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Short communication

T-type calcium channels regulate the acquisition and recall of conditioned fear in male, Wistar rats

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ABSTRACT

The T-type calcium channel blocker, Z944, has been used as a pharmacological tool to assess T-type calcium channel function and examined for use as an anti-epileptic. As Z944 affects fear learning and memory in a rodent model of absence epilepsy, it is important to determine the effect of Z944 on learning and memory in a non-disease outbred rodent strain. This study examined the dose-dependent effects (5 mg/kg, 10 mg/kg, i.p.) of acute systemic treatment with Z944 on the learning and memory of fear conditioning and extinction in male Wistar rats. Z944 administered prior to the acquisition of fear conditioning significantly increased freezing prior to acquisition and extinction, during acquisition, and impaired recall of fear memory 24 h later. These findings suggest that T-type calcium channel activity may be required during associative learning for intact long-term memory. Enhanced fear behaviour observed prior to acquisition and extinction, and during acquisition could reflect an increase in anxiety, however, further testing is needed to determine the effect of Z944 on anxiety during fear conditioning and extinction. The use of Z944 for therapeutic purposes should consider the potential effects of Z944 on learning and memory in clinical populations.

T-type calcium channels are low voltage-activated calcium channels that play a critical role in neuronal firing patterns [1]. The contribution of T-type calcium channels to normal physiological processes involved in activities such a sleep and pain, as well as the pathophysiological processes involved in disorders such as absence epilepsy have been studied extensively [2–4]. However, the modulatory role T-type calcium channels have in switching neurons between distinct modes of firing makes these channels well situated to also mediate the processes involved in complex neural activities such as learning and memory [1,5]. Indeed, recent studies show that T-type calcium channels mediate cognition [6], with evidence demonstrating a particular relevance to the processes of fear learning and memory [7].

In a recent study [7], we demonstrated that the potent and selective T-type calcium channel blocker Z944 [8,9] increases the acquisition of, yet reduces the recall of, fear conditioning in two rat strains, Genetic Absence Epilepsy Rats from Strasbourg (GAERS), and the non-epileptic control (NEC) strain. GAERS are a well-characterized rodent model of absence epilepsy derived from the selective inbreeding of an outbred Wistar colony of rats in Strasbourg whereas NECs are inbred rats derived from the same Strasbourg colony as GAERS that do not display absence seizures. Although NECs do not display absence seizures, they are behaviourally distinct from outbred Wistar rats on tests of anxiety [10], and startle reactivity [11] suggesting a disparity in the regulation of fear between the strains. Therefore, a systematic examination of the effects of T-type calcium channel regulation on the distinct learning and memory process of associative fear conditioning in an outbred rat strain is lacking. As Z944 is in clinical trials [12], this further highlights the importance in understanding how this drug impacts the basic processes of learning and memory. The purpose of the present study was to examine the dose-dependent effects of acute, systemic Z944 treatment on the learning and memory of fear conditioning acquisition and extinction in outbred Wistar rats.

Adult male Wistar rats (Charles River, Quebec, Canada) were housed in groups of 2 in polypropylene cages (W x D x H): 39.5 cm x 34.6 cm x 21.3 cm. The colony room was temperature controlled (21 °C) and maintained on a 12/12 h day-night cycle (lights on at 7am). Rats had *ad libitum* access to water and standard rat chow (Purina Rat Chow). All experimental procedures were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Saskatchewan Animal Research Ethics Board. Z944 was

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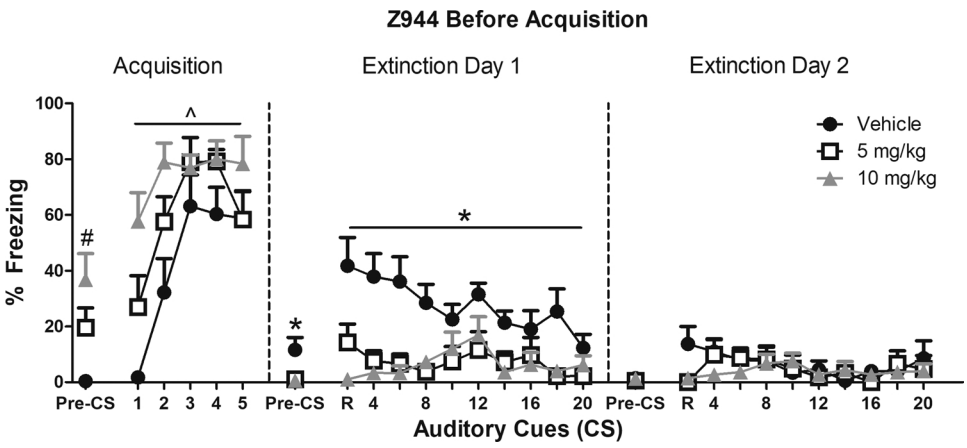

