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- Laboratorio di Neurogenetica e Neuroscienze, Istituto "G. Gaslini", Genova, Italia
- Dipartimento di Medicina Sperimentale (DiMeS), Università degli Studi di Genova, Genova, Italia
- Neuroscience Campus Amsterdam, Vrije Universiteit (VU), Amsterdam, The Netherlands
- In collaboration with: LICE collaborative group on STXBP1

TITOLO DEL PROGETTO

UN'INNOVATIVA STRATEGIA MOLECOLARE PER IL TRATTAMENTO DELL'ENCEFALOPATIA EPILETTICA DA MUTAZIONI IN *STXBP1*

SINOSSI

Mutazioni de novo in *STXBP1* sono causa di una grave encefalopatia epilettica ad esordio precoce. Syntaxin-binding-protein-1 è una proteina che partecipa all'ancoraggio e alla fusione delle vescicole sinaptiche [1]. Mutazioni con perdita di funzione di *STXBP1* determinano un deficit nel rilascio del neurotrasmettitore [2]. Questa encefalopatia è caratterizzata da crisi epilettiche di vario tipo, con esordio nei primi mesi di vita e spesso refrattarie ai farmaci. L'elettroencefalogramma presenta un'attività epilettica multifocale, ipsaritmia o burst-suppression [3]. I pazienti mostrano disabilità intellettiva, grave o profonda e, spesso, sintomi neurologici aggiuntivi, quali disturbi dello spettro autistico, disturbi motori (discinesia, distonia, tremore), ipotonia assiale ed atassia. La gravità del fenotipo e l'assenza di trattamenti efficaci richiedono un approccio terapeutico più mirato e radicale. Recentemente è stata identificata una nuova classe di RNA non-codificanti, naturali e sintetici, in grado di incrementare la traduzione gene-specifica di una proteina (SINEUPs), senza interferire con la quantità di mRNA del gene target. I SINEUPs sono attivatori della traduzione, composti da un Dominio di Legame (BD) antisenso all'mRNA codificante che determina la specificità e un Dominio Effettore (ED) che aumenta la sintesi proteica attraverso il reclutamento del macchinario molecolare necessario alla traduzione.

La nostra proposta mira a esplorare il potenziale terapeutico dei SINEUPs nell'encefalopatia da mutazioni in *STXBP1*. L'applicazione della tecnologia a RNA alle encefalopatie epilettiche mira ad aumentare l'attività dell'allele wild-type (WT) residuo e superare così la perdita di funzione dell'allele mutato. Lo scopo finale del progetto è di recuperare in vitro la quantità fisiologica della proteina target stxbp1 nelle cellule neurali derivate da pazienti con encefalopatia da *STXBP1*, utilizzando i SINEUPs.

A tal fine abbiamo definito i seguenti obiettivi specifici:

i) Selezione di pazienti per lo sviluppo di linee IPSCs mutazione-specifiche dai fibroblasti. Abbiamo raccolto una coorte di 23 pazienti portatori di mutazioni in *STXBP1* in collaborazione con diversi Centri italiani.

ii) Disegno di SINEUPs che legano in modo specifico l'mRNA STXBP1. Per testare l'effetto specifico dei SINEUPs abbiamo selezionato la linea cellulare umana SH-SY5Y che presenta una significativa espressione endogena di STXBP1. Dopo un'analisi bioinformatica del promotore *STXBP1* (Zenbu Genome Browser) abbiamo disegnato tre SINEUP con BDs diversi diretti contro lo stesso mRNA target e li abbiamo clonati in vettori di espressione.

iii) Valutazione dell'attività dei SINEUPs nella linea cellulare umana SH-SY5Y. I SINEUP-STXBP1 verranno testati nelle cellule SH-SY5Y trasfettate con SINEUP-STXBP1 o controlli negativi (SINEUP-ΔBD e SINEUP-Scramble). La proteina target dopo somministrazione di SINEUPs verrà valutata mediante Western blot.

iv) Generazione di neuroni derivati da cellule staminali pluripotenti indotte (IPSCs). IPSCs derivate da fibroblasti dei pazienti con mutazione con perdita di funzione in STXBP1 e da IPSCs di soggetti di controllo, verranno generati secondo il protocollo standardizzato nel nostro laboratorio.

v) Valutazione del recupero funzionale determinato dai SINEUPs nelle cellule derivate dai pazienti. Una volta confermata l'efficacia nella linea cellulare SH-SY5Y, i SINEUPs saranno testati in neuroni differenziati da cellule IPSC, derivate da fibroblasti di pazienti mutati in *STXBP1*. La quantificazione della proteina STXBP1 dopo la trasduzione con SINEUP verrà eseguita mediante Western blot. Il saggio funzionale sul rilascio e il riciclo delle vescicole sinaptiche sarà condotto in collaborazione con la Prof. A. Fassio (DiMeS, Università degli Studi di Genova) e con il Prof. M. Verhage (Vrije Universiteit Amsterdam).

Ci aspettiamo di identificare almeno un SINEUP che up-regola la traduzione della proteina stxbp1 e di recuperare il fenotipo sinaptico delle cellule derivate dai pazienti. Le molecole efficaci potranno essere ulteriormente testate in un modello animale eterozigote disponibile presso il Laboratorio del prof. Verhage e, in futuro, nei pazienti. I SINEUPs rappresentano potenzialmente un innovativo strumento terapeutico molecolare che abbraccia il paradigma della medicina di precisione.

RAZIONALE

Mutazioni de novo in *STXBP1* sono causa di una grave encefalopatia epilettica ad esordio precoce. Syntaxin-binding-protein-1 è una proteina che partecipa all'ancoraggio e alla fusione delle vescicole sinaptiche (1). Mutazioni con perdita di funzione di *STXBP1* portano a un deficit nel rilascio del neurotrasmettitore (2). Questa encefalopatia è caratterizzata da crisi epilettiche di vario tipo, con esordio nei primi mesi di vita e spesso refrattarie ai farmaci. L'elettroencefalogramma presenta un'attività epilettica multifocale, ipsaritmia o *burst-suppression* (3). I pazienti mostrano disabilità intellettiva, grave o profonda e, spesso, sintomi neurologici aggiuntivi, quali disturbi dello spettro autistico, disturbi motori (discinesia, distonia, tremore), ipotonia assiale ed atassia. Sono stati riportati anche pazienti con quadro neurologico grave senza epilessia. La prevalenza di encefalopatia con epilessia da mutazioni in *STXBP1* è stata stimata in 1:91.862 nati, nella popolazione danese, ma potrebbe essere più alta a causa dell'eterogeneità di questa patologia (3).

Recentemente è stata identificata una nuova classe di RNA non-codificanti, naturali e sintetici, in grado di incrementare la traduzione gene-specifica di una proteina (SINEUPs), senza interferire con la quantità di mRNA del gene target (4). I SINEUPs sono attivatori della traduzione, composti da un Dominio di Legame (BD) antisenso all'mRNA codificante, che determina la specificità e un Dominio Effettore (ED) che aumenta la sintesi proteica attraverso il reclutamento del macchinario molecolare necessario alla traduzione (4) (Fig. 1). La capacità dei SINEUPs di recuperare l'espressione fisiologica della proteina target è stata già provata in caso di aploinsufficienza (4)(5).

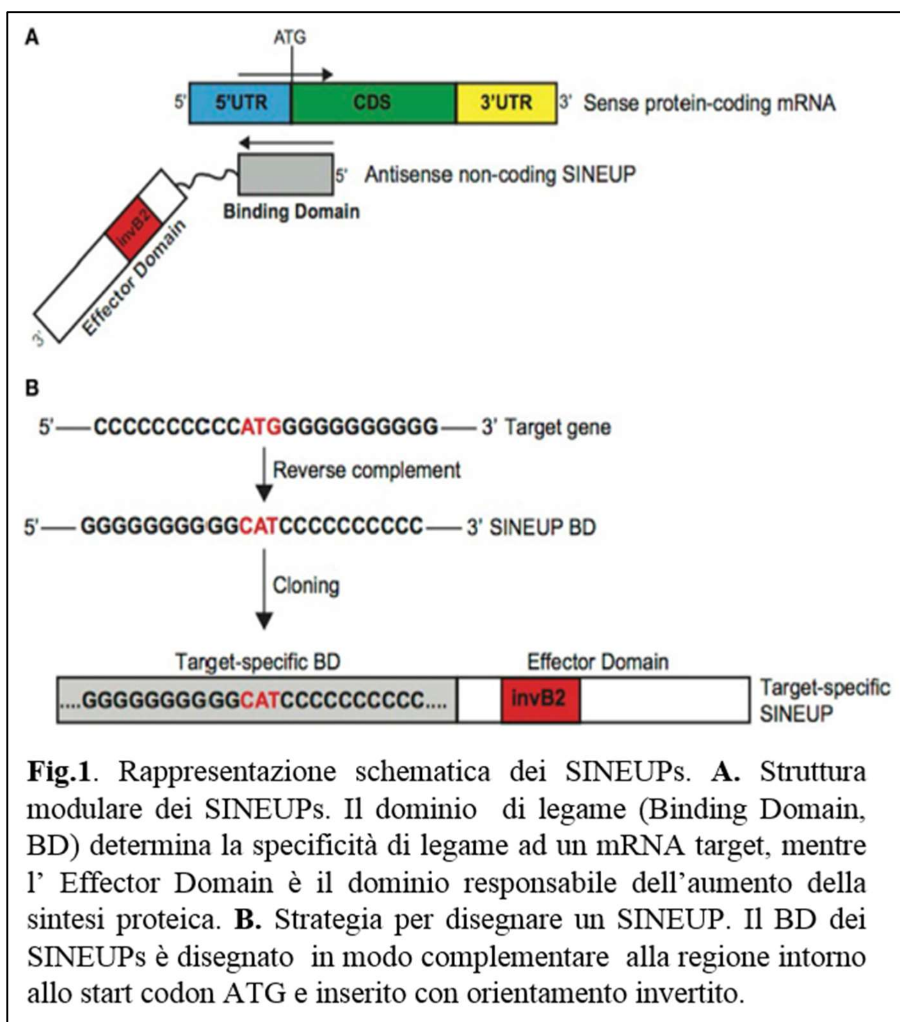

Fig.1. Rappresentazione schematica dei SINEUPs. **A.** Struttura modulare dei SINEUPs. Il dominio di legame (Binding Domain, BD) determina la specificità di legame ad un mRNA target, mentre l' Effector Domain è il dominio responsabile dell'aumento della sintesi proteica. **B.** Strategia per disegnare un SINEUP. Il BD dei SINEUPs è disegnato in modo complementare alla regione intorno allo start codon ATG e inserito con orientamento invertito.

