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

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric disorder, leading to re-experiencing of the traumatic memory, negative emotions and thoughts, avoidance, hypervigilance and hyperarousal in the months and years following severe trauma. PTSD has a prevalence of approximately 6–8% in the general population but can increase 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 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, the drugs have adverse effects, the response rate for these drugs is low, and less than 30% achieve full remission. Moreover, the treatment includes continued medication, addressing symptoms rather than the source of the problem which is the traumatic experience. We, therefore, aimed to develop a drug that can be useful for the treatment of PTSD. The drug should reduce the specific effects of the traumatic memory on the development and maintenance of PTSD. We designed and developed a new drug aimed to mitigate the development of PTSD when injected immediately after the traumatic event. It is also aimed to reduce the devastating effects of PTSD after it has been diagnosed when injected before retrieving the long-term traumatic memory during psychiatric treatment. Toward these ends, the drug should: 1) Be useful when applied immediately after the traumatic event to mitigate the risk of PTSD development. 2) Be useful when applied days (or months/years) after the traumatic event when PTSD has been already diagnosed to reduce the traumatic memory and the symptoms of PTSD. 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. PTSD-inducing trauma exposure is considered to be an example of human naturalistic fear conditioning (Ressler et al., Nat Rev Neurol. 2022). 4) Be effective when injected acutely and systemically. 5) Affect specifically fear conditioning memory related to the traumatic event and not other types of memories. 6) Be able to affect rats in the pre-clinical experiments suggested. 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. We designed an inhibitory ephrinA4 mimetic peptide targeted to EphA binding site. EphA tyrosine kinase receptors and its ephrinA ligand 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 the fear memory inhibitory peptide from ephrinA4 (pep-ephrinA4) because it has a very high affinity to EphA receptors and in particular to EphA4 (Bowden et al., Structure 2009). EphA4 is needed for synaptic plasticity in amygdala (Deininger et al., Proc Natl Acad Sci USA 2008), a brain area essential for fear memory formation (LeDoux, 2000) and is also a central brain region involved in PTSD (Etkin and Wager, Am J Psychiatry, 2007). The pep-ephrinA4 was designed to mimic the ephrinA4 binding domain (GH loop) (Dines et al., Translational Psychiatry, 2014). We have preliminary results showing the usefulness of the peptide: 1) Pep-ephrinA4 binds EphA (but not EphB or other proteins tested) and inhibits ephrinA4-induced EphA4 activation in amygdala. This result demonstrates the specificity of the peptide. 2) Injection of pep-ephrinA4 subcutaneously to rats (n=15) 1 h after fear conditioning training impaired fear long-term memory (large reduction of 31%) tested a day afterward when compared to vehicle-injected group (n=14) (F(1,27)= 8.6, p=0.007) (Dines et al., Translation Psychiatry, 2014). This shows the effectiveness of the peptide when injected systemically and after fear conditioning to mitigate fear memory and PTSD. 3) Acute subcutaneous systemic injection of pep-ephrinA4 into rats (n=18) 30 minutes before the fear traumatic memory retrieval, a day after the fear traumatic event, impaired long-term fear memory to the traumatic event (large reduction of 46%) tested a day afterward when compared to 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 to reduce fear memory and PTSD symptoms after PTSD has been established. These results are very encouraging regarding the effectiveness of pep-ephrinA4 but further tests need to be performed before moving to clinical tests. Toward that end, we will: 1) Use additional protocols with pep-ephrinA4 to increase its effectiveness even more. This will include: different modes of application (e.g. nasal application), different doses (e.g. increased dose), and different times of application (e.g. multiple times). 2) Study the effects of injection of the peptide systemically and acutely 1 hr after fear conditioning on long-term fear memory at more remote time points, for example, a week, month, 6 months, or a year after the traumatic event (our preliminary results tested 1 day after the traumatic event). We expect to achieve a long-lasting effect of the peptide. 3) Study the effects of injecting the peptide systemically and acutely before retrieval of fear memory at more remote time points after the traumatic event. Fear conditioning will be performed and memory will be retrieved a week, a month, 6 months or a year afterward. Pep-ephrinA4 will be injected 30 minutes before retrieval and long-term fear memory will be tested a week, a month, or half a year afterward. We expect to affect fear memory even when retrieved remotely after fear conditioning and that the effect of the peptide will last. 4) Study the effects of acute systemic injection of the peptide on other memories mediated by the amygdala. Rats will be trained for other memories (e.g. conditioned taste aversion (CTA)). Fear conditioning will be performed a day afterward. Pep-ephrinA4 will be injected systemically and acutely immediatelly after fear conditioning or before fear memory retrieval. Both fear conditioning and the other memory (e.g. CTA) will be tested a day after the pep-ephrinA4 application. We expect that the effect of pep-ephrinA4 will be specific to the fearful traumatic event. 5) Test the distribution of the pep-ephrinA4 in the brain and body after the acute systemic injection (standard pharmacokinetic). 6) Study the effect of acute injection on the health of the animals (weight, blood tests, tissue health etc.). We did not see any effects on the animals’ health in general in our preliminary studies.

