**Problem to Be Studied** (4000 characters maximum)

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric disorder that causes affected individuals to re-experience traumatic memories, negative emotions, and thoughts, contributing to avoidance, hypervigilance, and hyperarousal in the months and years following severe trauma. PTSD affects approximately 6–8% of the general population but this prevalence rate is up to 25% among groups who have experienced severe psychological trauma, such as combat veterans (Ressler et al., Nat Rev Neurol. 2022). Ample evidence suggests that PTSD can be viewed as a disorder that involves the dysregulation of normal fear processes (Mahan and Ressler, Trends Neurosci., 2012). Currently, the only FDA-approved treatments for PTSD are the serotonin reuptake inhibitors sertraline and paroxetine (Kelmendi et al. Clin Psychol., 2017; Ressler et al., Nat Rev Neurol., 2022). However, these drugs have adverse effects and exhibit low response rates, with less than 30% of patients achieving full remission. These treatments also center on continued medication, addressing symptoms rather than the source of the problem, which is the traumatic experience. We, therefore, sought to develop a drug capable of effectively treating the underlying causes of PTSD by reducing the specific effects of traumatic memory on the development and maintenance of PTSD. A drug that is effective in this context should be able to mitigate PTSD development when injected immediately after the traumatic event, while also providing the opportunity to alleviate the devastating effects of PTSD after it has been diagnosed when injected prior to the retrieval of the associated traumatic memory during psychiatric treatment. The ideal drug candidate would thus be one can fulfill the following criteria: 1) Mitigate the risk of PTSD development when applied immediately after the traumatic event; 2) Offer value when applied days, months, or even years after the traumatic event when PTSD has been already diagnosed by reducing the traumatic memory and associated PTSD symptom severity; 3) Affect the formation of long-term fear conditioning memory when injected immediately after the fear traumatic event or days after the fear traumatic event occurred before its retrieval, given that PTSD-inducing trauma exposure is an example of human naturalistic fear conditioning (Ressler et al., Nat Rev Neurol. 2022); 4) Be effective when injected acutely and systemically; 5) Specifically affect fear conditioning memory related to the traumatic event and not other types of memories; 6) Be able to affect rats in appropriately constructed pre-clinical experiments, as fear experiences elicit similar responses in rats and humans and the neural circuits underlying fear learning and memory are, at least to a first approximation, the same in rodents and humans (Fenster et al., Nat Rev Neurosci., 2018); 7) Have a specific target. With those criteria in mind, we previously designed an inhibitory ephrinA4 mimetic peptide that targets the EphA tyrosine kinase receptor binding site, as this receptor and its ligand ephrinA are involved in the regulation of neuronal morphology and synaptic transmission during development and in the adult brain. These neuronal events are shown to be involved in fear memory formation and PTSD.

**Theoretical Rationale, Scientific Methods, and Design** (4000 characters maximum)

We designed a fear memory inhibitory peptide from ephrinA4 (pep-ephrinA4) because it has a very high affinity for EphA receptors and for EphA4 in particular (Bowden et al., Structure 2009). EphA4 is required for synaptic plasticity in the amygdala (Deininger et al., Proc Natl Acad Sci USA 2008), a brain area essential for fear memory formation that is closely involved in PTSD (LeDoux, 2000; Etkin and Wager, Am J Psychiatry, 2007). Pep-ephrinA4 was designed to mimic the ephrinA4 binding domain (GH loop) (Dines et al., Translational Psychiatry, 2014), and our preliminary data attest to the utility of this peptide. Pep-ephrinA4 binds EphA, but not EphB or other proteins tested, and inhibits ephrinA4-induced EphA4 activation in the amygdala. This result demonstrates the specificity of the peptide. Strikingly, subcutaneous pep-ephrinA4 injection in rats (n=15) 1 h after fear conditioning training impaired fear long-term memory by 31% when tested one day later as compared to the vehicle control group (n=14) (F(1,27)= 8.6, P=0.007) (Dines et al., Translation Psychiatry, 2014). This highlights the efficacy of this peptide when injected systemically after fear conditioning, effectively mitigating fear memory development and PTSD. Acute subcutaneous injection of pep-ephrinA4 into rats (n=18) 30 minutes before fear traumatic memory retrieval on the day following initial fear conditioning also significantly impaired long-term fear memory associated with the traumatic event by 46% when tested one day later as compared to the injection of a non-binding control peptide (F(1,34) = 5.812, p = 0.021) (Mana et al., Scientific Reports, 2022). This demonstrates the effectiveness of the peptide as a tool that can reduce fear memory and PTSD symptoms after PTSD has been established. These encouraging results emphasize the potential efficacy of pep-ephrinA4, but further tests need to be performed before moving to clinical tests. Toward that end, in the proposed project we will: 1) Explore other dosing strategies, dosing schedules, and dosing routes (e.g. intranasal application) that may enhance pep-ephrinA4 efficacy; 2) Study the effects systemic peptide injection 1 hr after fear conditioning on long-term fear memory at later time points weeks, months, or years after the traumatic event to build on the preliminary testing performed 1 day after the traumatic event, as we expect to this peptide to exhibit long-lived activity; 3) Study the effects of systemic peptide injection 30 min prior to fear memory retrieval on long-term fear memory after 1 week, 1 month, or 6 months, as we expect pep-ephrinA4 to have durable effects in this setting; 4) Study the effects of acute systemic pep-ephrinA4 injection on other memories mediated by the amygdala by training rats for other memories (e.g. conditioned taste aversion (CTA)) and then performing fear conditioning one day later, with pep-ephrinA4 being injected immediately after fear conditioning or before fear memory retrieval followed by the testing of fear conditioning and the other memory (e.g. CTA) one day later, ideally demonstrating the specificity of pep-ephrinA4 to the fearful traumatic event; 5) Test the distribution of the pep-ephrinA4 in the brain and body after the acute systemic injection through standard pharmacokinetic studies; and 6) Study the effect of acute pep-ephrinA4 injection on the health of the animals (weight, blood tests, tissue health, etc.), although no adverse health effects were observed in our preliminary studies.

