconds.<sup>[32]</sup>

The novel object recognition (NOR) behavioural test was carried out in a wooden square chamber (60 × 60 × 45 cm) with non-glossy painted ply board walls and a white Formica floor divided by blue-black painted lines into 36 squares (10 × 10 cm). On PND 63, the rats were allowed 24 h habituation period to the NOR chamber, followed by the training and testing (PND 64) sessions as outlined by Marco et al.<sup>[33]</sup> with slight modification. During the habituation period, the rats were allowed to freely explore the chamber, under dim light conditions, for 300 s. The behaviour of the rat in the NOR chamber was video recorded for subsequent behavioural assessment. On PND 64, the procedure started with the training session. The rats were first exposed to two identical objects (two ceroplastic spherical balls) for them to explore the objects for 5 min. After a 5 h inter-trial interval, the test session began with the rats been exposed to one of the previously encountered objects (familiar object, F1 or F2) and to a novel, unfamiliar object (metallic red tinted spherical ball, N) for 5 min. The objects were placed in adjacent corners, at an approximate distance of 10 cm from the walls. At the commencement of each session, the rats were placed in the

middle of the chamber facing the objects. For each rat, the position of the objects was not changed between the training and test session. However, the objects' position was changed between the rats in order to prevent spatial preference. The NOR chamber and the objects were completely cleaned between tests on different rats with a 20% (v/v) ethanol solution. Both training and test sessions were video recorded (ANY-maze video tracking system; Stoelting; www.anymaze.co.uk) and the rats' behaviour was later evaluated by an experienced observer by means of event-recorder software (Observer<sup>®</sup>, Noldus, Netherlands). Exploration of an object was considered whenever the rats pointed their nose toward an object at a distance  $\leq 1.0$  cm, while turning around, climbing and/or biting the objects was not regarded as exploration. The time the rats' spent exploring the objects during the two sessions was recorded, and the discrimination index (DI) was calculated as the difference between the time spent exploring the novel object (N) and the familiar one (F1 or F2) in relation to the total time spent exploring the objects  $[(N-F) / (N+F)]$ . Rats that explored for less than 30 s during the training session and those only exploring just one of the objects during the test session were not included in the statistical analyses.

The rats then were deeply anesthetized with pentobarbital sodium and perfused transcardially with 50 ml normal saline and followed by 4% paraformaldehyde in tris buffer (pH 7.4) through the right cardiac ventricle and ascending aorta. The brains were then removed from the skulls of the rats, post-fixed in 10% phosphate-buffered formalin overnight, embedded in paraffin wax, and then sectioned at 5  $\mu$ m thickness on a microtome.

Sections obtained were used for Nissl staining and glial fibrillary acidic protein (GFAP) immunohistochemical staining.

Nissl staining was done according to the reported method of Hamburg et al.<sup>[34]</sup> Briefly, every 10th sections of the right halves of the mPFCs were mounted on glass slides, air dried, and immersed in 70% ethanol for 12 h. Treatment with 0.1% cresyl violet solution (Sigma-Aldrich, St. Louis, MO, USA) for 20 min was followed by differentiation in 70% ethanol for 10 min. Subsequently, the sections were dehydrated in ascending grades of ethanol (70% for 10 min, 96% for 20 min and 100% for 30 min), treated with two changes of xylene (100%, 10 min each) and covered with coverslips using DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Five periodic Nissl-stained sections at 4.7 to 2.7 mm ventral and 4.7 to 2.7 mm dorsal to the bregma were used for cell counts and the average was calculated. In the mPFC, neurons

were counted in two different regions delineated with a rectangle measuring  $0.47 \times 0.35$  mm<sup>2</sup>. The midpoints of both counting boxes were 5.0 mm apart from each other. The specimens were scanned at a magnification of  $\times 200$  within the identical areas in the mPFC, using a Zeiss AxioScope A1 with a camera scope (AxioCamMRc, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) attached to a computer interface. The intact neurons were outlined as non-basophilic neurons with both the pale nuclei and the discrete nuclei. Any neurons that had nucleolar fragments bigger than one-half of the average nucleolar diameter were included in the count as well as the neurons that had intact neuronal bodies.<sup>[35]</sup> The number of degenerating neurons was quantified as; (neuronal cell number in vehicle-treated rats) - (number of neuronal cells from the other respective-treated groups). All neuronal cell counting was performed by pathologist blinded to the grouping and treatment conditions.