Questo nuovo strumento presenta molti vantaggi, in quanto agisce sull'mRNA target endogeno; l'azione si limita all'mRNA prodotto fisiologicamente, per cui non sono attesi effetti *off-site* in cellule e tessuti che non esprimono il trascritto target; non determina alterazioni del genoma, poiché l'azione si esplica a livello post-trascrizionale sulla sintesi proteica. L'efficacia di questi costrutti *in vivo* è già stata dimostrata in un modello animale di microftalmia con difetti cutanei lineari (causata da aploinsufficienza di *cox7B*) (5). I SINEUPs potrebbero quindi essere un potenziale strumento terapeutico.

Questa proposta mira a indagare il potenziale terapeutico dei SINEUPs nell' encefalopatia da mutazioni in *STXBP1.* A questo scopo, utilizzeremo un modello umano *in vitro* della patologia, basato su neuroni derivati dalle cellule staminali pluripotenti indotte (IPSCs) da fibroblasti dei pazienti (6). Il vantaggio di questo modello è quello di produrre cellule neurali funzionanti, che conservano la mutazione in *STXBP1* e il background genetico del paziente.

Inoltre, la possibilità di sintetizzare specifici Domini di Legame rende i SINEUPs una tecnologia estremamente flessibile. Dal momento che centinaia di diversi geni in emizigosi sono correlati specificamente a epilessia, disabilità intellettiva e spettro autistico, l'implementazione di questo approccio può aprire la strada al suo uso in altre encefalopatie epilettiche causate dall'aploinsufficienza.

OBIETTIVI

Lo scopo del progetto è di recuperare l'espressione fisiologica della proteina stxbp1 nelle cellule neuronali derivate da fibroblasti di pazienti e di compensare la perdita di funzione dell'allele mutato, utilizzando i SINEUPs come attivatori gene-specifici della traduzione proteica.

Ci aspettiamo di identificare almeno un SINEUP che incrementi la traduzione di stxbp1 e recuperi il fenotipo sinaptico dei neuroni derivati da IPSCs.

L'attivazione gene-specifica della traduzione da parte dei SINEUPs fornirà una base sperimentale per lo sviluppo di un nuovo approccio terapeutico nell'ottica della medicina di precisione.

Il piano sperimentale è stato disegno sulla base dei seguenti obiettivi specifici:

i) Selezione di pazienti per lo sviluppo di linee IPSCs mutazione-specifiche dai fibroblasti.

ii) Disegno di SINEUPs che legano in modo specifico l'mRNA *STXBP1*.

iii) Valutazione dell'attività dei SINEUPs nella linea cellulare umana SH-SY5Y.

iv) Generazione di neuroni derivati da IPSCs dei pazienti con mutazione con perdita di funzione in STXBP1.

v) Valutazione del recupero funzionale determinato dai SINEUPs nei neuroni derivati da fibroblasti di pazienti.

PAROLE CHIAVE

Encefalopatia; Epilessia; SINEUP; STXBP1; medicina di precisione

METODI

Per il raggiungimento degli obiettivi abbiamo disegnato il seguente pino sperimentale:

i) Selezione di pazienti per lo sviluppo di linee IPSCs mutazione-specifiche dai fibroblasti.
Abbiamo già raccolto i dati clinici di una coorte di 23 pazienti portatori di mutazioni in *STXBP1* in collaborazione con diversi Centri italiani e collezionato fibroblasti di 3 soggetti.

ii) Disegno di SINEUPs che legano in modo specifico l'mRNA STXBP1.
Per testare l'effetto specifico dei SINEUPs abbiamo selezionato la linea cellulare umana di neuroblastoma SH-SY5Y, che presenta una significativa espressione endogena di stxbp1 (Fig.2). Dopo un'analisi bioinformatica del promotore *STXBP1* (ZenbuGenome Browser) abbiamo disegnato tre SINEUPs con BDs diversi diretti contro lo stesso mRNA *target* e li abbiamo clonati in vettori di espressione contenenti i seguenti BDs: -40/+32, -40/+4, -14/+4 antisenso all'mRNA *STXBP1* (dove +1 è la A del sito di inizio traduzione ATG). Come vettori per controllo negativo useremo il SINEUP senza BD (SINEUP-ΔBD) oppure con un BD *mismatched* (SINEUP-Scramble).

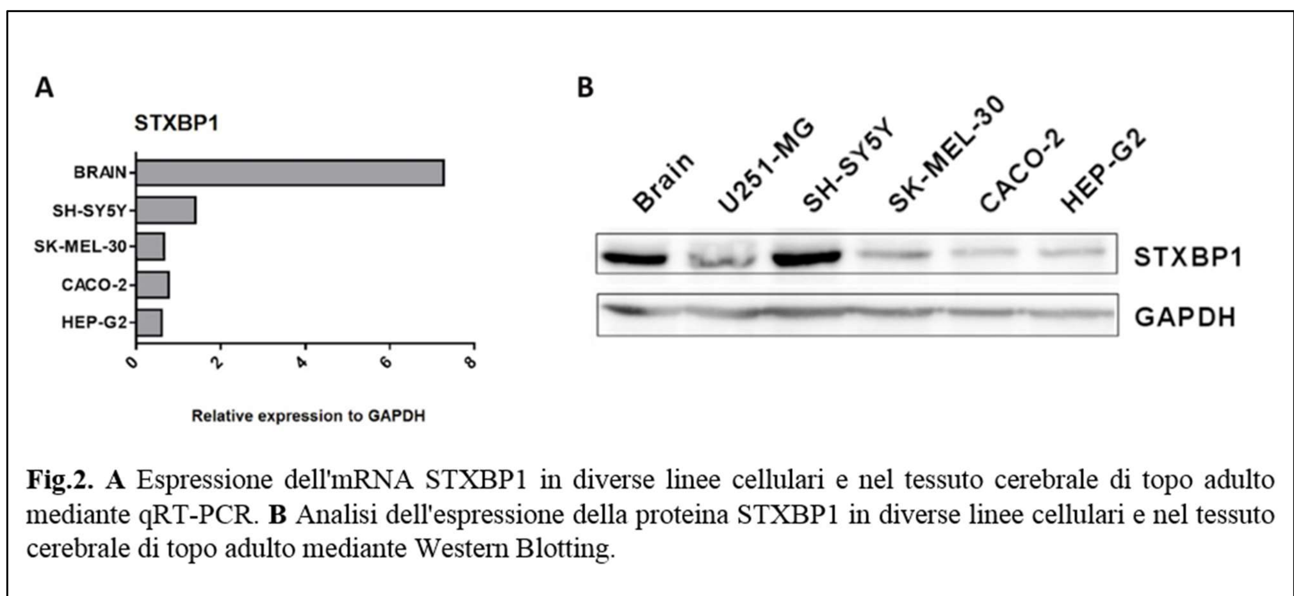

Fig.2. A Espressione dell'mRNA STXBP1 in diverse linee cellulari e nel tessuto cerebrale di topo adulto mediante qRT-PCR. **B** Analisi dell'espressione della proteina STXBP1 in diverse linee cellulari e nel tessuto cerebrale di topo adulto mediante Western Blotting.

iii) Valutazione dell'attività dei SINEUPs nella linea cellulare umana SH-SY5Y.

Abbiamo verificato l'efficienza di trasfezione e abbiamo confermato l'attività dei SINEUP sulla proteina GFP nelle cellule SH-SY5Y: le cellule trasfettate con SINEUP-GFP mostrano un aumento di 2,4-3,9 volte della proteina GFP a fronte delle cellule trasfettate solamente con il vettore GFP (Fig.3). Per prima cosa, i SINEUP-STXBP1 verranno testati nelle cellule SH-SY5Y trasfettate con SINEUP-STXBP1 o controlli negativi (SINEUP-ΔBD e SINEUP-Scramble). La proteina target dopo somministrazione di SINEUPs verrà valutata mediante Western blot e quantificata con il software UVItec. Utilizzando i SINEUPs, la trascrizione del gene target resta inalterata; i livelli di mRNA STXBP1 saranno valutati mediante qRT-PCR. Per il SINEUP selezionato e i controlli negativi