Fig. 1. Acquisition, extinction day 1, and extinction day 2 of conditioned fear in rats treated with Z944 prior to acquisition. Each CS data point represents the average of two CS presentations. On acquisition day, rats treated with 10 mg/kg of Z944 prior to acquisition froze significantly more than vehicle treated rats during the pre-conditioned stimulus (Pre-CS) period (#$p \leq 0.05$). During acquisition of CS cues, both the 5 and 10 mg/kg doses of Z944 significantly increased freezing relative to vehicle treatment (^$p \leq 0.05$). On extinction day 1, rats treated with 5 or 10 mg/kg of Z944 prior to acquisition showed significantly decreased freezing relative to vehicle treated rats during the Pre-CS period, recall (R), and during both the early and late extinction CS. (*$p \leq 0.05$).

prepared fresh each day in a 1 mg/ml or 2 mg/ml solution of 10 % dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO) and 90 % sodium carboxymethyl cellulose (0.5 % in saline, Sigma Aldrich), and administered at a volume of 5 ml/kg to yield doses of 5 mg/kg or 10 mg/kg respectively. Z944 was administered 15 min prior to either the acquisition day or first extinction day of fear conditioning.

Fear conditioning acquisition and extinction followed procedures previously published by our lab [7,13]. Briefly, rats were tested in standard operant conditioning chambers (ENV-008; Med Associates, St. Albans, VT). Each chamber comprised a grid wire floor connected to a shock generator and scrambler (ENV-414S), and a speaker (ENV-224BM) that presented high-frequency tones. A video camera placed opposite the conditioning chamber recorded all behaviours. Rats were handled for 5 min/day for 3 days prior to behavioural testing. Fear acquisition and extinction occurred over 3 days: acquisition (day 1), extinction day 1 (24 h after acquisition), and extinction day 2 (48 h after acquisition). During the acquisition of fear conditioning, rats received 5 conditioned stimulus-unconditioned stimulus (CS-US) trials. Each CS-US trial consisted of an auditory tone (4 kHz, 80 dB, 20 s) that co-terminated with a 1 s footshock (0.4 mA intensity). The inter-trial interval of each CS-US presentation was 180 s with a stimulus free 180 s period before the first and after the last trial. On extinction days 1 and 2, rats received 20 CS-only trials with the same tone frequency and inter-trial interval which occurred on acquisition day. The fear conditioning chambers were cleaned with a 40 % ethanol solution between subjects. Freezing behaviour, immobility with the exception of movement required for respiration and non-awake or rest body postures [14], was recorded based on a minimum duration of 1 s. Freezing behaviour was quantified by Video Freeze Software (Med Associates, St. Albans, VT), and was further manually checked for the occurrence of resting or sleep by a researcher blinded to the treatment conditions of each rat. The behaviour of rats found to be at rest or sleeping was converted to non-freezing behaviour for that time interval.

All data was analyzed using the Statistical Package for the Social Sciences version 20 for Windows (IBM). Statistical analyses of the acquisition and extinction sessions of conditioned fear were performed using separate mixed-factor repeated measures ANOVAs (CS as the repeated-measures factor, Drug Dose (vehicle, 5 mg/kg Z944, 10 mg/kg Z944) as the between-subjects factor). For the analysis of the acquisition of fear, CS examined freezing to each auditory cue over time. For the analysis of the extinction sessions, CS compared freezing behaviour during the average of the 3rd and 4th auditory cue presentations to the average of the 19th and 20th auditory cue presentations to assess the early versus late stages of extinction respectively [15]. One-way ANOVAs (Drug Dose as the between-subjects factor) were conducted to examine group differences in freezing behaviour during the 180 s pre-CS time periods, the recall of the acquisition of fear (the average of the 1st and 2nd auditory cue presentations on extinction day 1), and the recall of extinction (the average of the 1st and 2nd auditory cue presentations on extinction day 2). Separate analyses were conducted for animals that received Z944 prior to the acquisition of fear versus those that received Z944 prior to extinction day 1. As vehicle treated rats were used for both analyses, a Bonferroni correction was used to determine significance. Significant main effects of Drug Dose were followed up by Bonferroni post hoc comparisons. Partial η^2 was used to determine effect size, which represents the total variability in each dependent variable that can be attributed to the independent variables. Small, medium, and large effect sizes are considered partial η^2 values of 0.01, 0.06, and 0.014 respectively. Corrections were made for violations of sphericity where necessary. Only significant results are reported.