The IHC of glial fibrillary acidic protein (GFAP) was performed according to the method of Adekomi.<sup>[32]</sup> Briefly, immunohistochemical analysis of GFAP was carried out on every 10th section of the left halves of the mPFC from the rats in the respective treatment groups. 5  $\mu$ m-thick sections of the mPFC were fixed with 10% phosphate-buffered formalin for 6 hours. After blocking endogenous peroxidase, the sections were incubated with the primary antibodies; polyclonal anti-GFAP (1:100, Dako, Glostrup, Denmark). The peroxidase reaction was visualized using 0.03% DAB and 0.005% hydrogen peroxide. The immunostained sections were slightly counterstained with cresyl violet, dehydrated, cleared, and mounted in DPX (Dako, Glostrup, Denmark). The control sections for the IHC of GFAP were performed by excluding primary antibody and substituting it with a non-immune serum. The count of GFAP immunoreactive cells in the mPFC of the treated rats were quantified in 2.5 mm<sup>2</sup> fields of mPFC sections of five rats each from all the experimental groups using a X40 objective with a calibrated ocular micrometer system (Olympus Corporation, Tokyo, Japan). Counting of the GFAP immunoreactive cells in the mPFC were done by blinded microscopic observation by three pathologists who had no inkling of the study. Similar levels of mPFC (4.7 to 2.7 mm ventral and 4.7 to 2.7 mm dorsal to bregma) sections were maintained across the experimental groups according to the stereotaxic mouse brain atlas by Paxinos and Franklin.<sup>[36]</sup>

Data obtained are expressed as mean  $\pm$  SEM. The studied parameters were analysed using one-way analysis of variance. The statistical evaluation of the results was carried out using one- or two-way ANOVA with treatment

and number of trials (repeated measures) as the independent variables. Following significant ANOVAs, multiple post hoc comparisons were carried out using the Duncan test. After subjecting the data obtained from the cell count to one-way ANOVA, the data were further analysed using Kruskal–Wallis test. If any significance was observed, independent comparisons were made using Mann–Whitney's U test. A p-value <0.05 compared to vehicle values was considered statistically significant.

