**Scientific Background**:

ASD is a group of neurodevelopmental conditions characterized by persistent deficits in social behavior and the manifestation of repetitive and stereotypic patterns of behaviors with no pharmacological treatments available to treat these core symptoms 1. ASD is considered to be highly affected by genetic factors 2; About 5-10% of the ASD diagnoses can be identified with a specific etiology in clinical genetics and they are termed syndromic ASD 3. Nevertheless, distinct mutations of the same gene may cause different etiologies and symptoms 4. These may be related to the fact that many mutations not only lead to insufficient level of functional protein but also yield a truncated form of it, which may prevent appropriate activity of the functional form (dominant-negative effect). Therefore, it seems as if the most effective way to develop a treatment for syndromic ASD cases is through a personalized genetic approach, by targeting both the genetic haploinsufficiency and the possible dominant-negative effects. Coping with COVID19 pandemics demonstrated that mRNA holds the promise to transform clinical applications such as immunizations, therapy of genetic disorders, and protein replacement therapies 5. The delivery of mRNA and its expression in the brain are the key factors in optimizing specific therapeutic applications 6,7. Thus, syndromic ASD cases may benefit from the delivery of mRNA that encodes the functional protein as well as siRNA that inhibits the production of the truncated protein. Among the various strategies developed for efficient mRNA delivery to the brain *in vivo*, nanoparticles (NP) stand as the most frequently used non-viral means. It holds the merits of efficiently binding and condensing RNA, protecting against degradation in the extracellular space, and localizing the payload at the membrane of the desired target cell, followed by cellular uptake and endosomal escape into the cytosol 8. Zinger's research team recently designed neurons’ biomimetic NP to enhance selective targeting of nerve cells (Fig. 1). We hypothesize that such neurons’ biomimetic NP may provide an effective way to develop a tailored treatment for syndromic ASD patients, by delivering both mRNA and siRNA to the brain. This novel strategy may be tested by employing models which are directly related to the specific case under examination. Such models may be either patient-derived induced pluripotent stem cells (iPSCs) or animal models that mimic the specific mutation associated with the patient’s etiology. Here we plan to use both models in parallel.

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 as well as intellectual disabilities 9-16. 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 12,17. When the mutation is close to the ADNP start codon, the child will present a haploinsufficient loss-of-function phenotype. Indeed, Adnp+/− mice 18 suffer from slower axonal transport 19 and impaired dendritic spines, learning and memory deficiencies, muscle weakness, and communication problems, mimicking the human condition 20-25 (Table 1). 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 26. The ADNP microtubule-interacting fragment NAP (Davunetide, CP201) 27,28 resolves, in part, ADNP deficiencies and protects against ADNP pathogenic sequence variant abnormalities in mouse (above) and humanized cell models 10,29,30 (with mouse ADNP presenting 90% identity to the human ADNP) 31 (Tables 2 & 3). 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 (using siRNA) while replenishing this pathogenic allele with healthy ADNP mRNA. This approach will be compared to pharmacological intervention and will pave the path for treatments directed to other syndromes.

**Team**: 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 University), an expert in differentiating patient-derived pluripotent stem cells to neurons and organoids and electrophysiology and Dr. Assaf Zinger (AZ, Technion), an expert in producing 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 of the proposed study’s findings.

**Aim**: We propose a synergistic effort of our labs to develop and test a cutting-edge, gene-therapy strategy for the ADNP syndrome, based on RNA-encapsulating biomimetic NP. 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 cellular and animal models, and its results will be directly compared with pharmacological NAP treatment. We hypothesize that the deteriorating effects of ADNP mutations may arise from both a reduced dose of functional ADNP protein and dominant-negative effect 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 also be employed. To challenge this hypothesis, we will examine the effects of using NP for delivering either siRNA against the mutated gene, mRNA of the functional gene, or both RNA types together, to each one of the models.