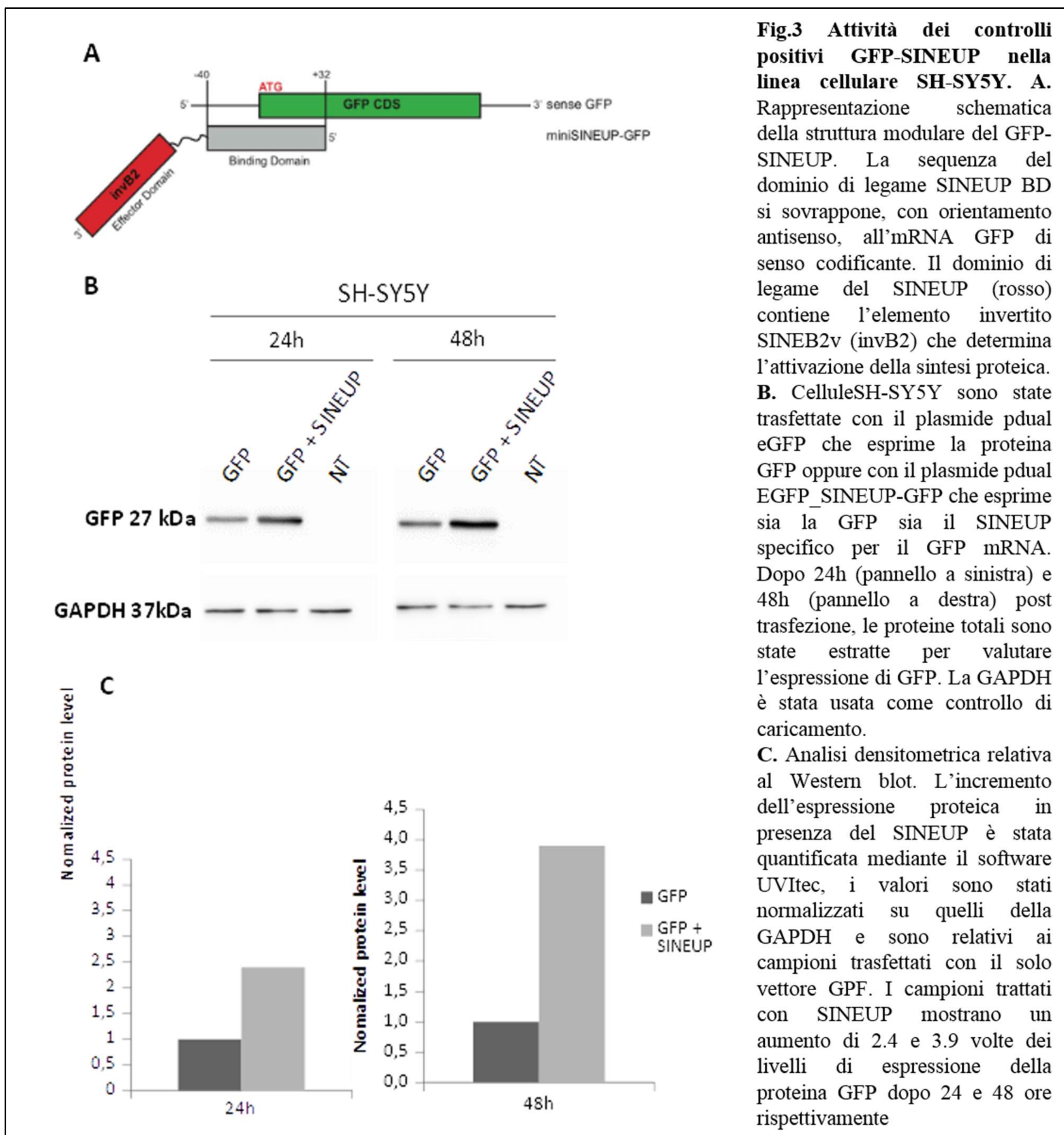

Fig.3 Attività dei controlli positivi GFP-SINEUP nella linea cellulare SH-SY5Y. A. Rappresentazione schematica della struttura modulare del GFP-SINEUP. La sequenza del dominio di legame SINEUP BD si sovrappone, con orientamento antisenso, all'mRNA GFP di senso codificante. Il dominio di legame del SINEUP (rosso) contiene l'elemento invertito SINEB2v (invB2) che determina l'attivazione della sintesi proteica.
B. CelluleSH-SY5Y sono state trasfettate con il plasmide pdual eGFP che esprime la proteina GFP oppure con il plasmide pdual EGFP_SINEUP-GFP che esprime sia la GFP sia il SINEUP specifico per il GFP mRNA. Dopo 24h (pannello a sinistra) e 48h (pannello a destra) post trasfezione, le proteine totali sono state estratte per valutare l'espressione di GFP. La GAPDH è stata usata come controllo di caricamento.
C. Analisi densitometrica relativa al Western blot. L'incremento dell'espressione proteica in presenza del SINEUP è stata quantificata mediante il software UVItec, i valori sono stati normalizzati su quelli della GAPDH e sono relativi ai campioni trasfettati con il solo vettore GPF. I campioni trattati con SINEUP mostrano un aumento di 2.4 e 3.9 volte dei livelli di espressione della proteina GFP dopo 24 e 48 ore rispettivamente

svilupperemo un vettore lentivirale, utilizzando il vettore lentivirus pLKO.1 puro, per trasdurre i neuroprecursori derivati dalle IPSCs dei pazienti.

iv) Generazione di neuroni derivati da IPSCs dei pazienti con mutazione con perdita di funzione in STXBP1.

Cellule IPSCs e neuroni differenziati sono già stati ottenuti con successo da fibroblasti di un paziente con mutazione troncante di STXBP1 e di tre controlli. I fibroblasti di altri due pazienti della nostra coorte con mutazioni con perdita di funzione verranno riprogrammati a IPSCs. Almeno 15 linee IPSCs indipendenti verranno ottenute da ciascuna coltura di fibroblasti e almeno tre cloni per individuo verranno processati per il differenziamento neuronale.

Le IPSCs verrano differenziate a rosette neurali, passando attraverso la fase di corpi embrioidi (Fig. 4).

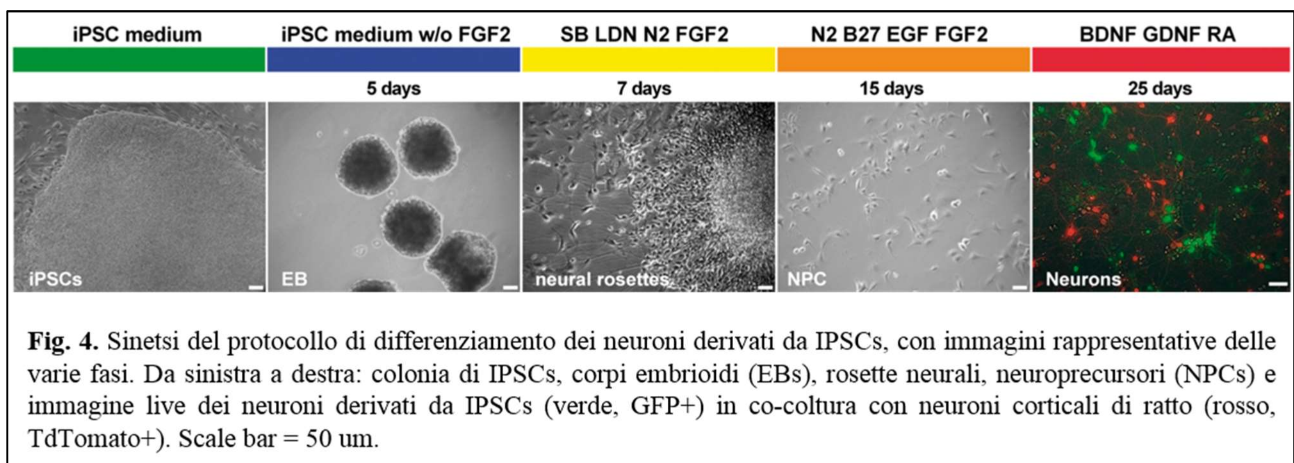

Fig. 4. Sinetsi del protocollo di differenziamento dei neuroni derivati da IPSCs, con immagini rappresentative delle varie fasi. Da sinistra a destra: colonia di IPSCs, corpi embrioidi (EBs), rosette neurali, neuroprecursori (NPCs) e immagine live dei neuroni derivati da IPSCs (verde, GFP+) in co-coltura con neuroni corticali di ratto (rosso, TdTomato+). Scale bar = 50 um.

Dalle rosette si ricaveranno i neuroprecursori, i quali andranno coltivati in terreno di differenziamento neuronale per almeno 35 giorni per ottenere neuroni maturi. (Fig. 5)

Ad ogni fase del protocollo, le cellule verranno testate per i marker della pluripotenza e delle varie fasi del differenziamento neuronale, utilizzando metodiche quali qRT-PCR, immuno-fluorescenza e Western blot.

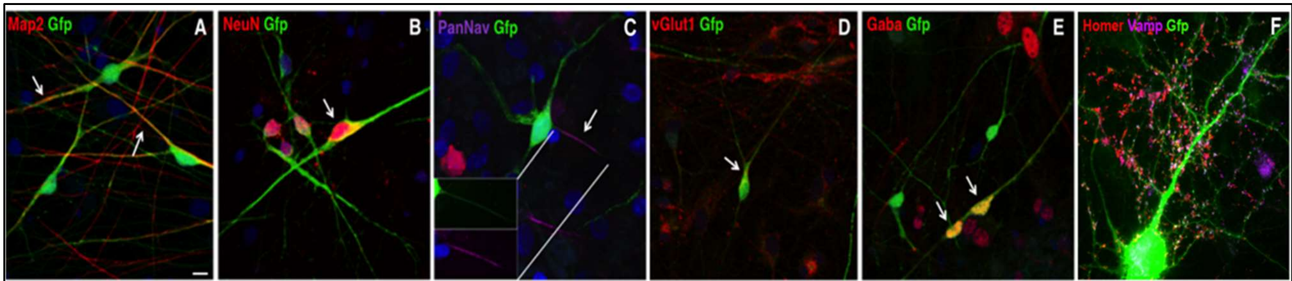

Fig. 5. Immagini in immuno-fluorescenza che rappresentano markers di neuroni corticali maturi in neuroni derivati da IPSCs dopo 4 settimane di differenziamento: MAP2 (**A**) e NEUN (B), canali Na+ voltaggio-dipendenti (PanNav) (**C**). L'inserto in C mostra la co-localizzazione di GFP e PanNav presso l'AIS. I Nuclei sono stati colorati con DAPI. In **D** è mostrato VGLUT-1 come marker glutammatergico pre-sinaptico. In **E** immunostaing del marker gabaergico Gaba; in **F** co-immunostainig del marker pre-sinaptico Vamp2 e del marker post-sinaptico Homer.

v) Valutazione del recupero funzionale determinato dai SINEUPs nei neuroni derivate da IPSCs di pazienti.

Una volta confermata l'efficacia nella linea cellulare SH-SY5Y, i SINEUPs saranno testati in neuroni differenziati da IPSCs, derivati da fibroblasti di pazienti mutati in *STXBP1*.

Quantificheremo la proteina stxbp1 dopo la trasduzione con SINEUP mediante Western blot. Il lisato cellulare estratto con RIPA lysis buffer sarà sottoposto a elettroforesi su gel SDS-poliacrilammide e trasferito su membrana di nitrocellulosa. La proteina verrà quantificata mediante il software UVItech (Cambridge, UK). Le cellule trasdotte con SINEUP-ΔBD e SINEUP-Scramble saranno utilizzate come controlli negativi. GAPDH verrà usata come controllo di carico per la normalizzazione delle proteine.

Come test funzionale utilizzeremo il saggio a fluorescenza pH-sensibile basato sulla *synaptophysin-pHluorin* nei neuroni derivati dalle IPSCs. Tale saggio permette di studiare il circolo delle vescicole sinaptiche (SV) a livello pre-sinaptico (6). A tal fine trasdurremo i neuroprecursori con SypHy e e le registreremo dopo differenziamento a 35 DIV. Tale metodo è già stato testato e abbiamo verificato che la capacità di induzione del circolo delle vescicole sinaptiche e del recycling pool nei neuroni derivati da IPSCs è risultata simile a quella registrata in neuroni corticali WT di topo e ratto (Fig. 6).

Per valutare il recupero della funzione di stxbp1 indotta dal SINEUP-STXBP1 nelle cellule dei pazienti, i puromycine-selected-SINEUP-STXBP1-NPCs, SINEUPΔBD, SINEUP-ScrambleNPCs e NPCs da controlli sani verranno trasdotte con vettore lentivirale esprimente SypHy. Le cellule trattate con SINEUP-STXBP1 saranno confrontate con le cellule trattate con SINEUPΔBD e SINEUP-Scramble e con i neuroni derivati da individui sani utilizzati come controlli positivi.

Il saggio funzionale sulle vescicole sinaptiche sarà condotto in collaborazione con la Prof.ssa A. Fassio (DiMeS, Università degli Studi di Genova) e il Prof. M. Verhage (CNCR, Vrije Universiteit, Amsterdam).