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

As described above the only FDA-approved treatments for PTSD are the serotonin reuptake inhibitors sertraline and paroxetine which are not very effective in treating PTSD and have adverse effects. Moreover, the treatment includes continued medication, addressing symptoms rather than the source of the problem which is the traumatic experience. Here we take a different approach to affect the source of PTSD- the fearful traumatic event. We also aimed for a new target, the ephrinA4 binding sites, which have not been used before. The preliminary results are very encouraging showing 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 a day after fear conditioning. Because we have used novel approaches, reducing directly the formation and maintenance of the memory of the traumatic event and using a new target the ephrinA4 binding site, we are hopeful that this drug can be extremely useful in mitigating the risk of developing PTSD when applied immediately after the traumatic event or reducing PTSD symptoms when it applied after PTSD has been diagnosed days (months/years) after the traumatic event. We predict that pep-ephrinA4 will be extremely useful in the prevention and treatment of PTSD. Other current approved drugs are not effective.

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

PTSD has a prevalence to occur in 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 because: 1) Our preliminary results show that administration of pep-ephrinA4 acutely and systemically reduces significantly fear memory and the risk of developing PTSD. 2) Our preliminary results show that injection of pep-ephrinA4 before fear memory retrieval reduces significantly fear memory tested a day later and therefore the maintenance of PTSD. Moreover, the effect is large after an acute injection without apparent side effects. Thus, pep-ephrinA4 has the great potential to serve as a very useful drug to mitigate the development of PTSD when given acutely and systemically to military personnel who experienced a traumatic event in combat or other situations. Moreover, pep-ephrinA4 can reduce PTSD in military personnel when given before memory retrieval in a psychiatry clinic during a treatment session. Pep-ephrinA4 therefore can reduce significantly PTSD in military personnel.

**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 are approved by the University of Haifa Institutional Committee for animal experiments in accordance with National Institutes of Health guidelines. We will inject systemically subcutaneously pep-ephrinA4 immediately after fear conditioning or before long-term fear memory retrieval (see above) and 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 the rat 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) to the presentation of tones without shock. We will run pharmacodynamics, pharmacokinetics (PK), ADME ("absorption, distribution, metabolism, and excretion") standard tests. The tissue health will be examined a month and 6 months after the injection by standard histopathology tests. Water and food consumption and the body weight of animals will be tested a month after the injection of the peptide.

**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 take it to the next level and start clinical trials with PTSD patients. The University of Haifa, through its Economic Corporation unit, is successful in locating companies interested in research products. Toward that end, we together with the University of Haifa patented the peptide for its use in PTSD and established a registered company based on the pep-ephrinA4 so it will be easier to offer it to another commercial company that has the capabilities to perform clinical trials. However, before we can offer it we need to complete the experiments suggested in the proposal. The production of pep-ephrinA4 as a commercially available and safe drug will be of outstanding benefit to military service personnel that will experience trauma or that have PTSD.