## Results

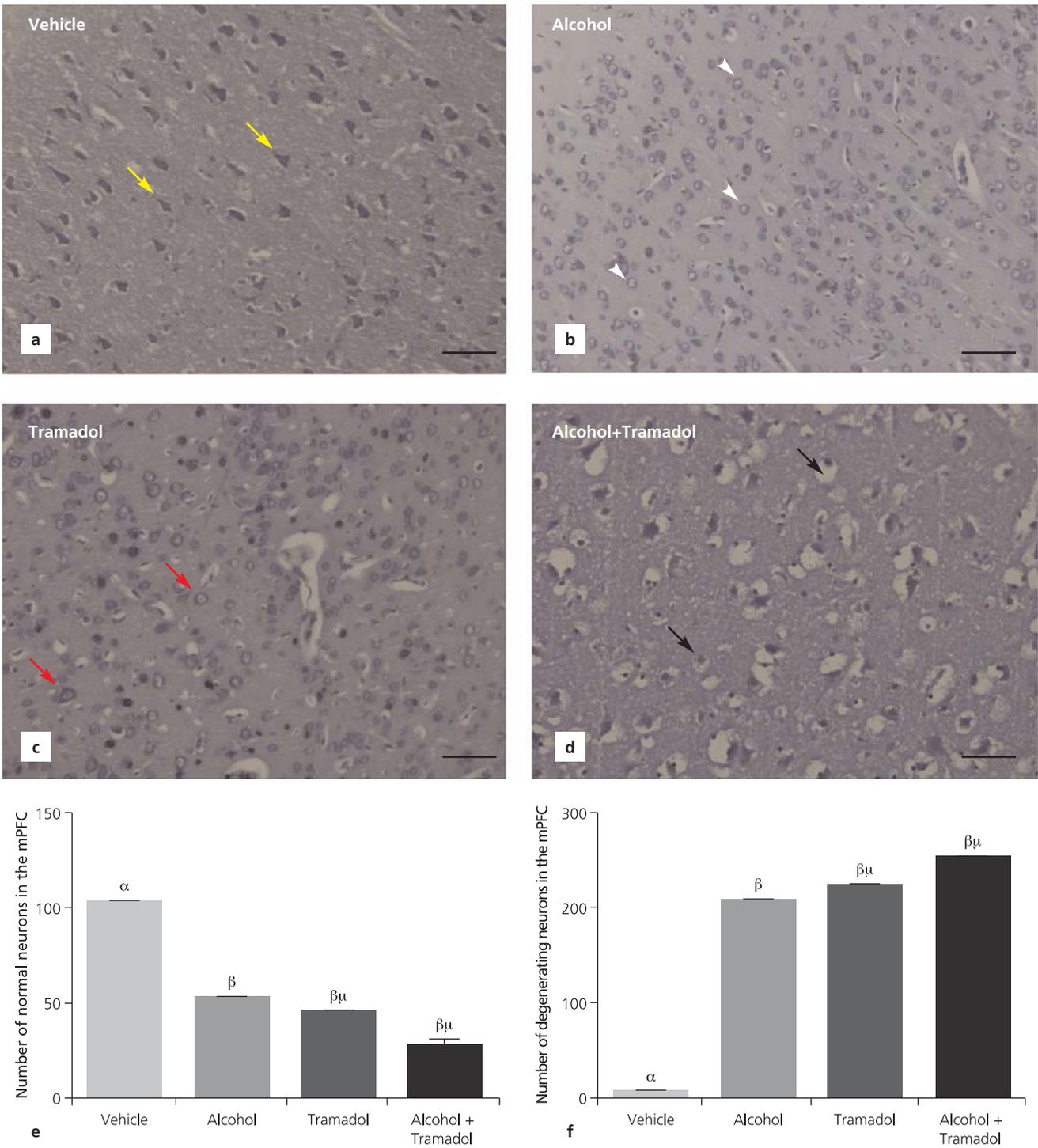
The general cytoarchitectural profile of the neurons in the cresyl violet stained sections of the mPFCs of the rats in the vehicle-treated group was well preserved. The mPFC of the rats in this group showed neurons with normal appearance, prominent basophilic cytoplasm, and small-sized neuroglia cells uniformly dispersed within the neuropil (**Figure 1a**). On the other hand, the cellular profile of the mPFC of the rats in the alcohol-treated group displayed intense deviations from the normal cytoarchitecture. These changes were characterized by heterogeneous pattern, including increased neuronal vacuolization and less cytoplasm, chromatolysis, loss of nissl substances/deposition of nissl substances at the perinuclear membranes of the neurons, and neuronal shrinkage with small pyknotic or karyorrhectic nuclei (**Figure 1b**). The cytoarchitectural profile of the mPFC in the tramadol-treated group had similar features, but the neuropil had varying sizes of vacuolations and a couple of perinuclear spaces were present around the monomorphic neurons (**Figure 1c**). Marked neuropathological characteristics were observed in the mPFCs of the rats treated with alcohol+tramadol combination. These characteristics include chromatolysis, accumulation of neurofibrils at the perinuclear rim of the neurons, heterogenous neuronal apoptotic appearance, fragmented cytoplasmic contents with deposition of nissl substances at the perinuclear membranes of the neurons, hypertrophy of the neurons (**Figure 1d**). There was a significant loss in the number of normal neurons obtained from the cresyl violet stained sections of the mPFCs of the rats in the alcohol-treated, tramadol-treated and alcohol+tramadol combined groups compared to those of control group ( $p < 0.05$ ). There was no significant difference in the number of normal neurons in the alcohol treated compared to the tramadol treated groups. Significant difference ( $p < 0.05$ ) was observed in the number of normal neuron among groups. Furthermore, the number of normal neurons in both alcohol-treated and tramadol-treated groups were significantly higher ( $p < 0.05$ ) than the number of normal neurons in the alcohol+tramadol co-treated group (**Figure 1e**). The number

of degenerating neurons in the vehicle group was significantly less ( $p < 0.05$ ) than the alcohol-treated, tramadol-treated, and alcohol+tramadol co-treated group, respectively (**Figure 1f**).

