Anticholinergic drug atropine diminishes newly formed fear memory in male rats

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Abstract: Post-traumatic stress disorder (PTSD) is a condition which is triggered shortly after experiencing traumatic events. PTSD is complicated by the fact that people with PTSD often develop additional disorders such as phobias, addiction, depression, panic disorder and obsessive–compulsive disorder. Beta-adrenergic and cholinergic system both are involved in memory formation as well as in emotional response associated with memory. It is reported that the administration of beta-adrenergic and cholinergic antagonist results in the impairment in memory formation. Here, we examined the potential of beta-adrenergic antagonist propranolol and muscarinic cholinergic antagonist atropine for impairing the recently formed fear memory associated with PTSD. Reconsolidation is the memory process during which labile memory converts into permanent memory. In this study it is hypothesized that if recently formed fear memory is disturbed during reconsolidation phase by pharmacological intervention then it could be possible to impair well-consolidated fear memory. Atropine and propranolol were injected in separate set of rats (n=6) just after the reactivation of fear memory. Short term memory and long term memory were monitored after 2 h and 24 h of reactivation respectively. Results of current study demonstrated that only atropine showed significant impairment of reconsolidation of newly formed fear memory whereas propranolol did not show fear memory disrupting effects. The results emphasize the significance of pharmacological intervention to impair reconsolidation of newly formed fear memory.

Keywords: Atropine, Pavlovian fear conditioning, propranolol, reconsolidation.

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a behavioral disorder which develops after a person encounters or witnesses an unexpected extreme traumatic stressor (Gardner and Griffiths, 2014) such as war, natural disasters, violent personal assault and terrorist attack (Iribarren *et al.,* 2005). It is vague yet why some people develop PTSD while others do not. Both the age and intensity of the trauma are essential risk factors (Javidi and Yadollahie, 2011). PTSD presents a significant burden not only to individuals but society at-large (Lonergan and Pitman, 2013). A major emphasis of PTSD research is to evaluate how these fear memories are developed, stored in brain and how they might be expunged. It has been demonstrated that fearful and traumatic memories are an essential feature of PTSD (Javidi and Yadollahie, 2011).

In Pavlovian conditioning, stimulus which can cause some kind of unlearned response, such as freezing due to electric shock, is regarded as unconditioned stimulus (US) whereas neutral stimulus which does not produce any response itself, for example bell, is considered as conditioned stimulus (CS). However, when US is paired with CS it elicits conditioned response that is freezing. To reconsolidate a memory of an emotional event, it should be retrieved first, which is done by "recalled trial". After repetitive pairings of CS with US and then the CS alone may reactivate the fear memory encountered earlier by US (Treanor *et al.,* 2017). In recent years, a small number of pharmacological compounds have appeared that are used in a clinical setup like antidepressants such as selective serotonin reuptake inhibitors (SSRIs). These are presently known for pharmacological treatment of PTSD (Monsey *et al.,* 2015) but practically their outcome on acute stress disorder is inadequate (Shalev, 2009). Recent research with respect to medical care of PTSD suggested the use of propranolol, which is a nonselective antagonist of beta-adrenergic receptors which can readily cross the blood brain barrier (Villain *et al.,* 2016). Increased level of epinephrine and norepinephrine in PTSD patients may cause stress (Sherin and Nemeroff, 2011). The binding of norepinephrine to beta-adrenergic receptors affects the memory consolidation (McIntyre *et al.,* 2012). Propranolol not only diminishes memory formation but also dissociates the emotional response from the retained memory. It has been suggested that propranolol helps the

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individual to forget their physical experiences and support them to dampen the emotions and fears associated with fear memories (Tully and Bolshakov, 2010). A growing number of researches supporting the idea that cholinergic system is involved in both consolidation and reconsolidation of memory processes (Blake *et al.,* 2012; Blake *et al.,* 2014). Administration of anti-cholinergic drug that can cross blood brain barrier such as atropine blocks muscarinic receptor and causes memory impairment (Tinsley *et al.,* 2004). Administration of muscarinic cholinergic antagonist impairs memory in different training tasks like inhibitory avoidance task, Pavlovian fear conditioning, conditioned place preference (Byrne, 2010).

We raised the question whether providing immediate treatment using pharmacological interventions after experiencing a traumatic event would disrupt fear memory associated with PTSD. So, the present study was designed to monitor the effects of atropine and propranolol on newly formed fear memory reconsolidation in rats using Pavlovian fear conditioning apparatus.