**Preliminary results**:

ADNP models and NAP treatment (IG): As stated in the Introduction, we are well positioned with cellular (Table 2) and unique animal models of the ADNP syndrome (Table 3) mimicking the human condition (Table 1) and responsive to pharmacological intranasal NAP treatment. We further have evidence that healthy ADNP overexpression is tolerated 29, and that ADNP self-regulates its own promoter 32,33. Using advanced RNA-seq, and gene expression profiling, we have shown that ADNP is a master regulator of critical gene expression, corrected, in part, by NAP treatment (Tables 2 & 3). While we have evidence of shared phenotype between distinct ADNP mutations, we also have indications of differences, given the size and multiple active sites on the ADNP molecule 34. Most importantly, we see gain of toxic function in the newly developed heterozygous *Adnp* p.Tyr718\* (Tyr) mice 26. These findings are extended to our novel CRISPR/Cas9 DNA-edited established neuroblastoma cell lines that express two different green fluorescent protein (GFP) – labeled mutated ADNP proteins (p.Tyr718\* and p.Ser403\*). These cells exhibit unique gain of toxic phenotypes, with p.Tyr718\* (mutated at the nuclear localization motif) showing diffused cellular distribution (unclear nuclear boundaries) in comparison to the control GFP-labeled ADNP as well as p.Ser403\*, even in DMSO-neuronal like cells. However, NAP + DMSO corrected this abnormal phenotype (Fig. 2). Complementing these cell cultures, IG has studied multiple patient-derived lymphoblatoid cells (LCLs, Tables 1-2), including, but not limited to the human homologue of p.Tyr718\*, namely, p.Tyr719\* e.g. 26, these cells are available to IG and SS through Simon Simplex Collection (SSC08311). Furthermore, the corresponding p.Tyr719\* iPSC line (and relevant controls) are available through collaboration with a leading group in Spain 30,35,36.

iPSCs (SS): Electrophysiological recordings were conducted at the SS lab from cortical and hippocampal neurons derived from patients with other ASD-associated mutations, such as the c.3679insG Shank3 and c.2065G>T GRIN2B (Fig. 2). Already at 2.5 wk post-differentiation, that represents an early developmental stage, we observed neurophysiological irregularities. The control lines at this stage are immature, have a small sodium current and cannot evoke action potentials. Both Shank3 and GRIN2B mutant neurons develop faster, exhibiting more significant potassium and sodium currents and more evoked action potentials. We have reported a similar phenotype in IQSEC2 mutant hippocampal neurons compared to the CRIPSR/Cas9 corrected controls 37 as well as in WWOX mutant cortical organoids compared to CRIPSR/Cas9 corrected controls 38. We saw similar phenotype in several lines derived from patients with ASD, suggesting a convergent phenotype of cortical and hippocampal neurons derived from ASD patients. We further explore these mutant lines, to see what are the changes at later stages and how does this early phenotype affect them. Importantly, IG has reported sex and age-dependent regulation of ion channels by ADNP, as specifically indicated in the hippocampus of the Adnp+/- mouse 19.

Animal models correlating electrophysiologial with social activity in live mice (SW): The SW lab used chronically-implanted electrode arrays (EAs) to explore the activity of 18 social behavior-associated brain regions in ICR mice (background strain to the Adnp+/- mice 20, now expanded to the p.Tyr718\* mice, IG). Conducting three distinct social discrimination tasks: social preference (SP), emotional state preference (ESP) and sex preference (SXP), SW found each task elicited a distinct profile of local field potential (LFP), as analyzed using power spectral density (PSD) analysis (Fig. 3A-C). By separately analyzing the theta (4-12 Hz) and gamma (30-80 Hz) rhythms, they observed a different pattern of power change across the various regions for each task (Fig. 3D-G). By analyzing the power change in synchronization with the distinct investigation bouts, separately for each of the stimuli, they found that distinct brain regions showed different pattern of stimulus-specific responses at the various tasks (Fig. 4A-D). Furthermore, analysis of the coherence between the various brain regions demonstrated a distinct pattern of coherence change during each task, although no difference between the tasks was observed before stimulus insertion (Fig. 4G-K). Altogether, these results reveal a unique pattern of brain activity at the system level for each of the tasks. To further explore this possibility, the SW team are now using Neuropixels probes to record single-unit from hundreds of neurons at a time from specific brain areas, during the various tasks (Fig. 5). Moreover, while conducting similar recordings from Cntnap2-/- mice, a well-established mouse model of ASD which exhibit impaired ESP behavior, they found general changes in both theta and gamma rhythmicity in the mutant (MT) mice, as compared to wild-type (WT) littermates. Specifically, they observed changes in neural activity in the nucleus accumbens, medial amygdala and infralimbic prefrontal cortex (Fig. 6). These data suggest that the neural activity associated with the impaired social behavior is differentially distributed in brain of MT and WT mice, specifically in these brain regions, which were previously implicated in ESP 39,40. IG reported deficits in visual evoked potentials because of the p.Tyr718\* mutation, corrected by NAP 26, the SW system will complement and extend these findings toward better understanding and future therapeutics.