**Significance and/or Uniqueness of the Proposed Effort** (4000 characters maximum)

As discussed above, the only FDA-approved treatments for PTSD are sertraline and paroxetine, and their efficacy is limited and associated with the potential for adverse effects. These drugs also focus on sustained medication to address symptoms, rather than seeking to target the underlying traumatic experience. In contrast, our developed pep-ephrinA4 drug candidate targets never before targeted ephrinA4 binding sites while providing a novel opportunity to affect the fearful traumatic event underlying the diagnosis of PTSD. Our preliminary results have been very encouraging, highlighting the usefulness of the peptide in targeting specifically ephrinA4 binding sites, mitigating fear memory formation when injected 1 hr after fear conditioning and reducing fear memory when injected before retrieval one day after fear conditioning. Given the novelty of this approach and the supporting data suggesting that it may ultimately offer clinical value, we believe that this drug has the potential to be extremely useful in mitigating the risk of PTSD development or alleviating established PTSD, thereby addressing a major unmet clinical need.

**Military Relevance and Impact** (4000 characters maximum)

PTSD affects 25-35% of combat soldiers who have experienced a severe traumatic event. Pep-ephrinA4 has the potential to significantly reduce the odds of developing and maintaining PTSD, given that our preliminary results show that the acute systemic administration of pep-ephrinA4 significantly reduces fear memory and the risk of developing PTSD while also offering an opportunity to disrupt PTSD maintenance when applied in the context of fear memory retrieval. Strikingly, our preliminary data indicate that the efficacy of this drug is substantial without apparent side effects. Thus, pep-ephrinA4 offers great potential as a tool that can help protect against PTSD development when provided to military personnel who have experienced a traumatic event in combat or other situations. Pep-ephrinA4 may also help reduce PTSD among military personnel and veterans when administered before memory retrieval in a psychiatry clinic in the context of treatment. Our novel peptide drug thus represents a potentially invaluable tool that may help alleviate the disproportionate burden of PTSD facing members of the armed forces and their loved ones.

**Brief Description of Research Involving Animals, Human Anatomical Substances and/or Human Subjects** (4000 characters maximum)

We will use rats in our experiments. Fear experiences elicit similar responses in rats and humans, and the neural circuits underlying fear learning are, at least to a first approximation, the same in rodents and humans (Fenster et al., Nat Rev Neurosci., 2018). All experiments have been approved by the University of Haifa Institutional Committee for animal experiments in accordance with National Institutes of Health guidelines. We will subcutaneously inject pep-ephrinA4 immediately after fear conditioning or before long-term fear memory retrieval as discussed above, and will then study its effects on long-term fear memory formation and maintenance, respectively. Fear conditioning is an established protocol described in many scientific papers and is performed by subjecting rats to a tone that is contaminated with a mild footshock. Long-term memory is tested by monitoring animal freezing (immobility is a typical fear response) in response to the presentation of the conditioned tone in the absence of any footshock. We will conduct standard pharmacodynamics, pharmacokinetics, and ADME ("absorption, distribution, metabolism, and excretion") tests using these model rats. Tissue health will be examined 1 and 6 months after injection using standard histopathological techniques. Water and food consumption and the body weight of experimental rats will be assessed one month after peptide injection.

**Plans and Strategy for Translation, Implementation, and/or Commercialization** (4000 characters maximum)

After the completion of the study, we will locate pharmaceutical companies that will help translate these results to the next stage of development and initiate clinical trials enrolling PTSD patients. The University of Haifa, through its Economic Corporation unit, has a track record of success in locating companies interested in research products. To that end, we, together with the University of Haifa, have patented this peptide for its use in PTSD and established a registered company based on the pep-ephrinA4 technology so that it will be easier to offer it to another commercial company that has the capacity to conduct clinical trials. Before this can occur, however, we must complete the experiments outlined in this proposal. If these efforts are successful, the production of pep-ephrinA4 as a safe, commercially available drug will be of outstanding benefit to military service personnel that experience trauma or suffer from PTSD.