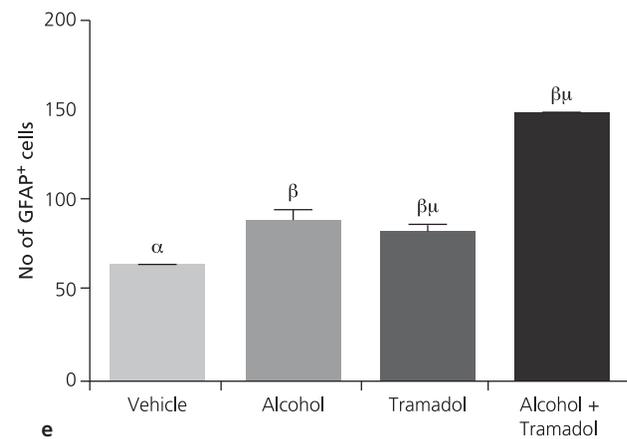
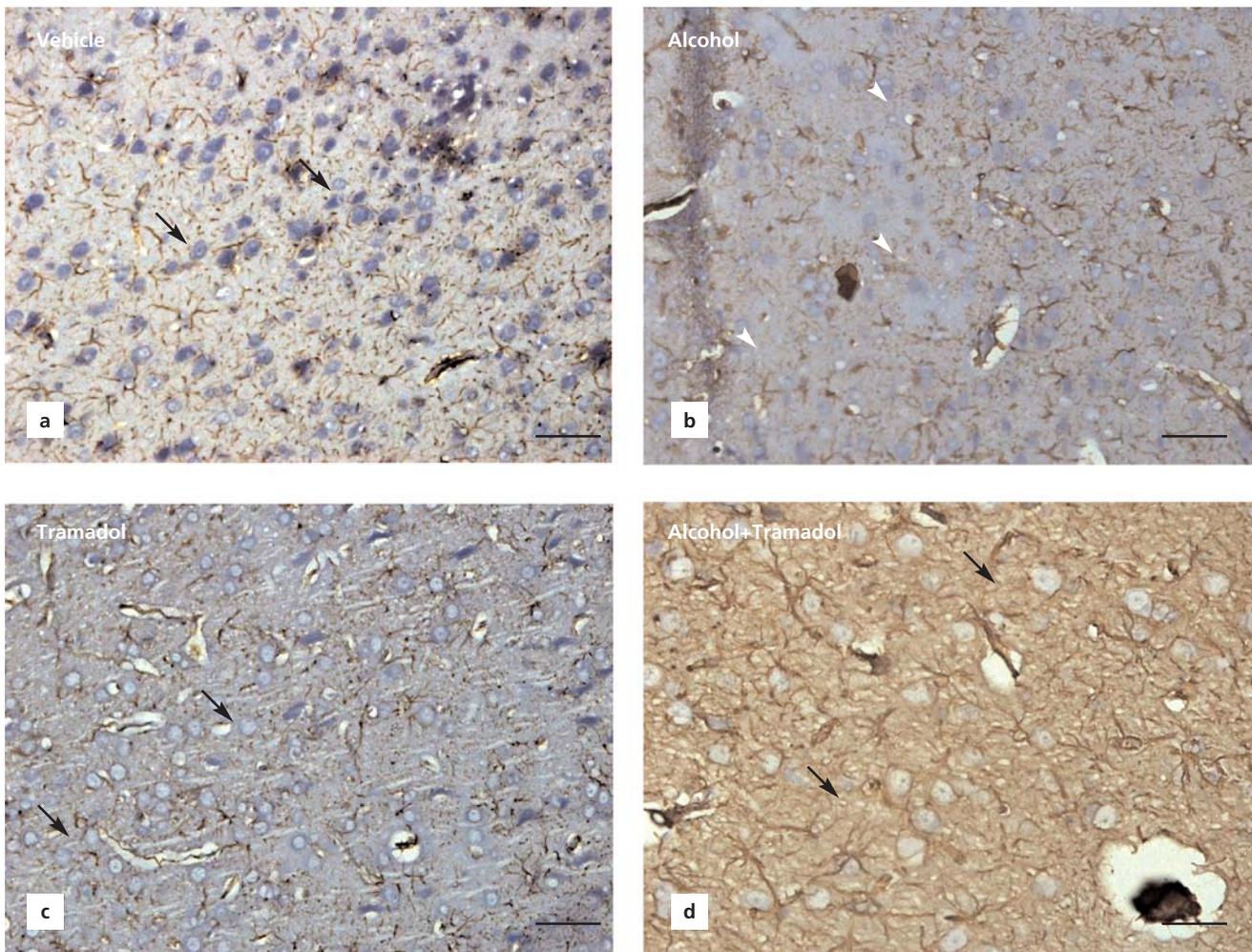
The activation of astrocytic cells following alcohol, tramadol and alcohol+tramadol-induced neuronal damage was demonstrated by using anti-GFAP antibody as a marker of astrocytic reaction in neuroinflammation (**Figures 2a–d**). In the vehicle treated group, immunohistochemical staining of the mPFC for GFAP displayed sparsely distributed GFAP-immunoreactive astrocytes with normal spatial arrangement, size, and dark brown cytoplasmic fibres which formed an organized array of network in the neuropil (**Figure 2a**). On the other hand, the astrocytes in the alcohol-treated group showed disruptive features, including astrogliosis with numerous small-sized astroglia ebbing around the pyknotic neurons (**Figure 2b**). The immunohistochemistry of GFAP in the tramadol-treated group had comparable cellular morphology with the alcohol-treated group. The mPFC was likewise characterized by reactive astrocytes traversing the entire neuropils (**Figure 2c**). GFAP-immunoreactive astrocytes increased across the mPFC section of the rats in the alcohol+tramadol co-treated group. The astrocytes were hypertrophied, and the neuropils had numerous glia scar around the degenerating neurons (**Figure 2d**).