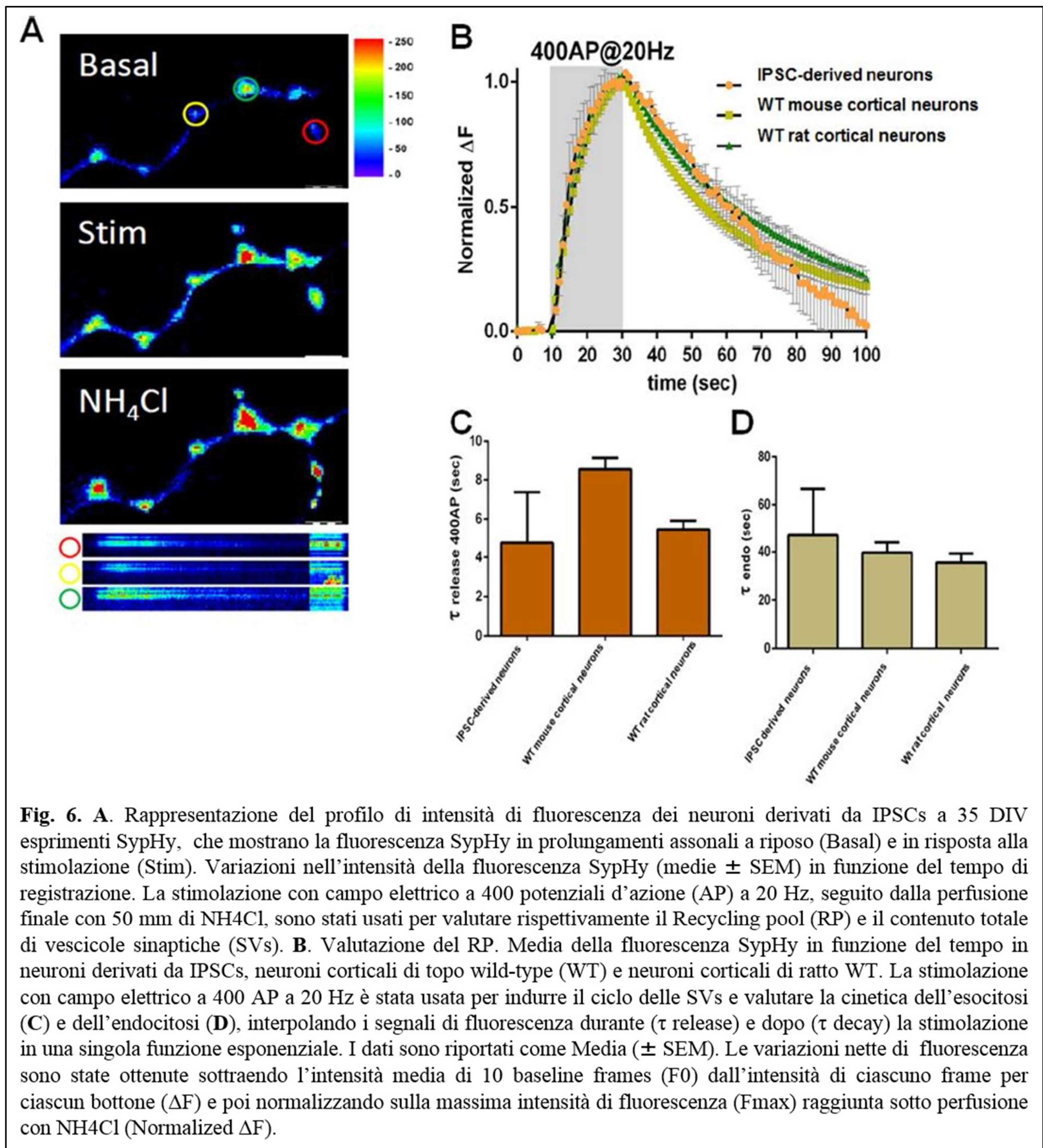

Fig. 6. A. Rappresentazione del profilo di intensità di fluorescenza dei neuroni derivati da IPSCs a 35 DIV esprimenti SypHy, che mostrano la fluorescenza SypHy in prolungamenti assonali a riposo (Basal) e in risposta alla stimolazione (Stim). Variazioni nell'intensità della fluorescenza SypHy (medie ± SEM) in funzione del tempo di registrazione. La stimolazione con campo elettrico a 400 potenziali d'azione (AP) a 20 Hz, seguito dalla perfusione finale con 50 mm di NH4Cl, sono stati usati per valutare rispettivamente il Recycling pool (RP) e il contenuto totale di vescicole sinaptiche (SVs). **B**. Valutazione del RP. Media della fluorescenza SypHy in funzione del tempo in neuroni derivati da IPSCs, neuroni corticali di topo wild-type (WT) e neuroni corticali di ratto WT. La stimolazione con campo elettrico a 400 AP a 20 Hz è stata usata per indurre il ciclo delle SVs e valutare la cinetica dell'esocitosi (**C**) e dell'endocitosi (**D**), interpolando i segnali di fluorescenza durante (τ release) e dopo (τ decay) la stimolazione in una singola funzione esponenziale. I dati sono riportati come Media (± SEM). Le variazioni nette di fluorescenza sono state ottenute sottraendo l'intensità media di 10 baseline frames (F0) dall'intensità di ciascuno frame per ciascun bottone (ΔF) e poi normalizzando sulla massima intensità di fluorescenza (Fmax) raggiunta sotto perfusione con NH4Cl (Normalized ΔF).

RISULTATI ATTESI

Il nostro scopo è di verificare le potenzialità terapeutiche della tecnologica ad RNA nell'ottica della medicina di precisione. Come primo obiettivo ci attendiamo di identificare almeno un SINEUP in

grado di incrementare l'espressione della proteina stxbp1 nella linea cellulare SH-SY5Y e quindi nei neuroni derivati dalle IPSCs dei pazienti. L'incremento di espressione è atteso correlare con il recupero funzionale del fenotipo sinaptico valutato nei neuroni derivati da IPSCs dei pazienti mediante una analisi comparativa con i neuroni derivati da IPSCs di individui sani.
I SINEUP efficaci potranno essere ulteriormente testati in un modello animale eterozigote disponibile presso il Laboratorio del prof. Verhage che collabora al progetto, ed, in futuro, nei pazienti.
Poiché molti di questi pazienti soffrono di epilessia farmaco-resistente e presentano un deterioramento motorio e neuro-cognitivo, nuovi approcci terapeutici sono necessari per raggiungere il controllo delle crisi e migliorare l'outcome di questa patologia.
La caratterizzazione dei fattori genetici e dei meccanismi patogenetici alla base delle encefalopatie epilettiche possono contribuire a sviluppare un trattamento individualizzato per prevenire l'esordio di processi fisiopatologici scatenati dalle mutazioni. In questo scenario, la struttura modulare dei SINEUPs permette di re-indirizzare l'incremento della traduzione su qualsiasi mRNA target attraverso il cambiamento del dominio di legame (BD) con sequenze antisenso appropriate, nell'ottica di una medicina personalizzata.
Il nostro studio mira ad avere un impatto significativo nel campo, fornendo una *proof-of-concept* dell'uso della tecnologia a RNA per il recupero funzionale in mutazioni con perdita di funzione in un grande numero di condizioni neurologiche incurabili nei bambini.

PIANO ECONOMICO RELATIVO AL PROGETTO IN TOTO E ALLE MODALITA' DI SPESA DEL CONTRIBUTO RICHIESTO

- 8.000 euro Contratti di ricerca
- 7.000 euro Materiale di consumo
- 15.000 Finanziamento totale richiesto

COLLABORAZIONI

- Dipartimento di Medicina Sperimentale, Università degli Studi di Genova, Genova (Prof. Anna Fassio): studio funzionale sulle vescicole sinaptiche
- CNCR, Neuroscience Campus Amsterdam, Vrije Universiteit, Amsterdam (Prof. Matthijs Verhage): studio funzionale sulle vescicole sinaptiche
- In collaborazione con: Gruppo LICE su STXBP1

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HOSTING CENTRE: Pediatric Neurology and Muscular Diseases Unit

SCIENTIFIC GUARANTEE: Prof. Pasquale Striano (Coordinator of Genetic Commission)

PARTICIPATING CENTRES:

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- Laboratory of Neurogenetics and Neuroscience, "G. Gaslini" Institute, Genova
- Department of Experimental Medicine (DiMeS), University of Genoa, Genova
- Neuroscience Campus Amsterdam, Vrije Universiteit (VU), Amsterdam, The Netherlands
- In collaboration with: LICE collaborative group on STXBP1

TITLE: AN INNOVATIVE MOLECULAR STRATEGY FOR TREATMENT OF EPILEPTIC ENCEPHALOPATHY CAUSED BY *STXBP-1* MUTATIONS

SYNOPSIS

STXBP1 encephalopathy is caused by de novo mutations in the Syntaxin-binding-protein-1 gene, a membrane-trafficking protein that plays an important role in the vesicular docking and fusion. Loss-of-function *STXBP1* mutations lead to a failure of neurotransmitter secretion from synaptic vesicles. The core clinical features are epileptic seizures of different types, with onset in the first months of life and typically difficult to control with standard treatment. EEG usually present multifocal epileptiform activity, hypsarrhythmia or burst-suppression patterns. All patients show intellectual disability, severe to profound in most cases. Additional neurologic features are often present, such as autistic features, movement disorder (dyskinesia, dystonia, tremor), axial hypotonia, and ataxia, indicating a broader neurologic impairment. According to this, a more tailored and radical therapeutic approach is necessary. Recently, a new class of natural and synthetic non-coding RNAs have been identified, enabling up-regulation of a protein translation in a gene-specific way (SINEUPs), without any impact on the mRNA quantity of the target gene. SINEUPs are translational activators composed by a Binding Domain (BD) that overlaps, in antisense orientation, to the sense protein-coding mRNA and determines target selection, and an Effector Domain (ED) that allows protein synthesis up-regulation through the recruitment of the machinery for translation.

The present proposal aims to explore the therapeutic potential of SINEUPs in STXBP1 Encephalopathy. The application of this RNA technology to Epileptic Encephalopathies is aimed to increase the activity of the residual wild-type (wt) allele and overcome the loss-of-function of the mutant allele. The final goal of the project is to rescue *in vitro* the physiological amount of the target protein stxbp1 in neuronal cells derived from patients with *STXBP1* encephalopathy, by using SINEUPs.

We define the following specific aims:

i) Selection of patients for development of mutation-specific induced pluripotent stem cells (IPSCs) lines from fibroblasts. To this aim, we have already collected an Italian cohort of 23 *STXBP1* mutated patients in collaboration with several Italian Centres.

ii) Design of SINEUPs that specifically bind STXBP1 mRNA. To test the specific effect of SINEUPs we identified the suitable human cell line SH-SY5Y with a significant endogenous expression of stxbp1. After bioinformatics analysis of *STXBP1* promoter (Zenbu Genome Browser), we have already designed three SINEUPs with different BDs for the same target mRNA and cloned them into expression vectors.

iii) Evaluation of translational activity of SINEUPs in human SH-SY5Y cell line. The test the effect of SINEUPs on stxbp1 protein, SH-SY5Y cells will be transfected with SINEUP-STXBP1 or Negative controls (SINEUPs-ΔBD and SINEUP-Scramble), and the expression will be evaluated by Western blot.

