**Background**: Activity-dependent neuroprotective protein (ADNP) syndrome, also known as Helsmoortel Van Der Aa syndrome, is a rare condition in children who exhibit signs of autism and suffer developmental delays and intellectual disabilities. The syndrome is due to de novo mutation of one of the two activity-dependent neuroprotector (*ADNP*) genes that results in STOP or other frameshift mutations and loss of normal function. When the mutation is close to the *ADNP* start codon, the child will present a haploinsufficient loss-of-function phenotype. Mice that are Adnp+/− mimic the human condition by suffering from slower axonal transport, impaired dendritic spines, learning and memory deficiencies, muscle weakness, and communication problems. Similarly, mice heterozygous for the most prevalent pTyr718\* mutation, which truncates ADNP by nearly half, show delayed development, altered gait, and early brain tauopathy coupled to aberrant visual evoked potential. The ADNP microtubule-interacting fragment NAP (Davunetide, CP201) partially resolves Adnp deficiencies and protects against Adnp pathogenic sequence variant abnormalities in the human cell and mouse models. We can now test a direct gene-therapy strategy for treating ADNP syndrome using these models. Our strategy will use RNA silencing to inhibit the expression of the potentially toxic *ADNP* allele while replacing its transcript with healthy *ADNP* mRNA. This approach will be compared to pharmacological intervention and establish a path to address other syndromes. Our team includes four PIs with different and complementary expertise. Professor Illana Gozes (IG, Tel Aviv University) is the discoverer of ADNP and NAP and an expert translational neuroscientist. Professor Shlomo Wagner (SW, Haifa University) is an expert in vivo electrophysiologist in social behavior and animal model autism. Dr. Shani Stern (SS, Haifa University) is an expert in patient-derived pluripotent stem cell to neuron differentiation. Dr. Assaf Zinger (AZ, Technion) is an expert in biomimetic nanoparticles (NPs) and their translational use for RNA delivery into the brain. Importantly, NAP (Dauvnetide) and related ADNP technologies are licensed exclusively to ATED Therapeutics Ltd (IG, Chief Scientific Officer), which is developing ADNP-based therapies, beginning with ADNP syndrome. With our discovery of the potential pipeline therapeutics proposed here, ATED will be ready for direct clinical translation.

**Aim**: We propose a synergistic effort among our laboratories to test a cutting-edge, two-step gene-therapy strategy for ADNP syndrome based on RNA-encapsulated biomimetic NPS. Using the most prevalent human de novo mutation of *ADNP* (Tyr719\*), we will test our strategy in the human cell, animal, and haploinsufficiency models. We will compare our results directly with pharmacological NAP treatment. We hypothesize that the deteriorating effects of *ADNP* mutations arise from a reduced dose of functional ADNP protein plus dominant-negative effects of the mutated protein. Thus, delivering mRNA for functional protein may be insufficient to compensate for the mutation. To test the hypothesis, we will incorporate an inhibitory RNA strategy which will enable us to examine the effects of NPs delivering siRNA against the mutated gene, mRNA of the functional gene, or both to the human cell and mouse models.

**Experimental approach**: We will utilize existing human cells and mouse models in the IG laboratory. These include cells from an ADNP syndrome patient with the Tyr719\* mutation, a paralog mouse model heterozygous for *Adnp* p.Tyr718\* (Tyr), cells from an *ADNP* haploinsufficiency patient, and an *ADNP*+/- mouse model (both mouse models were previously published).

*Specific Aim 1: Electrophysiological and transcriptional characterization of the human cellular models (SS and IG laboratories).*

We will reprogram patient-derived cells (pTyr718\* and pArg216\*, available at IG laboratory) to induce pluripotent stem cells (iPSCs), and their *ADNP* mutations will be corrected using CRISPR/Cas9 technology to create control lines resulting in two pairs of lines, each isogenic for the patient mutation and corrected control. We will then differentiate the isogenic iPSC lines into several types of hippocampal neurons (dentate gyrus granule and CA3 pyramidal neurons) and hippocampal brain organoids (SS laboratory). Each patient’s autosomal and network properties will be measured and compared to the CRISPR/Cas9 edited neurons and organoids at several time points throughout differentiation by patch-clamp, calcium imaging, and multi-electrode arrays. Specific receptor blockers will be applied to determine the types of changes in the case of synaptic transmission deficiencies. We will then determine the cellular composition of monolayer and 3D cultures by immunocytochemistry and immunohistochemistry for different neuronal populations using cell-specific markers, including PROX1, ELAVL2, CALB1, GABA, and MAP2. Bulk and single-cell RNA sequencing will be performed to determine differential gene expression in the monolayer cultures and brain organoids at three time points throughout differentiation to understand the dynamic gene regulation program in patients compared to their isogenic controls. The mutation-specific electrophysiological and transcriptional modifications we identify will be used in conjunction with Aim2 to assess potential ADNP syndrome treatments.