MATERIALS AND METHODS

Subjects

A total of 24 locally bred adult male albino Wistar rats, 2–3 months of age and weighing 250-300g were purchased from the HEJ Research Institute, University of Karachi. The animals were housed separately in plastic cages with free access to cubes of standard rodent's diet and tap water throughout the experiment. The experiment was approved by institutional Board of Advanced Studies and Research and executed in line with National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Experimental protocol

Rats were randomly divided into 3 groups. Control group received saline (0.9% NaCl) whereas, second and third groups were intraperitoneally injected with atropine at the dose of 1 mg/kg (Nootarki *et al.,* 2015) and propranolol at the dose of 10 mg/kg, respectively (Taherian *et al.,* 2014). Drugs were administered just after the reactivation of fear memory (Villain *et al.,* 2016). The drugs propranolol and atropine used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA) and saline was used as a vehicle to dissolve the drugs.

Pavlovian fear conditioning

Pavlovian fear conditioning is used to develop fear in animals and to evaluate the consequences of pharmacological interventions on fear memory reconsolidation. Experiments were conducted in two Plexiglas chambers 19×9×14 cm, contained in a sound-attenuating compartment. It consisted of four sessions. Habituation carried out on day 0 during which rats were acclimatized before training to chamber A, comprised of a brightly illuminated chamber equipped with a grid-iron floor, for 15 min (Monsey *et al.,* 2015). Conditioning session was carried out 24h after habituation during which rats were placed in the same conditioning chamber A. During conditioning three trials of CS-US pairings consisting of 20s, 5kHz, 75dB tone (CS) which was co-terminated with 1s, 0.5mA footshock (US) were given after 3 min baseline period. The inter-trial interval was 1 min and freezing behavior was measured during tone. Rats were kept in the chamber for additional 1 min after the last shock and then immediately removed from the apparatus (Liu *et al.,* 2004). After 24 h of conditioning session, reactivation session was performed in testing chamber (chamber B) comprised of flat black plastic floor that had been washed with a pepper mint scented soap. Following 3 min baseline period, rats exposed with a single presentation of a CS tone (20s, 5 kHz, 75dB). The testing procedure was performed after 2 h (PR-STM) and 24 h (PR-LTM) of reactivation of fear memory (fig. 1). During short term memory testing rats received three presentations of CS tones (20s, 5kHz, 75dB) with 1 min inter-trial interval. After 24h rats received 10 CS tone presentations (20s, 5kHz, 75dB) with 1 min inter-trial interval and freezing behavior was measured. If freezing behavior is absent or reduced, it shows impaired reconsolidation of fear memory and vice versa (Monsey *et al.,* 2015).

STATISTICAL ANALYSIS

Data was analyzed by two-way ANOVA with repeated measures whereas one-way ANOVA was used to analyze the data of reactivation using SPSS version 20. *Post-hoc* analysis was carried by Bonferroni test. p values <0.05 were considered as significant.

RESULTS

In our experiment, we evaluated the effects of pharmacological intervention that is propranolol and atropine.

Conditioning

All groups exhibited intact post shock freezing during the initial training session (fig. 2). The ANOVA for post shock freezing scores revealed a significant effect of trial ($F_{(2, 30)} = 31.041$, $p < 0.01$), but nonsignificant effects of treatment ($F_{(1, 15)} = 0.074$, $p > 0.05$) and the trial × treatment interaction ($F_{(4, 30)} = 0.257$, $p > 0.05$). Post-hoc analysis showed that freezing of animals of all three groups were significantly increased at trial 2 ($p<0.01$) and trial 3 ($p<0.01$) as compared to trial 1, representing the successful pairing of CS-US.

Reactivation

All groups exhibited intact and equivalent memory recall during the reactivation session (fig. 3) as evident by

comparable freezing in all groups. The ANOVA for the reactivation session revealed a non-significant difference between the groups ($F_{(4, 30)}$ =0.793, p>0.05).

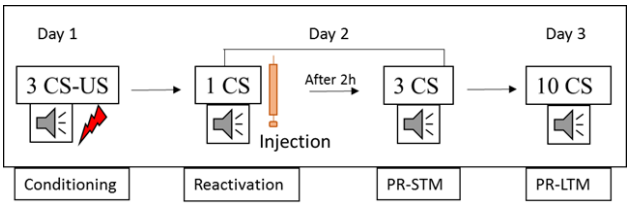

Fig. 1: Schematic representation of the behavioral protocol. US (unconditioned stimulus; footshock), CS (conditioned stimulus; tone), PR-STM (post-reactivation short term memory), PR-LTM (post-reactivation long term memory).