Biomimetic NPs and intranasal delivery (AZ): In preliminary studies, weI verified a method for incorporating membrane proteins extracted from membranal GFP expressing human neurons into lipid-based NP **(Fig. 2A)**. Following differentiation of the cells, their membrane proteins were extracted with an extraction kit (ProteoExtract®, Sigma) and quantified using a BCA protein assay kit (Pierce™, Thermo Fischer). As a negative control, we fabricated liposomes (i.e., lipid NP only, without integration of membrane proteins). This lipid formulation is composed of neutral lipids 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC, Avanti), 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC, Avanti), and cholesterol (Sigma), was chosen to enable the encapsulation of either hydrophilic41 or hydrophobic42 therapeutic cargo. Finally, we utilized our current microfluidic assembly protocol to fabricate NP, adapting the NanoAssemblerTM benchtop system for combining neuron cell-derived membrane proteins with lipids (Neurosomes). Neurosomes exhibited the same morphology as liposomes, as determined by cryo-transmission electron microscopy (TEM) **(Fig. 2B)**. We next defined the Neurosomes' physical characteristics and assessed reproducibility within three non-dependent batches using Dynamic Light Scattering Zetasizer Nano ZS (for NP size distribution and zeta potential) **(Fig. 2C)**. The decreased zeta potential of Neurosomes compared to liposomes verified the integration of the membrane proteins in the NP lipid membrane. Together, these studies confirmed our capacity to systematically incorporate membrane proteins into NP with dedicated formulation and characterize their physicochemical properties. To ensure the carry-over of unique proteins from neurons, we generated NP using neurons membrane proteins and assessed the presence of neuronal markers by western blot (WB). NCAM1 and membranal GFP were exclusively detected in Neurosome samples as evidence of the membrane protein carryover using WB **(Fig. 2D)**.

Generation of reporter mRNA (i.e., enhanced green fluorescent protein [EGFP]) encapsulating NPs. In preliminary studies, we have verified our ability to reproducibly fabricate and characterize mRNA encapsulating NPs, this time with distinct lipid formulation aimed to encapsulate mRNA: D-Lin-MC3-DMA; 1,2-distearoyl-sn-glycero-3-phosphocholine, and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethyleneglycol-2000 (Fig. 7A). The encapsulation efficiency of mRNA was determined using Quant-iT™ RiboGreen® RNA Reagent assay. As an initial proof-of-concept for our functional mRNA delivery approach, mRNA encoding EGFP was loaded into NPs and added to human triple-negative breast cancer (TNBC) cell line (MDA-MB-231, ATCC). The fluorescent protein expression as an indication for functional mRNA delivery was evaluated using IncuCyte Live-Cell Systems (Fig. 7B).

Validation of NP ability to accumulate in the brain, using two different routes of NP administration in vivo. In preliminary studies, we verified our ability to reproducibly and controllably administrate NP either locally or intranasally. For the local NP administration, the mice were anesthetized with isoflurane and placed on a stereotaxic frame with a built-in heating bed that maintains body temperature at 37 °C. First, a 10-mm midline incision was made over the skull to allow a 4 mm craniotomy on the left parietal bone. Next, 2 μl of cy5.5 labeled NP at a rate of 0.2 μl/min were injected using a mounted Hamilton syringe and automatic syringe. After the injection, the incision was closed with staples, and the mice were allowed to fully recover from anesthesia before transferring it to their home cages. For the intranasal cy5.5 NP administration, the mice were held, inverted, and intranasally administrated repeatably with 5 μl of NP into the left and right nostrils alternately until reaching a volume of 20 μl in each nostril, totally pf 40 μl. Finally, 24 h after administration, the mice and brains were imaged using *in vivo* imaging system (IVIS), and we successfully validated NP accumulation in the brain following local and intranasal delivery (Fig. 8). Notably, different distribution patterns were noticed.

In vivo transcriptomic analysis (IG): Previous published hippocampal Adnp+/- data is indicated in Tables 1-3 19. Results in complete RNA-seq of heterozygous *Adnp* p.Tyr718\* (Tyr) mice indicate a sex specific gene expression change in spleens, with focus on female *Foxo3*, a gene associated with synapse formation, autophagy and healthy aging 26. These finding are now extended to the Tyr hippocampus (under analysis), with *Foxo1*, a gene/protein regulated by Foxo3 43, in the female Tyr hippocampus (Fig. 9, Shapira, Karmon, Shomron and Gozes, in preparation, complete results RNA-seq analysis for model characterization and NAP influence will be available for the grant initiation time).