Quantitative astrocytic cell count was used to confirm the GFAP immunohistochemical staining in the mPFCs of the rats in the treatment groups. In the mPFC, quantification of the number of GFAP-immunoreactive cells showed that the mean number of astrocytes in mPFC of the rats in the alcohol-treated, tramadol-treated, and alcohol+tramadol combined groups was significantly increased ( $p < 0.05$ ) relative to the vehicle group (**Figure 2e**). Significant difference ( $p < 0.05$ ) was observed in the number of GFAP-positive cells between alcohol-treated, tramadol-treated and alcohol+tramadol co-treated groups. There was no significant difference ( $p > 0.05$ ) in the number of GFAP-immunoreactive cells in the alcohol treated compared with the tramadol treated groups. The alcohol-treated group had significant lower number of astrocytes than the alcohol+tramadol co-treated group ( $p < 0.05$ ). Similarly, the number of GFAP-positive cells in the tramadol-treated group was significantly reduced ( $p < 0.05$ ) than the number of GFAP-positive cells in the alcohol+tramadol co-treated group (**Figure 2e**).

In this study, we examined the ability of the rats to acquire (training session) and retrieve (test session), spatial information suggestive of learning and memory capabili-



**Figure 1.** Histochemical demonstration of Nissl's substances using cresyl violet stain. In the vehicle group (a), CV-stained neurons are with normal appearance and prominent basophilic cytoplasm (yellow arrows). Observable changes were prominent in the distribution of the Nissl substances in the neurons (arrowheads) in the mPFC of the alcohol treated rats (b). These includes; chromatolysis, tired cytoplasm and peri-nuclear Nissl deposits, pyknotic neurons, and neurons with ruptured membrane (white arrowhead). In the tramadol treated group (c), there were patho-anatomical features such as pyknosis, chromatolysis, and accumulation of Nissl's substances at the perinuclear surface of the neurons, and heterogenous neuronal apoptotic appearances (red arrows). In the alcohol+tramadol treated group (d), there was marked hypertrophied neuronal cell degeneration as well as increased apoptotic cells. Scale bar=100 μm. The mean number of normal (e) and degenerating (f) neurons respectively per group (n=5 per group; "α" p<0.05, significant difference from the vehicle group and the other groups; "β" p<0.05, significant difference between alcohol treated, tramadol and alcohol+tramadol groups while; "μ" p<0.05, significant difference between tramadol and tramadol groups). [Color figure can be viewed in the online issue, which is available at [www.anatomy.org.tr](http://www.anatomy.org.tr)]



**Figure 2.** Immunohistochemical demonstration of astrocytes using glial fibrillary acidic protein (GFAP) stain in the medial prefrontal cortex of the experimental rats. (a) Astrocyte processes are not overlapping, and many of the astrocytes do not express detectable levels of GFAP. (b) Moderately reactive astrogliosis, most of the astrocytes have upregulated expression of GFAP with noticeable astrocyte proliferation, as well as marked overlap of astrocyte processes. (c) Abundant reactive astrocytes processes after the loss of a large number of neurons. (d) Marked neuronal loss and severe diffuse reactive astrogliosis with significant upregulation of GFAP expression, astrocyte hypertrophy, astrocyte proliferation. Scale bar=100 μm. E. The graphical illustration of the number of GFAP-positive cells (e) per group (n=5 per group; “α” p<0.05, significant difference from the vehicle group and the other groups; “β” p<0.05, significant difference between alcohol treated, tramadol and alcohol+tramadol groups while; “μ” p<0.05, significant difference between tramadol and alcohol+tramadol groups). [Color figure can be viewed in the online issue, which is available at [www.anatomy.org.tr](http://www.anatomy.org.tr)]

ties. In the training session (Figure 3a) carried out on PND 59, two-way ANOVA (treatment against repeated measures) showed that a significant effect for the main factors. There was a significant difference (p<0.001) between vehicle and the alcohol group in all the trials except for trial 1 and 3. Also, there was significant difference