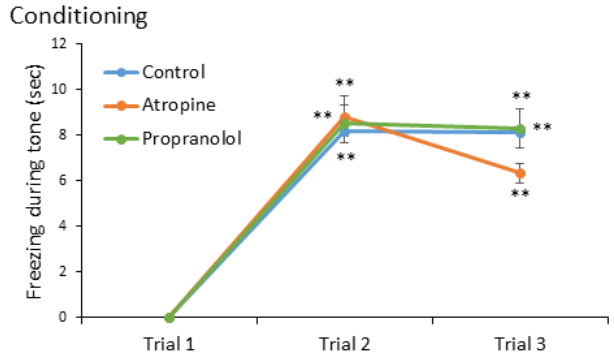

Fig. 2: Results in the conditioning session of Pavlovian conditioning. Post-shock freezing in control (n=6), atropine (n=6) and propranolol (n=6) groups was monitored immediately after the conditioning trials. Values are mean ±SEM. Significant differences were obtained by two-way ANOVA with repeated measure design. **p<0.01with respect to trail 1.

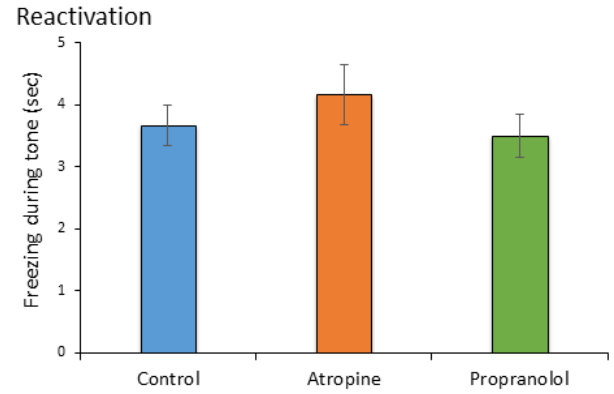

Fig. 3: Reactivation of fear memory. Values are mean ±SEM. Duration of freezing in each group in reactivation trial.

PR-STM

No effect of drug administration was observed on PR-STM tested 2h after the reactivation session (fig. 4). The ANOVA for PR-STM showed nonsignificant effects of treatment (F (2, 15) =0.358, p>0.05), trial (F (2, 30) =14.894, p>0.05), and the trial × treatment interaction (F (4, 30) =0.743, p>0.05). Post-hoc analysis showed that freezing of all three groups remained intact in each trial p>0.05.

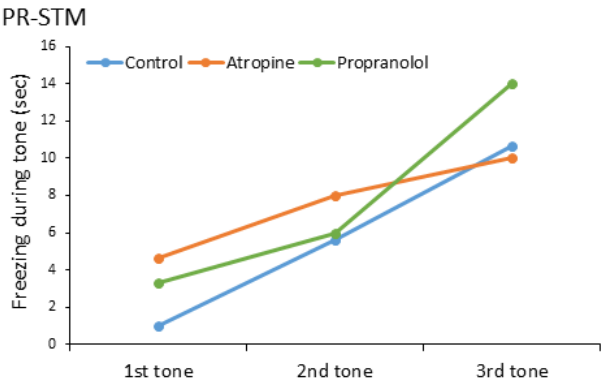

Fig. 4: Post-reactivation short-term memory (PR-STM) of fear memory. PR-STM was assessed 2h after the reactivation trial in each group. Data are expressed as mean ± SEM.

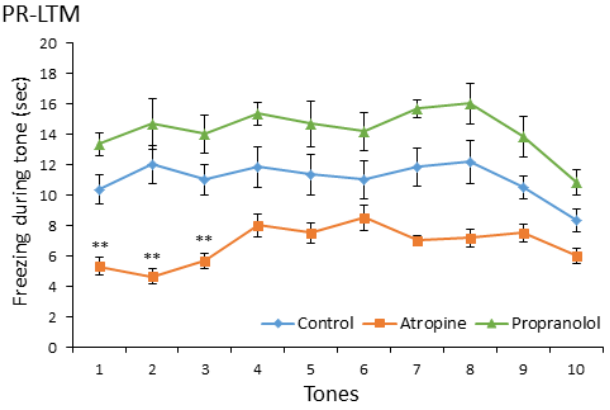

Fig. 5: Post-reactivation long term memory (PR-LTM) retention of fear memory. Atropine impairs reconsolidation of newly developed auditory fear conditioning. Values are mean ± SEM. PR-LTM assessed 24h after the reactivation trial in each group. Among the drug-injected rats, those receiving post-reactivation injections of atropine showed significantly less freezing during the memory test than those receiving saline injection. **p<0.01with respect controls.