At the end of this Aim, we expect to identify specific modifications in electrophysiological activity and transcription, which will be used later to assess the effects of the various treatments.

*Specific Aim 2: Electrophysiological characterization of the mouse models (SW, IG laboratories).*

We will use a battery of social discrimination tasks to identify specific behavioral and gait deficits using our heterozygous *Adnp* p.Tyr718\* (Tyr) and *ADNP*+/- mouse models, adding and comparing to existing data. Then we will apply chronic electrophysiological recordings from behaving mice using electrode arrays to characterize the neuronal activity simultaneously from up to 32 social-behavior associated brain regions, including the dorsal and ventral hippocampus (CA1, CA3, and DG), medial prefrontal cortex, nucleus accumbens, and multiple amygdaloid areas. Using this methodology, we will characterize the population neural activity in the brains of mutated animals during impaired social behavior at the system level compared to WT littermates. We will then analyze neural activity at specific brain areas displaying modified activity by electrode-array screen using Neurpixel probes. Specifically, we will examine hippocampal regions at the single-cell level. With the combined results of Aims 1 and 2, we will identify specific deficits in social behavior and specific signatures of brain activity modified in the mutated animals. These will be utilized in Aims 3 and 4 to examine the behavioral and electrophysiological effects of the treatments.

*Specific Aim 3: Establishing and testing RNA-encapsulating biomimetic NPs in the cellular models (AZ, IG, SS laboratories).*

We will fabricate four groups of NPS: one with functional Adnp mRNA, one with siRNA specifically designed for downregulating the truncated Adnp protein in the de novo mutated models, one with both RNAs, and one with shuffled siRNA as a control. After fabrication and characterization of their physiochemical and biomimetic properties (AZ laboratory), the NPs will be tested for toxicity, gene regulation, gene expression, association, uptake, and electrophysiology on cellular and organoid models produced by the SS laboratory. We will test all four particle types with the de novo mutation models. In contrast, only the mRNA-encapsulating and control NP will be tested on the mouse haploinsufficiency model, where there should be no significant dominant-negative effect of truncated ADNP. Our four human and mouse models will be tested using NAP pharmacological treatment. Furthermore, microtubule-Tau association will be determined by coimmunoprecipitation techniques (IG laboratory). This analysis will address potential pharmacological protection by NAP (Davunetide) compared to NP treatments. We will next examine whether any of the four treatments result in significant restoration of the electrophysiological deficits observed in Aim 1. In parallel, we will analyze the transcriptomic effects of the treatments on the various models (IG laboratory). In the results of Aims 1 to 3, we will provide comparative electrophysiological and transcriptomic analyses of the different treatments and their efficacy in our cellular models.

*Specific Aim 4: Testing RNA-encapsulating biomimetic NPs in animal models (AZ, IG, and SW laboratories).*

We will chronically administer intranasal NP three times per week during PN weeks 2-8 to two mice models (AZ laboratory). The whole body distribution of NPs will be tested using an *in vivo* imaging system (IVIS) at time intervals of 6-, 24-, and 48 hours following administration. Next, the mice will be transferred to SW laboratory and characterized behaviorally to assess if any of the four treatments alleviated impaired social behavior. We will compare the results to the pharmacological NAP (Davunetide) intervention (IG laboratory). For efficient treatments, we will use electrode microarrays and Neuropixel probes for in vivo electrophysiology to determine treatment outcomes. We will ask which treatments restored the modified electrophysiology during social behavior in the same brain regions identified in Aim 2, specifically in the hippocampal regions. In parallel, a single-cell transcriptomic analysis will examine transcriptomic changes induced by the treatment (IG laboratory). Overall, Aims 1 to 4 may identify a specific treatment that restores typical behavior and normalizes electrophysiology and gene transcription in the brains of the Adnp mutant mice.

Alternative approaches: If we fail to deliver the NP efficiently to the brain using intranasal administration, we will use direct injection to the lateral ventricles via a chronically implanted catheter.