**Specific aims**: This study will be based upon mouse models and human cells already existing in the IG laboratory. These include cells from ADNP-syndrome patients with the Tyr719\* mutation and a paralog mouse model - heterozygous Tyr mice, as well as haploinsufficient human ADNP cells and an Adnp+/- mouse model (Tables 1-3, and preliminary results).

*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 9 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 (DG) 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 and behavioral 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. Research deliverable will include couple unique deficits in social behavior with specific signatures of brain activity modified in the mutated animals. These will be used later to examine the treatments' behavioral and electrophysiological effects. These studies will be coupled to previous behavioral characterizations including learning and memory, gait and open field behavior measuring anxiety and aberrant activities (Table 3).

*Specific 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 in collaboration with IG 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 it is envisaged that there is no significant dominant-negative effect of truncated protein. All models will also be tested with a pharmacological treatment using NAP, for comparison. Furthermore, biochemical analysis of microtubule – Tau association will be determined by IG’s laboratory utilizing coimmunoprecipitation techniques, further addressing potential pharmacological protection by NAP (Davunetide) compared to NP treatments. We will examine whether any treatments yields 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.

*Specific 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 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, paralleling gait, motor, learning and memory that will be performed by the 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 study 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.

**Experimental design**:

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

* 1. **Reprogram PBMC into iPSCs.** Using Sendai reprogramming, we will reprogram PBMCs/lymphoblastoid cell lines (**SS,** LCLs, **IG**, Table 2) of one ADNP patient with the p.Tyr719\* (compared to available p.Tyr719\* iPSC, and one with p.Arg216\* mutations (IG). Additionally, iPSCs will be produced from two male children with the mutations in their ADNP gene, p.Arg216\*; and p.Arg419Glufs\*3, and two adults man with a p.Arg730\* mutation and a woman with a p.Val180Glyfs17\* mutation (ref Levine et al) iPSCs. Another cohort, also previously published by (Gozes et al ) will be reprogrammed with two ADNP-mutated LCLs that were purchased from the Simon Simplex Collection, SSC04121=ADNP (protein) p.Lys408Valfs\*31 and [SSC08311](https://www.ncbi.nlm.nih.gov/protein/SSC08311)= p.Tyr719\*. iPSCs  will be derived from PBMCs using a Cyto-Tune Sendai reprogramming kit (Invitrogen) or from LCLs using Episomal vectos. All iPSCs will be characterized as previously described 44. This will give a total of 8 patient lines. Some of these lines will be genetically engineered to produce isogenic controls.

Briefly, the non-integrating Sendai virus, a system we have found to be very efficient will be used to produce iPSCs (CytoTune Kit, Life Technologies). PBMCs will be transduced with the Sendai virus and re-plated 3 days later on Matrigel-coated plates. Approximately one week after the transduction, the medium will be gradually replaced with reproTeSR (without TGFβ). Once the colonies start to form, the reproTESR will be gradually replaced with mTESR. Colonies will be picked and replated in separate wells once the colonies are large enough. After the generation of the iPSC lines, we will confirm a normal karyotype and the clearance of the Sendai virus. Pluripotency will be confirmed by marker expression and the ability to differentiate into the three germ layers, endoderm, mesoderm, and ectoderm in embryoid bodies (EBs) assays. At least two independent, fully reprogrammed, pluripotent, and euploid iPSC lines will be established for each patient from which master and working cell banks will be generated. Reprogramming is regularly and successfully performed at the Stern lab. For ADNP haploinsufficiency, we will use CRIPSR/Cas9 as performed by **IG** 45.

For LCL reprogramming, LCLs will be transfected with Episomal vectors, and electroporation will be performed using the Neon Transfection System. Transfected LCLs will be plated in Matrigle coated 6 well plates. In the first two weeks the cells will be fed with DMEM with N2 and B27 supplaments. On the third week the media will be gradually replaced by mTeSR. Colonies will be picked and replated in separate wells once the colonies are large enough. After the generation of the iPSC lines, we will confirm a normal karyotype and the expression of pluripotency markers.**1.2 CRIPSR/Cas9 correction of the mutations.** In addition to the patient lines that will be reprogrammed, control lines will be generated by CRISPR/Cas9 editing and genetically correcting the mutations. These edited lines will establish mutant lines with control isogenic lines with a similar background. This reduces the diversity in the measurements and helps to establish a causative relationship between the genotype-phenotype. sgRNAs will be designed to specifically target each of the genes. We will confirm the mutation and its correction with Sanger Sequencing in the hiPSC clones. Two corrected clones will be selected for each of the mutations. The expression of pluripotency markers will be measured with immunocytochemistry. Potential off-target sites will be checked by Sanger sequencing.