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v) Evaluation of the functional rescue elicited by SINEUPs in patients' cells. Once we have assessed the efficacy in SH-SY5Y cell line, the SINEUPs will be tested in neurons differentiated from IPSCs, derived directly from patient carrying loss-of-function-muttion in *STXBP1*. The increased protein expression will be assessed by Western blot. The functional assays on synaptic vesicle release and recycling will be carried in collaboration with Prof. A. Fassio (DiMeS, Univeristy of Genoa) and Prof. M. Verhage (Vrije Universiteit, Amsterdam).

We expect to identify at least one SINEUP which up-regulates stxbp1 protein translation and to revert the synaptic phenotype of the patient-derived neuronal cells. As a future development, the effective SINEUP will be tested in an existing heterozygous animal model that is available in the Laboratory of prof. Verhage and, in the future, in humans. SINEUPs represents an innovative potential molecular therapeutic tool that embraces the precision medicine paradigm.

RATIONALE

STXBP1 encephalopathy is caused by *de novo* mutations in *Syntaxin-binding-protein-1* gene, encoding a membrane-trafficking protein that plays an important role in vesicular docking and fusion (1). Loss-of-function *STXBP* mutations lead to a failure of neurotransmitter secretion from synaptic vesicles (2). Core clinical features include early-onset epilepsy with EEG showing hypsarrhythmia, or burst-suppression pattern, or multifocal epileptiform activity (3). Seizures are typically difficult to control with standard treatments. Patients show intellectual disability, severe to profound in most cases. Additional neurologic features may include ASD, movement disorders (dyskinesia, dystonia, tremor), axial hypotonia, and ataxia, indicating a broader neurologic impairment. Patients with severe neuro-cognitive features but without epilepsy have been reported. The prevalence of *STXBP1* encephalopathy has been estimated to be ~1:92000 in a 10-year Danish birth cohort (1), but it is likely to be higher because of the heterogeneity of this disorder.

Recently, a new class of natural and synthetic non-coding RNAs have been identified, enabling the up-regulation of a protein translation in a gene-specific way (SINEUPs), without any impact on the mRNA quantity of the target gene. SINEUPs are translational activators composed by a Binding Domain (BD) that overlaps, in antisense orientation, to the sense protein-coding mRNA, and determines target selection; and an Effector Domain (ED), that allows protein synthesis up-regulation through the recruitment of the machinery for translation. (4) (Fig. 1). SINEUPs have been shown to restore the physiological expression of a protein in case of haplo-insufficiency (4)(5).

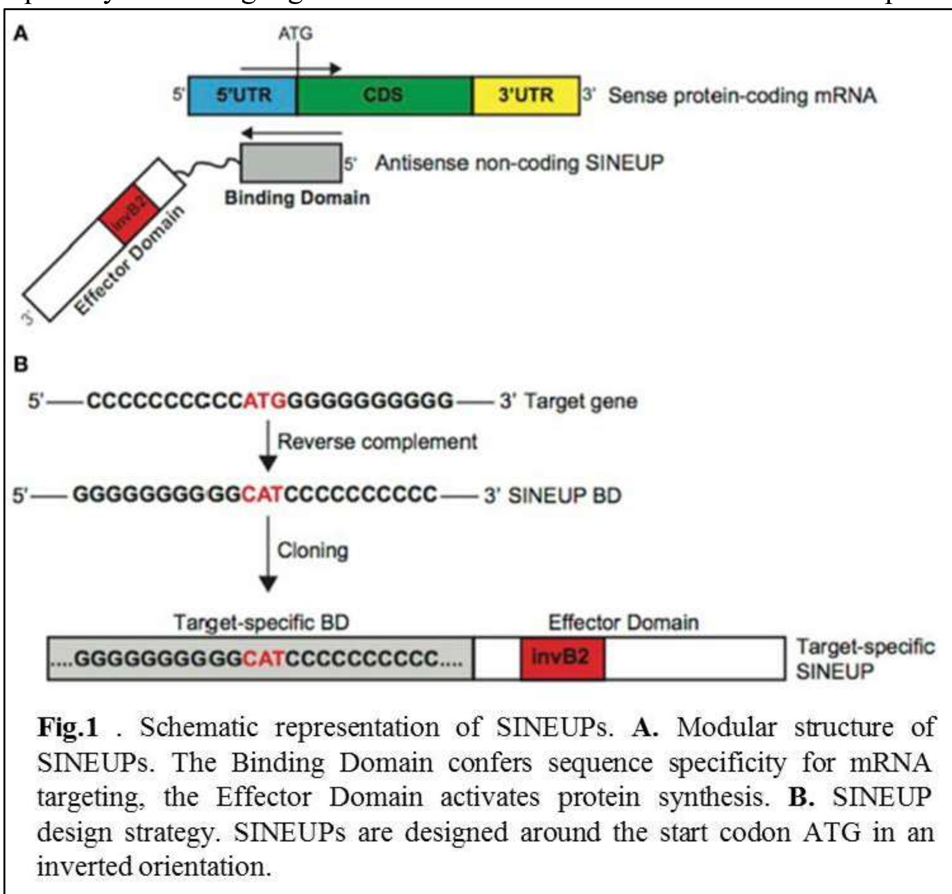

Fig.1 . Schematic representation of SINEUPs. **A.** Modular structure of SINEUPs. The Binding Domain confers sequence specificity for mRNA targeting, the Effector Domain activates protein synthesis. **B.** SINEUP design strategy. SINEUPs are designed around the start codon ATG in an inverted orientation.

This technology brings many advantages as it mainly acts on endogenous target mRNAs produced *in situ*; this action is limited to mRNA under physiological regulation, therefore no off-site effects can be expected in cells and tissues that do not express the target transcript; by acting only on a post-transcriptional level, SINEUPs do not trigger hereditable genome editing.

The efficacy of these constructs to up-regulate protein translation has already been proved in animal model of microphthalmia with linear skin defects (*cox7B* haploinsufficiency) [5]. Therefore, SINEUPs may be potentially used as pharmaceutical tools.

Our proposal aims to explore the therapeutic potential of SINEUPs in patients with *STXBP1* encephalopathy. Toward this aim, we will use a unique *in vitro* human model of the disease based on the neurons derived from the patients' induced pluripotent stem cells (IPSCs) (6). The advantage of this model is to produce functioning neural cells which carry the patient's mutation in *STXBP1* and keep his genetic background.

Furthermore, the possibility to synthesize specific Binding Domains makes SINEUPs an extremely flexible technology. As hundreds of distinct gene hemizygosities are specifically linked to epilepsy, intellectual disability, and autistic spectrum disorder, the implementation of this approach can open the path to its use in other developmental epileptic encephalopathies caused by gene haploinsufficiency.

OBJECTIVES

The goal of the project is to rescue physiological expression of Stxbp1 in neuronal cells derived from fibroblasts of patients and overcome the loss-of-function of the mutant allele, by using SINEUPs, a new class of non-coding RNA that are able to increase a gene-specific protein translation.

We expect to identify at least one SINEUP which up-regulates stxbp1 protein translation and to revert the synaptic phenotype of the neurons derived from the patients' IPSCs.

Gene-specific activation of translation by SINEUPs will provide the experimental basis to develop a new potential therapeutic tool looking towards precision medicine.

We define the following specific aims:

i) Selection of patients for development of mutation-specific IPSCSs lines from fibroblasts.

ii) Design of SINEUPs that specifically bind *STXBP1* mRNA.

iii) Evaluation of translational activity of SINEUPs in human neuroblastoma cell line SH-SY5Y.

iv) Generation of IPSCS-derived neurons from patients' fibroblasts with loss-of-function mutations in *STXBP1*.

v) Evaluation of the functional rescue elicited by SINEUPs in neuronal cells derived from patients' fibroblasts.

KEYWORDS

Encephalopathy; Epilepsy; SINEUP; *STXBP1*; precision-medicine.

METHODS

We defined the following experimental plan:

i) Selection of patients for development of mutation-specific IPSCSs lines from fibroblasts.

We have already collected clinical data of of 23 patients with loss-of-function mutations in *STXBP1* in collaboration with several Italian Centres from the LICE network and collected fibroblasts from three subjects.

ii) Design of SINEUPs that specifically bind STXBP1 mRNA.

To test the specific effect of SINEUPs we identified a suitable human neuroblastoma cell line, SH-SY5Y, with a significant endogenous expression of stxbp1 (Fig.2).

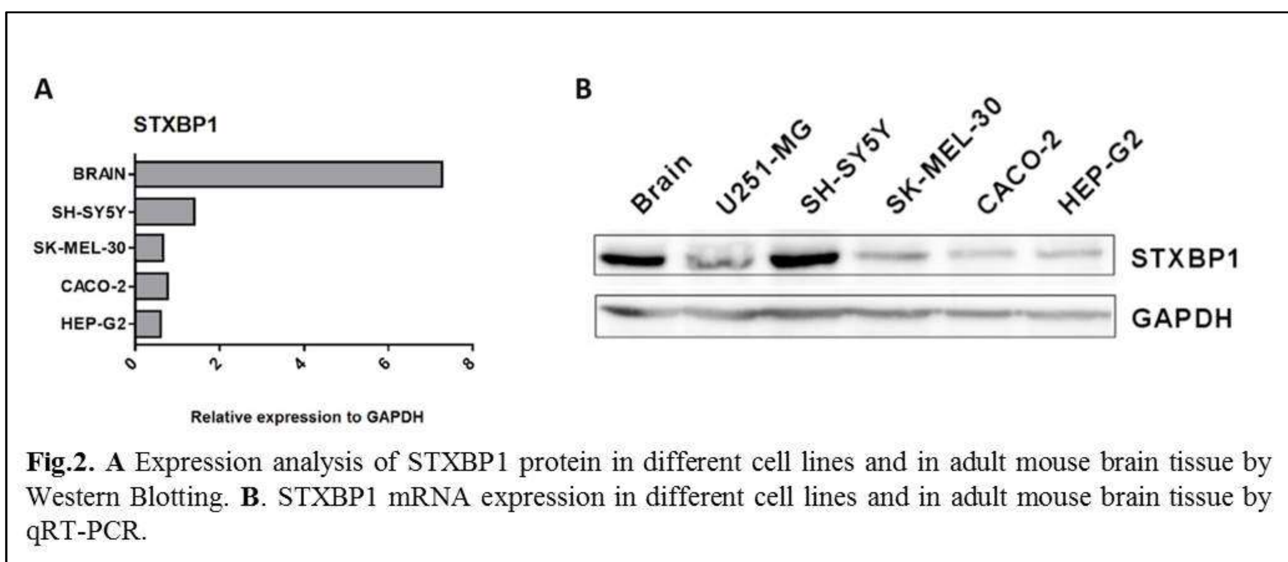

Fig.2. A Expression analysis of STXBP1 protein in different cell lines and in adult mouse brain tissue by Western Blotting. **B**. STXBP1 mRNA expression in different cell lines and in adult mouse brain tissue by qRT-PCR.