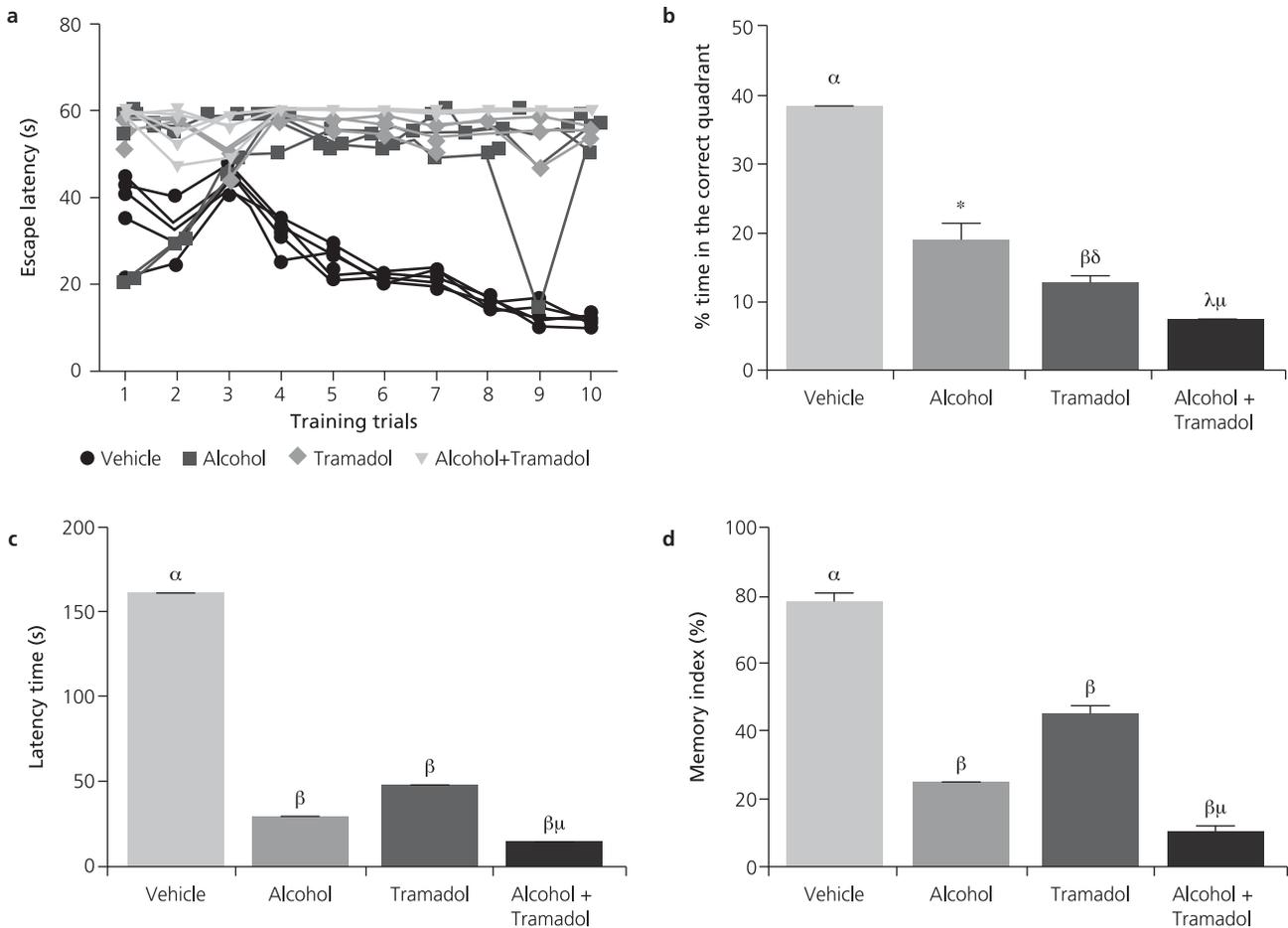
(p<0.001) between vehicle and tramadol in all trials except during the 3rd trial. Meanwhile, there was significant difference (p<0.001) in all the trials between vehicle and alcohol+tramadol. In the test session, performed 24 h after the training session, one-way ANOVA showed a significant effect for the treatments. Subsequent post-hoc compar-

isons showed that treatment with alcohol, tramadol and the combination of alcohol+tramadol showed a significant decline in both learning and memory as revealed by longer latencies (Figure 3a) compared to the vehicle, and reduced target quadrant preference during the probe trial (Figure 3b).

In the passive avoidance behavioural test, exposure to alcohol, tramadol and the combination of alcohol+tramadol altered the latency time when compared with the vehicle group. In the alcohol-treated group, the latency time in the passive avoidance test was significantly reduced ( $p < 0.05$ ) compared with the vehicle group. In addition, there was also a significant reduction ( $p < 0.05$ ) in the latency time between alcohol-treated group and

the alcohol+tramadol co-treated group. However, the latency time was reversed in the tramadol-treated group compared with the alcohol-treated group. There was also significant difference ( $p < 0.05$ ) in latency time between the tramadol-treated group and the alcohol+tramadol co-treated group (Figure 3c).