**1.3 Hippocampal differentiation.** Our hippocampal differentiation protocol is robust and produces over 50% DG granule neurons and over 80% CA3 pyramidal neurons 46,47. The neurons are functional with active synapses, and display network bursts. Briefly, starting from iPSC colonies, we will produce hippocampal neural progenitor cells (NPCs) and then differentiate them into DG granule neurons. Cell identity will be confirmed by immunocytochemistry. In these protocols, approximately 15% of the neurons are GABAergic and 40-50% are Prox1 positive, indicating DG granule neurons. To enhance the synaptic connectivity, the neurons will be dissociated at 2-3 weeks of age and re-plated. Experiments will be performed at approximately 8 weeks’ post-differentiation. At this age, the neurons exhibit mature firing patterns as well as synaptic connectivity that we measure in voltage-clamp mode as Excitatory Post Synaptic Currents (EPSCs). Electrophysiology, as well as single cell RNA sequencing, will be performed on the patient, gene-edited, and control neuronal cultures.

**1.4 Hippocampal organoids differentiation.** Using published protocols, we will differentiate DG hippocampal neurons and hippocampal organoids 48. Mature organoids will be fixed, cryosected, and immunostained to verify cell identities and distribution. Electrophysiology, as well as single cell RNA sequencing, will be performed on the patient and control organoids.

**1.5 Cell morphology and cell numbers.** Differentiated cultures will be analyzed for neuronal morphology including soma size, neurite growth, branching, and relative numbers of differentiated cell types generated (glutamatergic neurons, GABAergic neurons, and astrocytes). We note, in our experience, the ratios of differentiated cell types in the mixed cultures we generate can differ from line to line; however, significantly skewed ratios of glutamatergic and GABAergic neurons have been reported for iPSC derived neurons from ASD, supporting the idea that such a potential phenotype should be carefully examined.

**1.6 Electrophysiology- Seeking neurophysiological alterations in ADNP patients’ neurons and orgnaoids.** SS expertise is functional assays of patient-derived neurons and organoids 37. Whole-cell patch-clamp provides the opportunity to find specific ionic currents that may be altered in the patient’s neurons. These may include changes in small currents that cannot otherwise be measured. Patch-clamp will also allow us to identify changes in neurotransmitter-specific receptors. Briefly, for whole-cell patch-clamp, neurons on coverslips or brain organoids will be transferred to a recording chamber in a standard recording medium (reference 46) Signals will be amplified and recorded and data will be analyzed using Clampfit-10 and the software package MATLAB (release 2019b; The MathWorks, Natick, MA, USA). For calcium imaging, we will incubate the neuronal cultures or organoids with Fluo-5AM for 45 minutes and then perform the recordings with a sensitive camera. The analysis will be performed with Matlab scripts that were developed at the Stern lab.

**1.7 Changes in gene regulation program in ADNP patients organoids.** We have previously used gene expression analysis to find dysregulated pathways in different neurological disorders 49,50 and even specific genes that are severely dysregulated in the ADNP syndrome (Tables 1-3). We will perform single-cell RNA sequencing for the brain organoids to seek changes in specific cellular populations. Analysis of the data will give us information on the affected pathways that are common or specific for each patient. Known ADNP-related pathways (Tables 1-3) responsive to RNA treatment will validate our methodology.

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

**2.1** We will employ our published experimental and analysis systems 51 which enable highly detailed automatic and unbiased analysis of subject mouse behavior during various tasks of social discrimination. Using this methodology, we will thoroughly characterize the social behavior of male and female WT and heterozygous (MT) mice from both the haploinsufficiency and pTyr718\* mouse models. To that end, we will use a battery of social discrimination tasks 52 including: (SP), Social novelty preference (SNP), SXP and social vs. food preference (SvF)53. We will also use three flavors of the ESP paradigm 39,52, in which the subject mouse is exposed to same-sex two novel social stimuli – a neutral stimulus (Control) and an aroused stimulus. We use three types of aroused stimuli: (1) a stimulus that received access to water for 60 min before the test, after 23 hours of water deprivation (Relieved), (2) a stimulus held in a restrainer for 15 min just before the test (Stressed) and (3) a 7-d isolated stimulus (Isolated). The behavioral analysis will characterize not only the investigation time for each stimulus but also how it changes across the time course of the session and its distribution to short and long investigation bouts, which reflect curiosity and interactive behavior, respectively 54. We previously showed that the preference of C57BL/6J mice to a specific stimulus is reflected mainly by long-bouts 52. Another parameter we will analyze is the transitions between stimuli, which are likely to mark time points of social decision-making 53. At the end of this stage, we hope to identify specific deficits in the social behavior of each one of the mouse models, including sex-dependent differences.