Nine naive rats were used for each treatment group, which consisted of the following: Vehicle, 5 mg/kg Z944 before acquisition day, 10 mg/kg Z944 before acquisition day, 5 mg/kg Z944 before extinction day 1, and 10 mg/kg Z944 before extinction day 1. Z944 treatment prior to the acquisition of fear conditioning significantly increased freezing during the Pre-CS period ($F(1,24) = 23.30$, $p = 0.004$, partial $\eta^2 = 0.375$; Fig. 1), and during the CS presentations ($F(2,24) = 15.13$, $p < 0.001$, partial $\eta^2 = 0.558$; Fig. 1) on acquisition day. Post hoc analyses showed a significant increase in freezing in rats treated with 10 mg/kg Z944 relative to vehicle treatment during the Pre-CS period ($p = 0.003$); whereas, both the 5 mg/kg ($p = 0.019$) and 10 mg/kg ($p < 0.001$) dose of Z944 increased freezing to the CS cues during acquisition. All rats, regardless of whether they were treated with Z944 prior to the acquisition phase ($F(4,96) = 12.93$, $p < 0.001$, partial $\eta^2 = 0.350$; Fig. 1), or prior to extinction day 1 ($F(4,96) = 24.91$, $p < 0.001$, partial $\eta^2 = 0.509$; Fig. 2), showed increased freezing over the CS cue presentations on acquisition day.

During the Pre-CS period on extinction day 1, rats treated with Z944 prior to acquisition showed a significant decrease in freezing ($F(2,24) = 5.64$, $p = 0.010$, partial $\eta^2 = 0.320$; Fig. 1) with both the 5 mg/kg ($p = 0.027$) and the 10 mg/kg ($p = 0.020$) dose of Z944 significantly reducing freezing relative to vehicle treated rats. Rats treated with Z944 prior to the acquisition of fear conditioning continued to exhibit reduced freezing during not only the fear recall phase on extinction day 1 ($F(2,24) = 8.78$, $p = 0.001$, partial $\eta^2 = 0.423$; Fig. 1), but also during the extinction phases on extinction day 1 ($F(2,24) = 11.14$, $p < 0.001$, partial $\eta^2 = 0.481$; Fig. 1). Post hoc analyses showed that both the 5 mg/kg and the 10 mg/kg doses of Z944 significantly reduced freezing during the recall and extinction phases on extinction day 1 relative to vehicle treatment (all $p \leq 0.032$). A significant CS by Drug Dose interaction ($F(2,24) = 6.59$, $p = 0.005$, partial $\eta^2 = 0.355$) was also found for rats treated with Z944 prior to acquisition on extinction day 1. Examination of the profile plot of this interaction determined that significant extinction of freezing occurred in the vehicle treated rats from early to late extinction, whereas Z944 treated rats displayed very

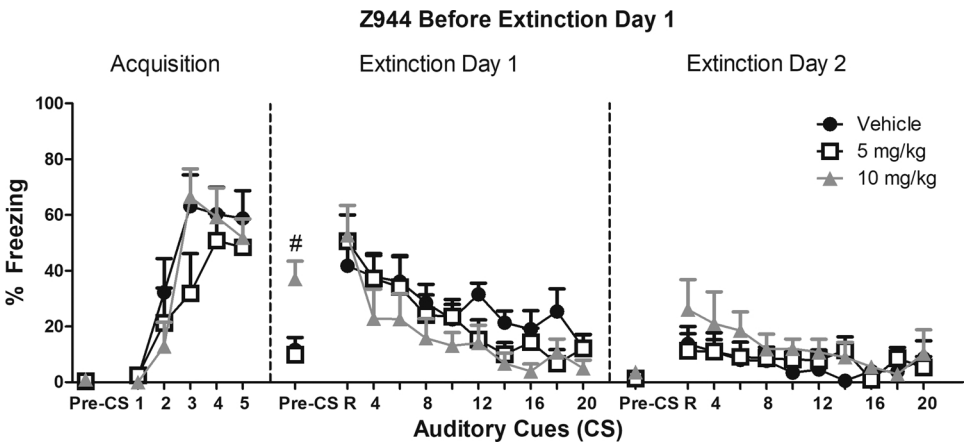

Fig. 2. Acquisition, extinction day 1, and extinction day 2 of conditioned fear in rats treated with Z944 prior to extinction day 1. Each CS data point represents the average of two CS presentations. During the Pre-conditioned stimulus (Pre-CS) period on extinction day 1, rats treated with 10 mg/kg of Z944 prior to extinction day 1 showed significantly increased freezing relative to vehicle treated rats ($\#p \leq 0.05$).

little freezing behaviour during both early and late extinction time bins.