Novel-object recognition test was done to evaluate non-spatial working memory in the experimental rats. Exposure to alcohol, tramadol, and alcohol+tramadol treatments triggers significant alterations in memory function relative to the vehicle-treated. Treatment with alcohol resulted in significant decrease ( $p < 0.05$ ) in memory function relative to the control. The tramadol-treated group displayed a less significant decrease in memory function



**Figure 3.** Exposure to alcohol, tramadol and the combination of alcohol+tramadol triggered cognitive deficits in the experimental rats. (a) Training trials were performed on PND 59. Values are expressed as mean  $\pm$  SEM latency in seconds for escape to a hidden platform (n=5 for each group). (b) The probe test session was carried out 24 h after the training trials. Values are expressed as mean  $\pm$  SEM of the time spent in the in the correct quadrant. (c) The latency of time of passive avoidance test across the groups. (n=5 per group at  $p < 0.001$ ). " $\alpha$ " represents significant difference between control and the other groups,  $\beta$  represent significant difference between alcohol, tramadol and alcohol+tramadol groups while " $\mu$ " represents significance difference between tramadol and alcohol+tramadol groups. (d) The memory index of novel object recognition test across the experimental groups. (n=5 per group at  $p$ -value  $< 0.001$ ). " $\mu$ " represents significant difference between vehicle and the other groups,  $\beta$  represent significant difference between alcohol, tramadol and alcohol+tramadol groups while " $\mu$ " represents significance difference between tramadol and alcohol+tramadol groups.

against the vehicle-treated ( $p < 0.05$ ), when considered with alcohol against the vehicle ( $p < 0.05$ ) and alcohol+tramadol against the vehicle ( $p < 0.05$ ). Furthermore, exposure to alcohol+tramadol co-treatment induced memory consolidation defect compared with the vehicle-treated (Figure 3d).

## Discussion

In this study, we examined the effects of alcohol, tramadol, and the combination of alcohol and tramadol on some cognitive behavioural parameters using MWM, passive avoidance test, and novel object recognition test and sub-cellular neuropathological procedures using histological and immunohistochemical protocol in the mPFCs of juvenile male rats.

Data obtained in this study demonstrated that alcohol+tramadol co-treatment triggers shortfalls in the MWM test. Compared to the vehicle-treated group, alcohol-, tramadol-, alcohol+tramadol-treated groups spent significantly more time locating the escape platform in the swimming chamber. Observations from experiments in which animals and humans were exposed to alcohol and opioids suggest that stimulants and opioids are capable of conferring memory consolidation defect through alteration of the functional integrity of the opioid-neurotransmitter combination pathway(s), thereby triggering alcohol-opioid-related brain damage.<sup>[32,37-39]</sup>

In the MWM test, the vehicle-treated group easily found the location of the platform. No significant difference was observed when statistical comparison was made on the latency time to locate the escape platform between the alcohol and tramadol-treated groups. In addition, in the passive avoidance test, treatment with alcohol, tramadol, and, alcohol+tramadol impaired the latency time spent in the passive avoidance test (PAT) compared with the vehicle. These behavioral deviations are suggestive of the deleterious effects of alcohol, tramadol, and, alcohol+tramadol on cognition. There was a slight attenuation in the latency time in the PAT in the tramadol-treated group compared with the alcohol treated and the alcohol+tramadol groups, respectively. Also, there was a slight attenuation in the latency time in the PAT between alcohol and alcohol+tramadol treated groups. Therefore, based on these results, it could be suggested that the combined use of alcohol+tramadol could impair learning and memory in juvenile male rats. This observation is similar to a published report from our laboratory in which we observed that exposure to morphine-alcohol combination confers deleterious effects on cognition in juvenile male rats.<sup>[32]</sup> These impairments could also be linked to the dele-

terious effects of the substances administered on motivational and sensorimotor centers in the brain.

A large number of authors studied the singular effects of alcohol, and tramadol on neurobehavioral paradigm; however, to our knowledge, this is the first study to examine the effects of co-administration of alcohol+tramadol on cognitive behaviours in juvenile male rats.

Evidence from the histochemical and immunohistochemical data showed that the administration of alcohol, tramadol and the combination of alcohol+tramadol alters the cytoarchitectural profile of the mPFC of the treated juvenile male rats. Undoubtedly, adolescent and juvenile in the developing and developed world are significantly abusing drugs on daily basis. These drugs are either abused alone or in addition with other substances.

Studies have shown that tramadol abuse confers deleterious effects on the functional integrities of the CNS.<sup>[9,40,41]</sup>

In rats, tramadol preferentially gains access to the brain tissues compared to its active metabolite.<sup>[42]</sup> It was postulated that tramadol potentially induced neurotoxicity in rabbits by decreasing membrane fluidity of the blood brain barrier secondary to loss of unsaturation and fundamental changes in the structural concentrations and number of fatty acids.<sup>[43]</sup>