**2.2** We will conduct mesoscale electrophysiological recordings from behaving mice 55,56 and characterize the distributed neural activity that takes place in 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 several amygdaloid areas]. This will be done in male and female (WT) and MT mice from both the haploinsufficiency and pTyr718\* mouse models while they are performing the specific social discrimination tasks found to be impaired in each one of the models (**2.1**). We will analyze changes in multi-unit activity (MUA) frequency and the power at various frequencies of local field potential (LFP) signals (e.g. theta, gamma) in the distinct brain areas, as well as in the coherence between them (Fig. ?-??). For each social discrimination session, we will analyze the data first by comparing the 5-min session (test period) to the 5-min period before it (baseline period). We will then analyze the same parameters in synchrony with the investigation bouts of the distinct stimuli and the transitions between them (Fig. ???). We will look for brain areas (or couples of brain areas in the case of coherence) which show statistically significant changes between the WT and MT mice for each Adnp model, in any of the examined parameters during the various tasks. At the end of this stage, we will define specific signatures of brain activity at the system level, which are modified in the two Adnp-syndrome models during impaired social behavior. We also hope to identify specific brain regions showing the most significant changes in neural activity in the mutated mice, as compared to their WT littermates.

**2.3** We will characterize WT and MT mouse neural activity that takes place during the impaired tasks (**2.2**) at the single-cell level. To that end, we will use Neuropixels 1.0 probes, which enable recordings of hundreds of single-units during behavior 57. Specifically, we will record activity from the various hippocampal areas, as well as from mPFC infralimbic (IL) and prelimbic (PrL) areas and accumbens core (AcbC) and shell (AcbSh) areas, some of which may be recorded simultaneously using a single probe. Our preliminary results suggest that these areas show modified population activity in Cntnap2-KO mice during affective-state recognition behavior (Fig. ???). We will analyze the activity of the recorded units across the session, as well as in synchrony with specific behavioral events, such as short and long investigation bouts for each stimulus, as well as the transitions between stimuli. At the end of this stage, we hope to identify specific hubs of modified activity in the brain and to characterize how the various mutations change their neural activity during impaired social discrimination tasks.

Overall, at the end of this aim we will have quantitative behavioral and electrophysiological signatures of the distinct Adnp mutations, which will allow us to examine the effects of the various treatments in Aim 4.

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

**3.1** Here, we will generate three distinct groups of NP: (1) *Adnp*-mRNA, (2) Adnp Si-RNA based on the CRISPR/Cas9 generation of the p.Tyr718\* mice 26, and (3) Adnp-mRNA+Si-RNA group. The Adnp+/- haploinsufficient mouse model will serve as a control for *Adnp* mRNA delivery. mouseEncapsulating biomimetic nanoparticles (ASD-NP) using the NanoAssemblr™ Benchtop by applying our optimized expertise in synthesizing biomimetic NP using microfluidics 58-60. Next, we will optimize ASD-NP formulations by adjusting the NP lipid backbone for improved stability, mRNA encapsulation efficiency, mRNA release, and the NP membrane proteins composition to enhance ASD-NP neurons specific targeting.

**3.2 Adjusting ASD-NP lipid formulation.** Two combinations of lipid formulations capable of delivering mRNA will be tested. These formulations were chosen based on their known ability to load and deliver mRNA 61,62, and our lab's previous experience with their ability to integrate membrane proteins and to support the loaded NP structure **(Table 4)**. Moreover, these two NP formulations allow us to adjust the cholesterol content (0, 15, or 30 mol %) to test its effect on the mRNA release rate at a physiological temperature of 37°C.