After bioinformatics analysis of *STXBP1* promoter (Zenbu Genome Browser) we have already designed three SINEUPs with different BDs for the same target mRNA and cloned them into expression vectors containing one of the following BDs: -40/+32, -40/+4, -14/+4, antisense to *STXBP1* mRNA. +1 is considered the A of the ATG Translation Start Site. As negative control vectors, we will use SINEUPS lacking the BD region (SINEUP-ΔBD) or harbouring a mismatched BD sequence (SINEUP-Scramble).

iii) Evaluation of translational activity of SINEUPs in human neuroblastoma cell line SH-SY5Y.

We have already tested if the selected SH-SY5Y cell line could be suitable for our experiments by transfecting cells with the positive controls, which consist of pDUAL/eGFP and pDUAL/eGFP_eGFP-BD/ED plasmid pair. The pDUAL/eGFP plasmid expresses a GFP protein downstream of a CMV promoter. The pDUAL/eGFP_GFP-BD/ED plasmid expresses GFP protein downstream of a CMV promoter and also a GFP-BD, followed by the SINEUP ED downstream of an H1 promoter. We verified the transfection efficacy and confirmed the SINEUP activity for GFP protein in SH-SY5Y cells: the cells transfected with SINEUP-GFP shows 2,4-3,9-fold increased GFP protein compared to cells transfected only with GFP vector (Fig.3).

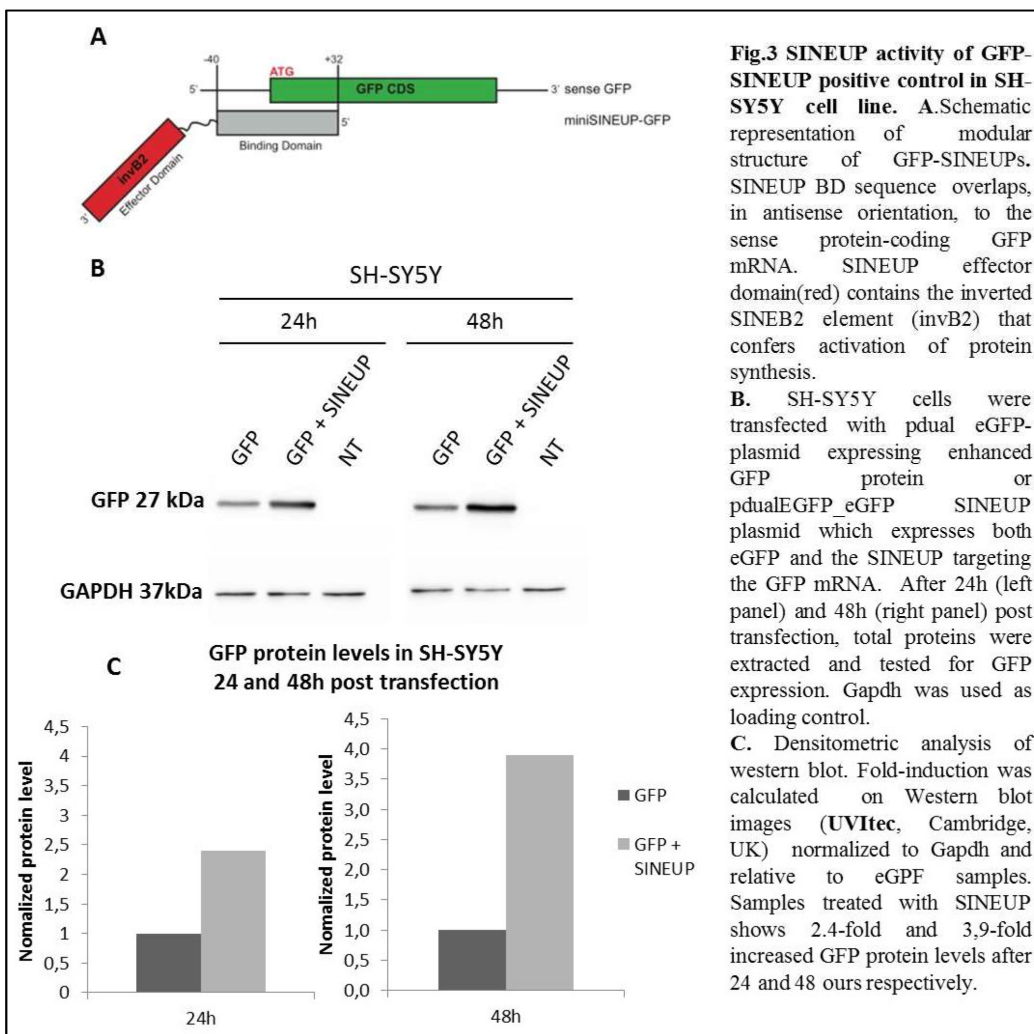

Fig.3 SINEUP activity of GFP-SINEUP positive control in SH-SY5Y cell line. A. Schematic representation of modular structure of GFP-SINEUPs. SINEUP BD sequence overlaps, in antisense orientation, to the sense protein-coding GFP mRNA. SINEUP effector domain(red) contains the inverted SINEB2 element (invB2) that confers activation of protein synthesis.
B. SH-SY5Y cells were transfected with pdual eGFP-plasmid expressing enhanced GFP protein or pdualEGFP_eGFP SINEUP plasmid which expresses both eGFP and the SINEUP targeting the GFP mRNA. After 24h (left panel) and 48h (right panel) post transfection, total proteins were extracted and tested for GFP expression. Gapdh was used as loading control.
C. Densitometric analysis of western blot. Fold-induction was calculated on Western blot images (**UVItec**, Cambridge, UK) normalized to Gapdh and relative to eGPF samples. Samples treated with SINEUP shows 2.4-fold and 3,9-fold increased GFP protein levels after 24 and 48 ours respectively.

First, the STXBP1-SINEUPs will be tested in SH-

SY5Y transfected with SINEUP-STXBP1 or Negative controls (SINEUPs-ΔBD and SINEUP-Scramble). After SINEUPs administration, the target protein will be evaluated by Western blot and quantified using UVItec software. Using SINEUPs, the transcription of the target gene is unaltered. However, the level of *STXBP1* mRNA will be verified by qRT-PCR.

For selected SINEUP and negative controls we will develop lentiviral vectors, with lentivirus pLKO.1 puro vector, to transduce the patient's IPSCSs-derived NPCs.

iv) Generation of IPSCS-derived neurons from patients' fibroblast with loss-of-function (LOF) mutations in STXBP1.

We have successfully generated IPSCs lines and differentiated neurons from fibroblasts of a patient with *STXBP1* truncating mutation and three controls. We will reprogram fibroblasts of two additional patients carrying LOF mutations from our cohort.

We will obtain at least 15 independent IPSCSs lines from each fibroblasts culture and we will process at least three clones for each individual for neural differentiation. IPSCSs will be differentiated into Neural Rosettes-like structure, through embryoid bodies step (Fig. 4).

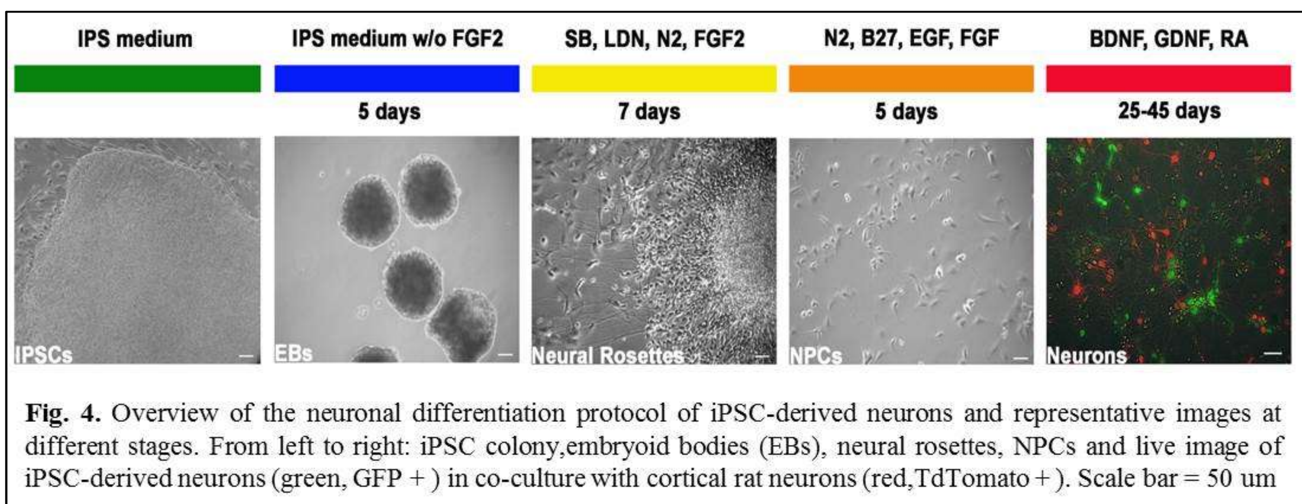

Fig. 4. Overview of the neuronal differentiation protocol of iPSC-derived neurons and representative images at different stages. From left to right: iPSC colony,embryoid bodies (EBs), neural rosettes, NPCs and live image of iPSC-derived neurons (green, GFP +) in co-culture with cortical rat neurons (red,TdTomato +). Scale bar = 50 um

The rosettes will be grown to obtain a homogeneous neural precursor cells (NPCs) culture. NPCs will be then grown in neuronal differentiation medium for at least 35 days to obtain mature neurons (Fig.5).

At every step of the protocol, the cells will be characterized for markers of pluripotency and of each neural differentiation step, using qRT-PCR, immuno-fluorescence and Western blot.

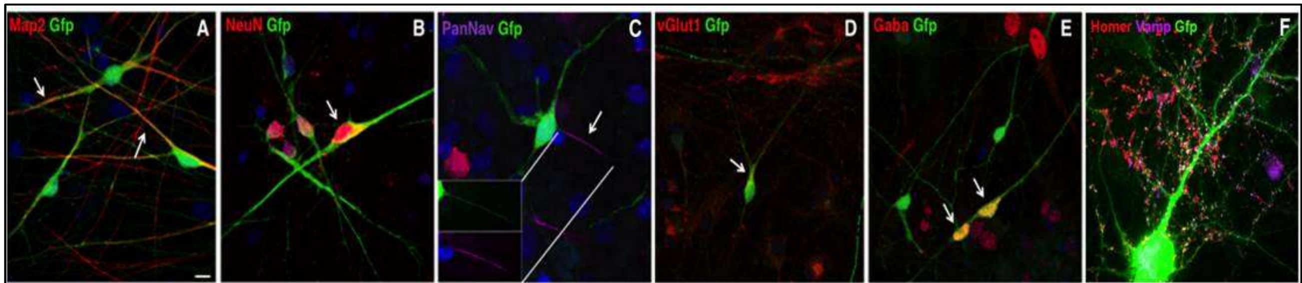

Fig. 5. Representative immunofluorescence images of the mature neuronal markers in iPSC-derived neurons after 4 weeks of differentiation: MAP2(**A**) and NEUN , voltage-gated Na+ channels (**C**).The inset in C shows co-localization of GFP and PanNav at the AIS. Nuclei were stained with DAPI. In **D** is shown VGLUT-1 as a glutamatergic pre-synaptic neuron marker; In **E** immunostaing of gabaergic neuron marker Gabaa nd in **F** co-immunostainig of presynaptic marker Vamp2 and post-synaptic neuron marker Homer.

v) Evaluation of the functional rescue elicited by SINEUPs in patients' cells.