PR-LTM

Atropine was observed to significantly impair PR-LTM (fig. 5). The ANOVA for PR-LTM revealed a significant effect of treatment ($F_{(2, 15)}$ =59.588, p<0.01) and trial ($F_{(9,135)}$ =3.863, p<0.01), but a nonsignificant trial × treatment interaction ($F_{(18,135)}$ =0.959, p>0.05). Post-hoc analysis revealed significant decreased freezing (p<0.01) in atropine treated group at tone 1, tone 2 and tone 3 as compared to control.

DISCUSSION

Recently formed fear memories may go through a process of consolidation instantly after training (Nader *et al.,* 2000). For reconsolidation of an emotional memory, it should be retrieved first, which is done by "retrieval trial" (giving a reminder of the original experience) (Graham *et al.,* 2011). New insights suggested that pharmacological interventions and behavioral methods are suited to modify or remove fear memory by targeting reconsolidation process whether it is a newly formed or an older formed fear memory (Steinfurth *et al.,* 2014). The present study investigated whether atropine and propranolol are appropriate to disrupt or impair newly formed fear memory by targeting reconsolidation process.

Previous study showed that, when propranolol was administered after 1 or 36 day of training without memory reactivation, no outcome was established on either new or old memory, which suggested that disruptive effect of propranolol was dependent upon reactivation of fear memories (Taherian *et al.,* 2014). It has been suggested that beside particular pharmacological agents, some factors may also influence the anticipated result of reconsolidation of new fear memory (Cai *et al.,* 2006; McKenzie, 2011). Fear memory impairing effects of propranolol are not consistent in previous studies. Most studies showed impairment of fear memory reconsolidation following the administration of propranolol (Sherin and Nemeroff, 2011; Tully and Bolshakov, 2010), however, scientists are unable to replicate the results in human subjects (Tollenaar *et al.*, 2009). It has been suggested that propranolol reduces emotional expression of fear but cannot disrupt fear memory (Muravieva *et al.,* 2010). Our study is parallel with studies which were unsuccessful to replicate the promising effects of propranolol. Bos and co-workers found no fear-impairing effect of propranolol when applied during reconsolidation (Bos *et al.,* 2014). It has been suggested that for memory reconsolidation, a prediction error is a precondition, which is generated by a mismatch between what is predictable and what actually occurs upon memory retrieval (Exton-McGuinness *et al.,* 2015). The studies which did not show promising effects of propranolol suggest that eraser of fear memory depends upon the strength and age of the memory. Higher dose of amnesic agent or multiple reactivation trials may be required to impair stronger fear memory. Moreover, level of anxiety in treating subjects is also suggested to be responsible for the false negative effects of propranolol (Villain *et al.,* 2016). Therefore, in this study we can suggest that the dose of propranolol used was unable to impair newly formed fear memory by the procedure followed in this study.

In current study atropine was also tested to impair the newly formed fear memory. Cholinergic activity blockade with acetylcholine receptor antagonist scopolamine is shown to impair memory consolidation (Tinsley *et al.,* 2004; Zelikowsky *et al.,* 2013). Administration of muscarinic receptor antagonist impaired the retrieval of memory developed from contextual fear conditioning (Soma *et al.,* 2014) and the impairments of learning have been shown in humans, monkeys, rabbits and rodents (Anagnostaras, 1999). Administration of scopolamine either before or after fear conditioning has shown to impair context conditioning indicating impairing effects of muscarinic cholinergic antagonist on memory consolidation and reconsolidation (Tinsley *et al.,* 2004). In this study although we did not find any fear memory impairing effects of propranolol, but atropine administration after the session of reactivation significantly impaired long term memory retention of fear expression in rats. Newly formed memories go through a consolidation process and this consolidated memory becomes stable by moving from short term to long term state. This transition can be altered by blocking consolidation process (Nader *et al.,* 2000). During reconsolidation synthesis of new proteins are required to re-stabilize an already existing reactivated synapse. In this study we found that STM is intact, because after reactivation the reactivated memory traces remain functional approximately about for 4h. Furthermore, when we tested PR-LTM there was a significant impairment of fear memory following the administration of atropine. When memory enters into labile state there is a need of protein synthesis through the activation of phospholipase C and protein kinase C cascade mechanism (Hernandez and Abel, 2008). It may therefore be suggested that inhibition of muscarinic receptor during this crucial step may cause disruption of signal transduction pathway and thus impair reconsolidation of fear memory as observed in this study.

CONCLUSION

In conclusion the present study suggests the potential benefits of pharmacological interventions directed at cholinergic neurotransmission instead of adrenergic system to disrupt reconsolidation of fear memories associated with environmental cues. Impairing the well-consolidated fear memory within a clock by effective treatment may reduce the intensity of fear memories and also reduce vividness of these memories. Further studies are needed to elucidate the findings of current study.

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