**3.4 Adjusting ASD-NP protein formulation.** Three different membrane protein compositions will be tested to enhance specific ASD-NP neurons targeting based on homotypic cell-cell adhesion (i.e., the attachment of a cell to a second cell of the identical type via adhesion molecules). *NCAM1-ASD-NP* incorporating in their membrane only neural cell adhesion molecule 1 (NCAM1) human recombinant membrane protein; *L1CAM-ASD-NP* incorporating only L1 Cell Adhesion Molecule.

(L1CAM) human recombinant membrane protein and *Neuro-ASD-NP* integrating the whole neuron membrane proteins extract. NCAM1 and L1CAM were chosen based on their natural abundancy on neurons’ surfaces to increase ASD-NP probability to target neurons. In addition, using one type of membrane protein presented on the ASD-NP surface might shorten future food and drug administration (FDA) approval. The use of whole neurons’ membrane proteins extract for NP generation was chosen based on our previous published data, demonstrating specific neuron targeting field 58 and, together with liposomes (i.e., NP without membrane proteins), will be used as experimental controls to assess ASD-NP groups neurons’ targeting ability.

**3.5 ASD-NP protein characterization.** The ASD-NP groups will be thoroughly characterized and tested for homogeneity, stability, mRNA encapsulation efficiency and mRNA release tests, and the integration and orientation of the specific proteins on their surface. In addition, we will rigorously characterize each of the ASD-NP groups to address reproducibility (batch-to-batch and consistent protein presentation) and ability to retain surface proteins using the following approaches. We will assess the reproducibility of the physiochemical properties of NP by monitoring variations in size, polydispersity index (PDI), zeta potential, and concentration using a Zetasizer Ultra particle analyzer, and cryo-transmission electron microscopy (TEM). **Protein consistency** will be analyzed using micro-BCA assay, denaturing polyacrylamide gel electrophoresis (SDS-PAGE) mass spectrometry, Western blot (WB), and cryo-TEM using with specific fluorescent and metal-labeled antibodies, respectively. **Stability** will be tested by evaluating the changes in size, zeta potential, NP concentration, and the expression of particular biomarkers using WB for up to 21 days. We will assess the stability at two different temperatures: 4°C (storage temperature) and 37°C (physiological temperature). At the end of this stage, we expect to determine the optimal formulation of ASD-NP that yields a reproducible size, zeta potential, PDI, optimal mRNA encapsulation efficiency and release, and membrane protein consistency.

**3.6 ASD-NP treatment for the human organoids.** The NP treatment will be supplied to the human organoids three times a week immediately when starting the differentiation. We have shown previously that transcriptional changes occur within days of starting the differentiation 49 (Schafer et al 2019 Nature Neuroscience). We, therefore, need to start the genetic intervention immediately when starting the differentiation. Early phenotypes such as electrophysiological, morphological, and transcriptional changes will be measured after the treatment to seek rescue. Additionally, later stage phenotypes such as synaptic degradation, will be measured to see if rescuing the neuronal changes early will also stop the synaptic degradation that appear in many ASD-related patient-derived neurons.

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

**4.1 Assessing neuronal targeting, organs distribution, and mRNA functional delivery by ASD-NP *in vivo* using the Adnp+/- and the Adnp Tyr (p.Tyr718\*) mice.** Two routes of administration, locally and intranasally, will be used for exploring ASD-NP potential as a ASD treatment. Local injection of ASD-NP will allow us to determine the exact injection site and, due to higher ASD-NP in the target location, to have a better probability for higher Adnp expression. However, this administration method might harm healthy tissue during the injection and demand a future osmotic pump or catheter installation if chronic treatment is chosen. The intranasal administration allows a non-invasive delivery and is more appealing for future treatment. However, in these cases, a lower amount of NP will reach the target area due to their bio-distribution. First, we will use the ASD-NP group that demonstrated the highest neuronal targeting and optimal mRNA expression *in vitro*. Here, we will fluorescently label this NP by DSPE-rhodamine (absorbance:560, emission:583), encapsulate either eGFP expressing mRNA (absorbance:488, emission:507) or *luciferase* mRNA, and will either locally inject it to the brain (i.e., cortex) or deliver it intranasally at pre-determined time intervals: 1,3,6,8 and 24 hr. After 24 hr., the mice will be euthanized perfused, and the major filtering organs: lungs, liver, spleen, and kidneys, along with the target organ (i.e., brain), will be harvested and imaged using in vivo imaging system (IVIS). Finally, the organs will be fixed using 4% neutral buffered formalin overnight and processed for paraffinization and sectioning except for the brains that will be sent to *expressive neuroscience* for assessing ASD-NP specific neurons targeting and desired protein expression (e.g., both eGFP and Adnp) *in vivo* **(Table 5)***.* ASD-NP effectiveness will be tested compared to liposomes (e.g., lipid NP without membrane proteins that will serve as a negative control for the ASD-NP). At the end of this stage, we expect that different NP groups and routes of administration will exhibit different levels of targeting and functional mRNA delivery to neurons in the brain due to different bio-distribution patterns.