Once we have assessed the efficacy in SH-SY5Y cell line, the SINEUPs, cloned into lentiviral vectors, will be tested by infection in neurons differentiated from IPSCSs, derived from fibroblasts of mutant patients.

Endogenous stxbp1 protein after SINEUPs transduction will be evaluated by Western blotting. Whole-cell lysates will be extracted with RIPA lysis buffer, electrophoresed separated in SDS– polyacrylamide gel and transferred to nitrocellulose membrane. Protein will be quantified by Uvitec software (Cambrige, Uk). Cells transduced with SINEUPD-□BD and a SINEUP-Scramble will be used as negative controls. GAPDH will be used as loading control protein for normalization.

We already developed a functional test to assess neuronal function, evaluating the dynamics of exocytosis isand endocytosis of synaptic vesicles (SV) in IPSCs derived neurons. This assay is based on the fluorescent probe synaptophysin-pHluorin, a pH-sensitive fluorescent probe which allows the monitoring of SV cycling at presynaptic site (6). To test the efficacy of this functional test on human IPSCS-derived neurons, we already transduced neuroprecursors with SypHy, and recorded at 35 DIV Human IPSCs-derived neurons. We found that derived-neurons showed the ability to induce the synaptic vesicle cycle and the recycling pool as WT mouse and rat cortical neurons (Figure 6).

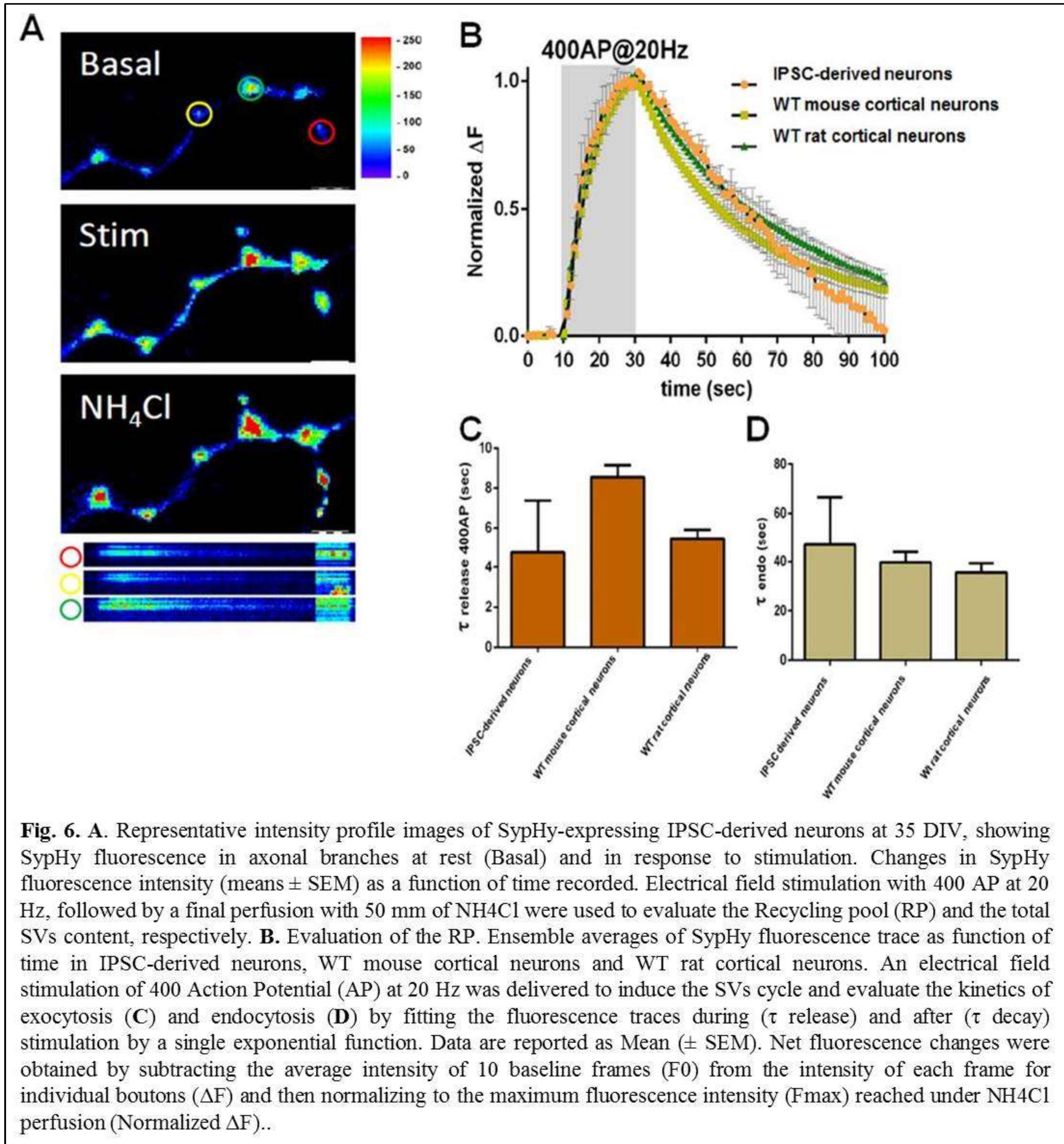

Fig. 6. A. Representative intensity profile images of SypHy-expressing IPSC-derived neurons at 35 DIV, showing SypHy fluorescence in axonal branches at rest (Basal) and in response to stimulation. Changes in SypHy fluorescence intensity (means ± SEM) as a function of time recorded. Electrical field stimulation with 400 AP at 20 Hz, followed by a final perfusion with 50 mm of NH4Cl were used to evaluate the Recycling pool (RP) and the total SVs content, respectively. **B.** Evaluation of the RP. Ensemble averages of SypHy fluorescence trace as function of time in IPSC-derived neurons, WT mouse cortical neurons and WT rat cortical neurons. An electrical field stimulation of 400 Action Potential (AP) at 20 Hz was delivered to induce the SVs cycle and evaluate the kinetics of exocytosis (**C**) and endocytosis (**D**) by fitting the fluorescence traces during (τ release) and after (τ decay) stimulation by a single exponential function. Data are reported as Mean (± SEM). Net fluorescence changes were obtained by subtracting the average intensity of 10 baseline frames (F0) from the intensity of each frame for individual boutons (ΔF) and then normalizing to the maximum fluorescence intensity (Fmax) reached under NH4Cl perfusion (Normalized ΔF)..

To evaluate the rescue of *STXBP1* function in patients' cells induced by SINEUP-STXBP1, we will transduce puromycine-selected-SINEUP-STXBP1-NPCs, SINEUPΔBD, SINEUP-ScrambleNPCs, and NPCs from healthy controls with lentiviral vectors expressing pHluorin-based probes, SypHy.SINEUP-STXBP1treated cells will be compared with SINEUP□BD and SINEUP-Scramble-treated cells and with IPSCs-derived neurons from healthy individuals used as positive control.

The functional assays on synaptic vesicle release and recycling will be carried in collaboration with Prof. A. Fassio (Univeristy of Genoa) and Prof. M. Verhage (Vrije Universiteit, Amsterdam).

EXPECTED RESULTS

We aim to develop a new potential therapeutic tool that embraces the precision medicine paradigm.
First, we expect to identify at least one SINEUP which up-regulates Stxbp1protein translation from the endogenous mRNA in SH-SY5Y cell line and then in patients' IPSCs-derived neurons. The increment of stxbp1 expression is expected to lead to a functional rescue which will be evaluated through a comparative analysis between patient and control fibroblast-derived neurons.
The effective compounds may be further tested in an existing heterozygous animal model which is available at the Laboratory of prof. Verhage and, in the future, in humans.
Because many of these patients suffer from drug-resistant epilepsy and manifest motor and/or neuropsychological deterioration, novel therapeutic tools are urgently needed to achieve seizure control and improve the outcome of these devastating disorders.
Genetic discoveries hold promise for the development of individualized treatments, which may prevent the initiation of specific physio-pathological processes triggered by genetic mutations and underlying the many different forms of epileptic encephalopathies. In this scenario, the modular architecture of SINEUPs allows to redirect translation enhancement activity to any target mRNA, by swapping its BD with the appropriate antisense sequence.
Our study is expected to significantly impact the field, by providing a valuable proof-of-concept of the use of RNA-technology SINEUPs for functional recovering of loss-of-function mutations in a large group of intractable neurological conditions of children.

ECONOMIC PLAN OF THE CURRENT PROJECT:

- 8.000 euro Research Contracts
- 7.000 euro Consumables
- 15.000 Total requested budget

COLLABORATIONS

• Department of Experimental Medicine, University of Genoa, Genoa (Prof. Anna Fassio): functional study of recirculation of synaptic vesicles

• Neuroscience Campus Amsterdam, Vrije Universiteit, Amsterdam, The Netherlands (Prof. Matthijs Verhage): functional study of recirculation of synaptic vesicles

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CANDIDATE RESPONSIBLE FOR THE PROJECT: DR. MARIA STELLA VARI

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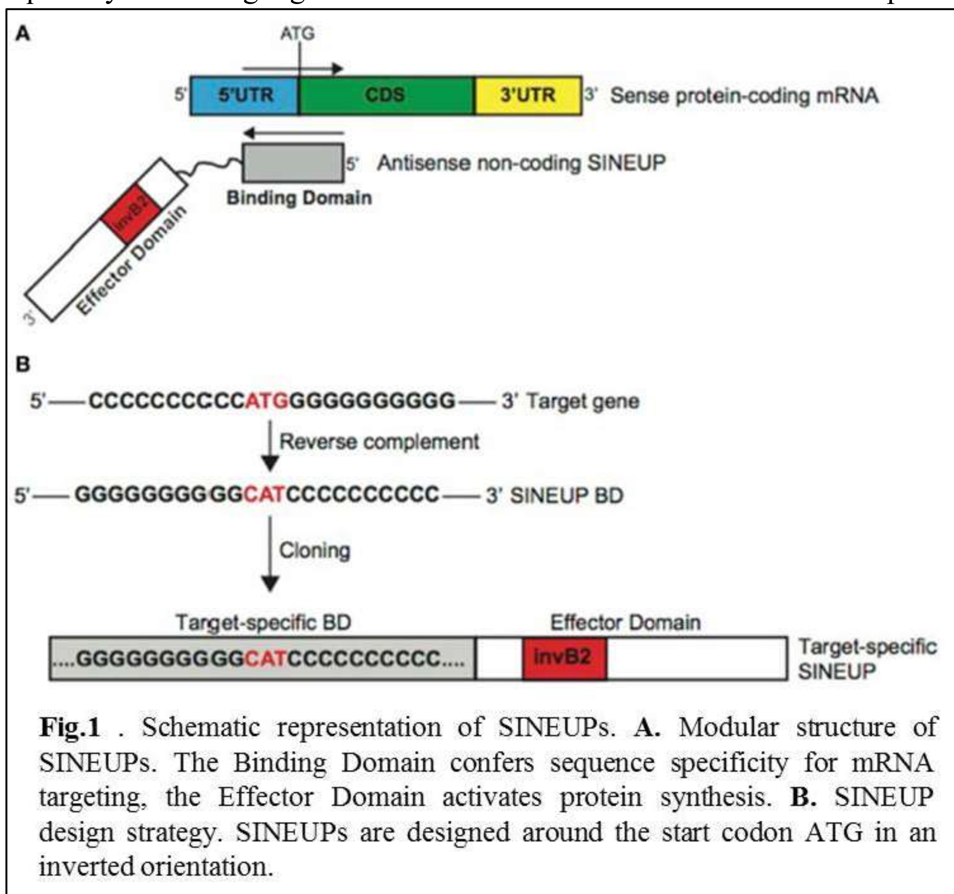

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Encephalopathy; Epilepsy; SINEUP; *STXBP1*; precision-medicine.