Evidences of neuronal degeneration and inflammation were observed under light microscopy in the mPFC of the rats receiving alcohol, tramadol, and the combination of alcohol+tramadol. In the mPFC, the degenerating neurons were characterized by fragmented cytoplasm, nuclear pyknosis, vacuolated neuropil, astrogliosis and glial scar surrounding the degenerating neurons. These features were further confirmed by the count of the normal and degenerating neurons, as well the number of astrocytes observed following the administration of the respective substances. Furthermore, the invasion of GFAP-immunoreactive cells in the mPFC of the rats in the alcohol, tramadol, and alcohol+tramadol treated groups was presumably the result of the chemotactic factors generated by the degenerating neuronal cells, and this further suggests that astrocytes can serve as facultative phagocytes in drug related neurotoxicity. In this context, the juvenile model of tramadol and alcohol-induced neuronal degeneration observed in this study provides a good system to study the neuron-glia interactions in response neuronal injury following exposure to alcohol and opioid combination. These observations are in consonant with the findings of Kofke et al.<sup>[44,45]</sup> and Miao et al.<sup>[46]</sup>

The significant difference in the numerical density of degenerating neurons in the mPFC of the experimental

rats could be an indication of severe damage to the neuronal cytoplasmic contents which contain the cellular machinery required for the functional biology of the cells.<sup>[47]</sup>

Numerous studies have identified that dysfunction of specific PFC subregions including the medial, anterior cingulate, and orbitofrontal cortices can promote drug- or ethanol-seeking behaviour.<sup>[48,49]</sup> Additional experiments in rodent models of ethanol consumption also further highlight the importance of the medial PFC. For example, lower numbers of GFAP-immunoreactive astrocytes have been observed in the prelimbic cortex of rats genetically bred for their high preference for ethanol over water and infusion of gliotoxins or a gap junction blocker into the rat prelimbic cortex increased preference for ethanol.<sup>[50]</sup>

Despite the changes in the number of GFAP-immunoreactive astrocytes within the mPFC of the alcohol treated rats, no significant changes were detected in the number of GFAP-immunoreactive astrocytes within the mPFC of the alcohol and tramadol treated rats. The nissl stain showed some changes that may have occurred within neuronal cell populations. It is known that neuronal loss occurs in the orbitofrontal cortex of rats following repeated ethanol consumption.<sup>[51]</sup> Regardless, the observed alterations in GFAP-immunoreactive cells could have profound impact on neural plasticity since astrocytes can actively modulate neuronal activity.<sup>[52]</sup>

Degenerating neuronal cells seem to be drained by the sustained augmented activity in response to the administration of alcohol+tramadol. It was shown that rats exposed to stress factors, including chemical substance, developed disturbance in the function of serotonin receptors in nerve cells and other tissues with subsequent occurrence of uncontrolled cholinergic action causing vasoconstriction and ischemia.<sup>[53]</sup> Moreover, stress-induced modulation of dopamine D1 and serotonin receptors functions through hyperactivation of cyclic adenosine 3-5-monophosphate which triggered neuronal degeneration as suggested by Tsukada et al.<sup>[54]</sup> Evidences from the cresyl violet stain further suggest that opioids may be involved in neurodegeneration. Although there are conflicting data in the scientific literature concerning the effects of opioids on programme cell death, *in vitro* experiments using specific cell lines revealed that opioids are capable of stimulating apoptosis.<sup>[9,55]</sup>

Alcohol-opioid combination may be involved in programmed cell death. There are conflicting results in the literature concerning the effects of opioids on apoptosis. *In vitro* studies using specific cell lines showed that opioids might induce or enhance apoptosis.<sup>[55]</sup> Another likely

and/or possible mechanism of alcohol+tramadol induced brain damage is the decrement in the rat brain activities of Na<sup>+</sup>/K<sup>+</sup>-, Mg<sup>2+</sup>- and Ca<sup>2+</sup>-dependent ATPases with subsequent decrease in ATP turnover and energy metabolism, as well as loss of mitochondrial membrane transport functions.<sup>[56]</sup> In addition, tramadol and/or its active metabolite may react with alcohol to trigger the release of excessive ROS leading to DNA breakage.<sup>[57]</sup> Quantitative and pathological observations from this study is in support of the claims of Hauser et al.<sup>[58]</sup> and Eisch et al.<sup>[59]</sup> which suggested that opiate exposure can decrease the proliferation and survival of new neurons in the mature adult brain by acting directly on the neurocytes progenitor population, so decrease their proliferation and DNA synthesis via an opioid action at the  $\mu$ -opioid receptor.

## Conclusion

Exposure to alcohol and/or tramadol impaired cognition, learning and memory, and astrocytic activation became more prominent in the mPFC of the rats compared with the vehicle-treated rats. Furthermore, administration of these substances also conferred deleterious and toxic effects on the cellular profile of the mPFC of juvenile male rats.

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*Conflict of interest statement:* No conflicts declared.

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