**4.2** We will use the same methodology as in **4.1** to compare the behavior of animals from the various treatment groups. Parameters to be measured are gait (motor) and learning and memory previously impacted in the Adnp MT mice (Tables 1-3). We will then test social behavior coupled to electrophysiological assessment (Aim 2 above) to test if any of the treatments will cause an improvement in the impaired behavior of the MT animals. In parallel, transcriptomic analyses will be conducted by IG as before (Tables 1-3) 19,26 to examine the transcriptomic changes induced by the treatment.

**Pitfalls and alternative strategies**:

Aim 1: We do not envisage problems in the generation of the iPSCs, with published literature, and collaborative science 35 and available expertise (SS). However, given the critical ADNP function during development, the differentiation of these cells may be stunted, requiring early intervention, as an alternative strategy.

Aim 2: While the Adnp+/- mice show social deficits (IG, Tables 1 & 3), handling of the Tyr mice results in loss of some social deficits 26. In the proposed studies, we will use Tyr mice on ICR background complementing the same background strain of the Adnp+/- mice and the available expertise (SS). Furthermore, IG will also perform gait, motor and learning and memory analyses as before (Tables 1 & 3), further establishing the models and the RNA efficacy.

Aim 3: Suppose the size of the different ASD-NP groups will cross the 250 nm diameter. In that case, we will assess the effect of increasing the polyethylene glycol (PEG) molar ratio in the NP formulation and use shorter PEG chains. In case the stability of ASD-NP at 4ºC is inadequate, i.e., ±40 nm size, ±5 mV zeta potential, and ±0.2 polydispersity index (PDI), we will explore (1) adjusting the formulation to incorporate longer lipid chains that will stabilize the formulation, (2) increase the molar concentration of saturated lipid chains, or (3) increase the concentration of cholesterol. Suppose encapsulated mRNA concentration is low, lower cationic lipid:mRNA amount (w/w) ratios will be tested, and longer phospholipid chains will also be used.

Aim 4: 4a - One major obstacle could be that NP would not be detected in the brain using IVIS due to either low fluorophore emission or poor NP concentration. In this case, we will increase the fluorescent molecule concentration during the fabrication process as demonstrated in our previous publication 60 or treat the mice with a higher concentration of NP followed by an IVIS distribution assessment. Another alternative approach will be, replacing the eGFP-mRNA with Luciferase-mRNA and increasing the chance that IVIS will detect the expressed protein. 4b - 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. Of note, IG developed a highly accessible nose-brain formulation, easily tested before 24, which could be used together with the suggested nasal delivery mode.

**Project significance and relevance to autism:**

This is a proof of principal study, with ADNP being one of the most abundant genes de novo mutated in autism 63. Most importantly, recent data suggests that ADNP interacts with other key autism genes, for example, SHANK3 34 and POGZ 64,65, with NAP being effective in treating Shank3-mutated mice 34. Furthermore and also for example, with ADNP being part of the SWI/SNF chromatin remodeling complex 66, it is also associated with autism inflicted by mutations/dysregulation in these genes with multiple literature citations (<https://pubmed.ncbi.nlm.nih.gov/?term=SWI%2FSNF+autism&sort=date>). Thus, we will prove: 1] if Adnp mRNA treatment is viable for haploisufficiency, 2] if silencing of mutated Adnp mRNA expression is viable for the treatment of gain of toxic function, which will be applicable for the ADNP syndrome and serve of a prototype for other syndromes. Additionally, *ADNP* mRNA, may be unbalanced in the related syndromes, or idiopathic autism (which could be assessed in the future by serum/CSF evaluations) and could supplement directed treatment modalities. Finally, ATED Therapeutics is centered on ADNP and neurodevelopmental, neuropsychistric and neurodegenerative diseases, as such, developing NAP (davunetide) as the lead compound for the ADNP syndrome. As such, ATED is well poised for further development, accelerating translational science for the benefit of autism, currently an unmet need.

**Timeline and milestones:**

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