**Background**: Activity-dependent neuroprotective protein (ADNP) syndrome, also known as the Helsmoortel Van Der Aa syndrome, is a rare condition diagnosed in children exhibiting signs of autism and suffering from developmental delays and intellectual disabilities. The syndrome occurs when one of the two copies of the *ADNP* gene is de novo mutated (mostly STOP or frameshift mutation), resulting in loss of normal functions. When the mutation is close to the ADNP start codon, the child will present a haploinsufficient loss-of-function phenotype. Indeed, Adnp+/− mice suffer from slower axonal transport and impaired dendritic spines, learning and memory deficiencies, muscle weakness, and communication problems, mimicking the human condition. Similarly, heterozygous Adnp Tyr mice carrying the most prevalent pTyr718\* mutation (almost halving the mutated protein) show delayed development, aberrant gait, and early brain tauopathy coupled to aberrant visual evoked potential. The ADNP microtubule-interacting fragment NAP (Davunetide, CP201) resolves, in part, Adnp deficiencies and protects against Adnp pathogenic sequence variant abnormalities in mouse and cell models. Having all the models at hand, we present an advantage to test a direct gene-therapy strategy for treating Adnp syndrome cases by inhibiting the potential toxic allele expression by RNA silencing while replenishing this pathogenic allele with healthy ADNP mRNA. This approach will be compared to pharmacological intervention and will pave the path to other syndromes. Our team includes four PIs with different and complementary expertise as follows. Professor Illana Gozes (IG, Tel Aviv University), the discoverer of ADNP and NAP, an expert translational neuroscientist. Professor Shlomo Wagner (SW, Haifa University), an expert for in vivo electrophysiology in social behavior and animal models of autism. Dr. Shani Stern (SS, Haifa Univesity), an expert in differentiating patient-derived pluripotent stem cells to neurons and Dr. Assaf Zinger (AZ, Technion), an expert in biomimetic nanoparticles and their translational use for RNA delivery into the brain. Importantly, NAP (Dauvnetide) and related ADNP technologies are exclusively licensed to ATED Therapeutics Ltd (IG, Chief Scientific Officer), which is developing ADNP-based therapies, targeting first the ADNP syndrome. With the discovery of potential pipeline therapeutics proposed here, ATED will be ready for direct clinical translation.

**Aim**: We propose a synergistic effort of our labs to develop and test a cutting-edge, two-step gene-therapy strategy for the ADNP syndrome based on RNA-encapsulating biomimetic nanoparticles. This strategy will be tested on cellular and animal models of the most prevalent human de novo mutation of the ADNP gene (Tyr719\*), as well as on haploinsufficiency models, and its results will be directly compared with pharmacological NAP treatment. Moreover, we hypothesize that the deteriorating effects of ADNP mutations may arise from a reduced dose of functional ADNP protein and dominant-negative effects of the mutated protein. Thus, delivering mRNA of functional protein may not be enough to correct all the effects of the mutation, and an inhibitory RNA strategy should be for that. To challenge this hypothesis, we will examine the effects of using the nanoparticles for delivering either siRNA against the mutated gene, mRNA of the functional gene, or both to each one of the models.

**Experimental approach**: This study will be based upon mouse models and human cells already existing in the IG laboratory. These include cells from an ADNP-syndrome patient with the Tyr719\* mutation and a paralog mouse model - heterozygous *Adnp* p.Tyr718\* (Tyr) mice, as well as cells from 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 labs).*

Batches of patient-derived cells (pTyr718\*, and pArg216\*, available at IG lab) will be reprogrammed to iPSCs, and the mutations will be corrected using CRISPR/Cas9 technology to create two pairs of isogenic lines with the patient and the control with the same genetic background. The mutant and the corrected control iPSC lines will then be differentiated into several types of hippocampal neurons (dentate gyrus granule neurons as well as CA3 pyramidal neurons) and hippocampal brain organoids at the SS lab. Using patch-clamp, calcium imaging, and multi-electrode arrays, 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 the differentiation. Specific receptor blockers will be applied to determine the types of changes in the case of synaptic transmission deficiencies.

The cell composition of the monolayer and 3D cultures will be determined using 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 the differentiation to better understand the dynamic gene regulation program in the patients and how it changed compared to the isogenic controls.

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 labs).*

We will first use a battery of social discrimination tasks to identify specific behavioral as well as gait deficits in both 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 from multiple (up to 32 at a time) social-behavior associated brain regions, including the various areas (CA1, CA3, and DG) of both dorsal and ventral hippocampus, medial prefrontal cortex, nucleus accumbens, and multiple amygdaloid areas. Using this methodology, we will first 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 use Neurpixel probes to analyze the neural activity at specific brain areas that show modified activity by the electrode-array screen, most specifically hippocampal areas, at the single-cell level. At the end of this proposal, we hope to identify specific deficits in social behavior and specific signatures of brain activity modified in the mutated animals. These will be used later to examine the treatments' behavioral and electrophysiological effects.

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

Four groups of nanoparticles (NP) will be fabricated: 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 that will serve as a control. After fabricating and characterizing these NP physiochemical and biomimetic properties by the Zinger lab, they will be tested for toxicity, gene regulation, gene expression, association, uptake, and electrophysiology on cellular and organoid models produced by the SS lab with all types of particles tested with the de novo mutation models. In contrast, only the mRNA-encapsulating and control NP will be tested on the haploinsufficiency models, where there is no significant dominant-negative effect of truncated protein. All models will also be tested with a pharmacological treatment using NAP. Furthermore, biochemical analysis of microtubule – Tau association will be determined by IG’s laboratory utilizing coimmunoprecipitation techniques, thus addressing potential pharmacological protection by NAP (Davunetide) compared to NP treatments. We will examine whether any treatments yield a significant restoration of the electrophysiological deficits observed in Aim 1. In parallel, IG lab will analyze the treatments' transcriptomic effect on the various models. At the end of this aim, we will compare the electrophysiological and transcriptomic analyses of the different treatments and examine their efficacy regarding the cellular models.

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

The two animal models will be chronically treated with intranasal NP administration three times a week during PN weeks 2-8 by AZ lab. The NP whole body distribution will also be tested using *in vivo* imaging system (IVIS) at pre-determined time intervals: 6, 24, and 48 hours following administration. Next, the mice will be transferred to SW lab and behaviorally characterized to assess if any of the treatments alleviated the impaired social behavior. The results will be compared to the pharmacological NAP (davunetide) intervention conducted in IG lab. For the efficient treatments, we will use the in vivo electrophysiology (both electrode microarrays and Neuropixel probes), to examine if the treatment also restored the modified electrophysiology during social behavior in the same brain regions identified in Aim 2 and specifically in the hippocampal regions. In parallel, a single-cell transcriptomic analysis will be conducted by IG to examine the transcriptomic changes induced by the treatment. At the end of this aim we hope to identify a specific treatment that restores typical behavior and normalize electrophysiology and gene transcription in the brains of the Adnp mutant mice.

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