When the rats treated with Z944 prior to extinction day 1 are considered, a significant effect of Drug Dose was found for freezing behaviour during the Pre-CS period on extinction day 1 ($F(2,24) = 10.26$, $p = 0.001$, partial $\eta^2 = 0.461$; Fig. 2). Post hoc analysis revealed a significant increase in freezing in 10 mg/kg Z944 treated rats relative to vehicle treatment during this testing period ($p = 0.003$). Further analysis of the behaviour of the rats treated with Z944 prior to extinction day 1 showed that both the vehicle and Z944 treated rats significantly decreased in freezing behaviour from the early to late extinction time bins ($F(1,24) = 24.09$, $p < 0.001$, partial $\eta^2 = 0.501$). Analysis of freezing behaviour for all rats, regardless of whether they were treated with Z944 prior to the acquisition of fear conditioning, or prior to extinction day 1, revealed non-significant results for all measures on extinction day 2.

In this study, we demonstrate that acute systemic treatment with the T-type calcium channel blocker, Z944, administered prior to fear conditioning increases freezing during acquisition, but impairs the recall of conditioned fear in male, Wistar rats. Although Z944 administered prior to extinction day 1 significantly increased freezing during the pre-CS period, no other effects of the drug were observed. These results are similar to those previously found in the GAERS and NEC strains [7].

Converging lines of evidence suggest that T-type calcium channel activity during the acquisition of associative fear learning is required for the subsequent long-term memory of these associations [7]. The results from this study, in addition to previous findings, also suggest that T-type calcium channel activity is not required for the acquisition, expression, extinction, or consolidation phases of conditioned fear [7]. One potential explanation for the increase in freezing produced by Z944 during acquisition is that acute Z944 treatment increases anxiety. Previous studies have demonstrated an increase in anxiety produced by Z944 in NEC rats without affecting overall locomotor activity at the 5 mg/kg dose; however, the effects of Z944 on anxiety have been inconsistent [16]. Further, an increase in freezing was not observed in rats treated with Z944 prior to extinction day 1 during the CS presentations, suggesting that Z944 does not consistently increase anxiety. Alternatively, the increase in freezing produced by Z944 during acquisition may be the result of an enhancement in the learning of conditioned fear associations rather than an increase in anxiety. Further studies are needed to determine whether increased freezing during acquisition produced by Z944 is the result of increased anxiety or enhanced fear learning in the Wistar strain.

The findings of this study are supported by the literature which implicates T-type calcium channel activity during associative learning for intact long-term memory. Wild-type mice infused with the mixed T-type and high voltage-activated calcium channel blocker, Mibefradil, into the hippocampus prior to the acquisition of trace fear conditioning showed impaired recall of context-cues 25 h following training [17]. In a separate study, Cav3.2 T-type KO mice show disrupted recall of novel and spatial object recognition memory 24 h after retention [18]. NEC rats treated with Z944 prior to crossmodal object recognition learning also exhibit impaired recall of objects when recall relied on visual or tactile-to-visual memory [6]. As object recognition memory is also impaired by reduced T-type calcium channel activity, these studies suggest that the effects of T-type calcium channel activity on memory are not specific to associative fear learning. It is important to note, however, that we did not observe a significant effect of Z944 on the acquisition or recall of extinction learning and memory. As the acquisition and extinction of conditioned fear are dependent on distinct neural circuits [19], further research is required to investigate the potential dissociative effect of T-type calcium channel activity on fear conditioning versus fear extinction.

There is evidence to suggest that the activation of T-type calcium channels contributes to the mechanisms of long-term potentiation (LTP) [20]. The acquisition of conditioned fear has been shown to induce LTP in the lateral amygdala [21], thus disrupted LTP caused by Z944 treatment is a potential mechanism for the disruptions in recall observed in this study. This possibility is supported by research which shows that acute inactivation of T-type calcium channels in wild-type mice by TTA-P2 prevented LTP induction in parallel fiber-Purkinje cells of the cerebellum, as well as impaired cerebellar learning [22]. In a separate study, Cav3.2 KO mice demonstrated altered late-phase hippocampal LTP, as well as disrupted hippocampal dependent recall of trace fear conditioned contextual cues [17]. Given these findings, future studies should determine whether impairments in recall produced by Z944 are LTP-dependent. It is important to note, however, that the increase in freezing observed during the acquisition of fear produced by Z944 may indicate an enhancement of learning and, thus LTP. Further, extinction learning was not impaired following Z944 treatment indicating that Z944 does not affect all phases of fear learning and memory. However, fear extinction learning and memory is thought to involve both LTP and long-term depression-dependent mechanisms which are distinct from the mechanisms of fear recall, and thus, may be differentially affected by Z944 treatment [23]. As the effects of Z944 on synaptic plasticity within this study are strictly speculative, the potential contribution of Z944 to the synaptic plasticity associated with fear learning and memory should be further investigated.

In this study, we observed robust memory impairments produced by acute Z944 treatment prior to the learning of fear-associated memory in male rats, as well as significant increases in freezing during acquisition. As Z944 is being used in clinical trials [12], the use of Z944 for therapeutic purposes should consider the potential effects of Z944 on learning and memory in clinical populations.

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