METHODS

We defined the following experimental plan:

i) Selection of patients for development of mutation-specific IPSCSs lines from fibroblasts.

We have already collected clinical data of of 23 patients with loss-of-function mutations in *STXBP1* in collaboration with several Italian Centres from the LICE network and collected fibroblasts from three subjects.

ii) Design of SINEUPs that specifically bind STXBP1 mRNA.

To test the specific effect of SINEUPs we identified a suitable human neuroblastoma cell line, SH-SY5Y, with a significant endogenous expression of stxbp1 (Fig.2).

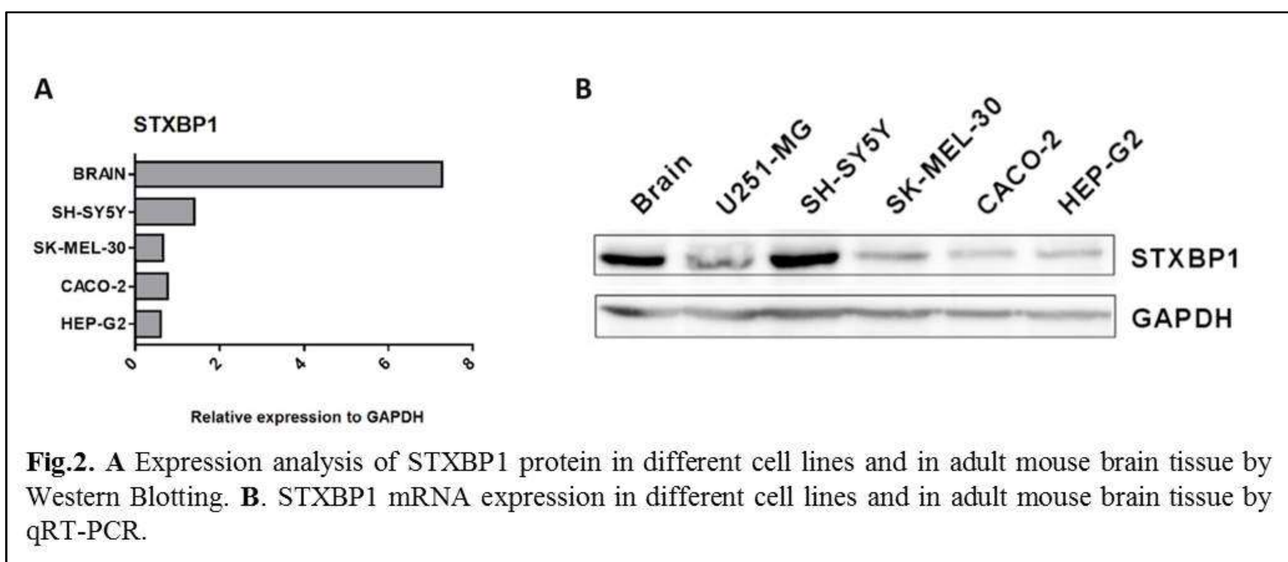

Fig.2. **A** Expression analysis of STXBP1 protein in different cell lines and in adult mouse brain tissue by Western Blotting. **B**. STXBP1 mRNA expression in different cell lines and in adult mouse brain tissue by qRT-PCR.

After bioinformatics analysis of *STXBP1* promoter (Zenbu Genome Browser) we have already designed three SINEUPs with different BDs for the same target mRNA and cloned them into expression vectors containing one of the following BDs: -40/+32, -40/+4, -14/+4, antisense to *STXBP1* mRNA. +1 is considered the A of the ATG Translation Start Site. As negative control vectors, we will use SINEUPS lacking the BD region (SINEUP-ΔBD) or harbouring a mismatched BD sequence (SINEUP-Scramble).

iii) Evaluation of translational activity of SINEUPs in human neuroblastoma cell line SH-SY5Y.

We have already tested if the selected SH-SY5Y cell line could be suitable for our experiments by transfecting cells with the positive controls, which consist of pDUAL/eGFP and pDUAL/eGFP_eGFP-BD/ED plasmid pair. The pDUAL/eGFP plasmid expresses a GFP protein downstream of a CMV promoter. The pDUAL/eGFP_GFP-BD/ED plasmid expresses GFP protein downstream of a CMV promoter and also a GFP-BD, followed by the SINEUP ED downstream of an H1 promoter. We verified the transfection efficacy and confirmed the SINEUP activity for GFP protein in SH-SY5Y cells: the cells transfected with SINEUP-GFP shows 2,4-3,9-fold increased GFP protein compared to cells transfected only with GFP vector (Fig.3). First, the STXBP1-SINEUPs will be tested in SH-

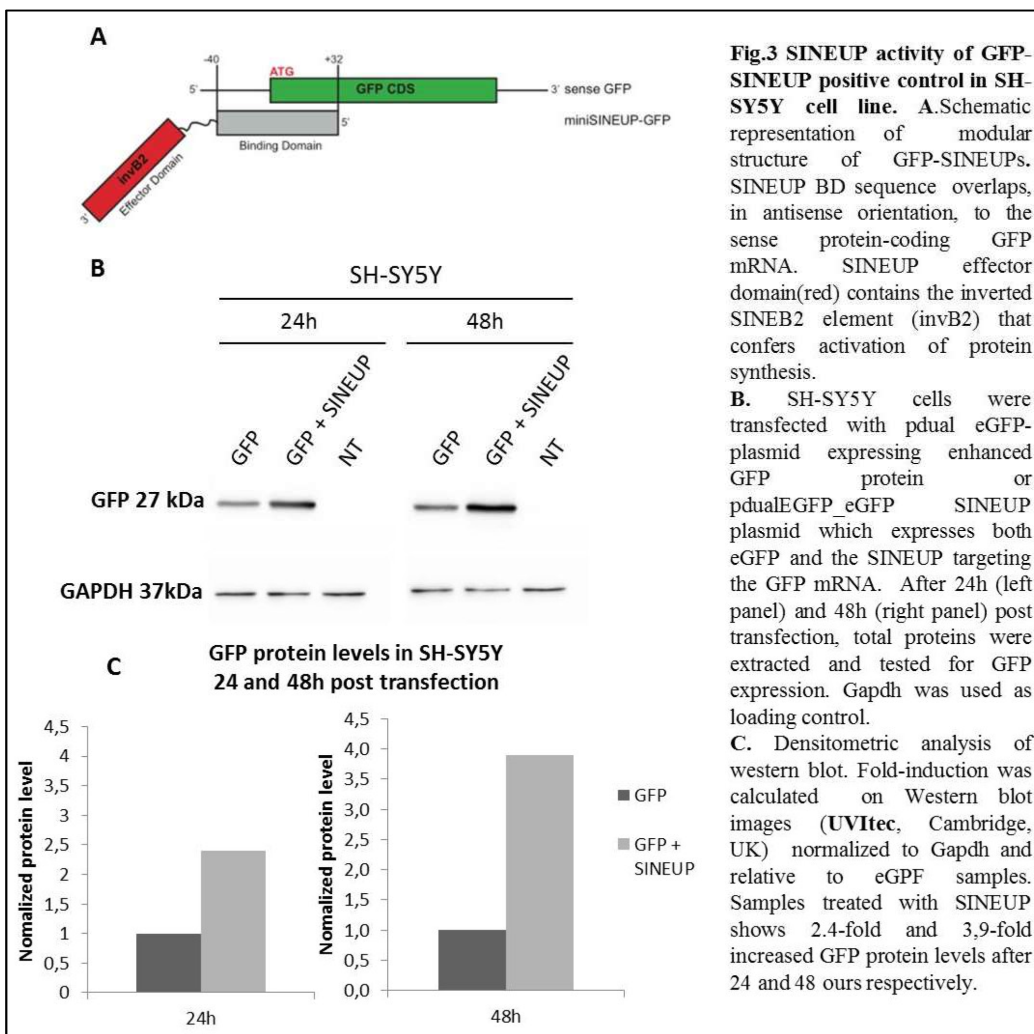

Fig.3 SINEUP activity of GFP-SINEUP positive control in SH-SY5Y cell line. A. Schematic representation of modular structure of GFP-SINEUPs. SINEUP BD sequence overlaps, in antisense orientation, to the sense protein-coding GFP mRNA. SINEUP effector domain(red) contains the inverted SINEB2 element (invB2) that confers activation of protein synthesis.
B. SH-SY5Y cells were transfected with pdual eGFP-plasmid expressing enhanced GFP protein or pdualEGFP_eGFP SINEUP plasmid which expresses both eGFP and the SINEUP targeting the GFP mRNA. After 24h (left panel) and 48h (right panel) post transfection, total proteins were extracted and tested for GFP expression. Gapdh was used as loading control.
C. Densitometric analysis of western blot. Fold-induction was calculated on Western blot images (**UVItec**, Cambridge, UK) normalized to Gapdh and relative to eGPF samples. Samples treated with SINEUP shows 2.4-fold and 3,9-fold increased GFP protein levels after 24 and 48 ours respectively.

SY5Y transfected with SINEUP-STXBP1 or Negative controls (SINEUPs-ΔBD and SINEUP-Scramble). After SINEUPs administration, the target protein will be evaluated by Western blot and quantified using UVItec software. Using SINEUPs, the transcription of the target gene is unaltered. However, the level of *STXBP1* mRNA will be verified by qRT-PCR.

For selected SINEUP and negative controls we will develop lentiviral vectors, with lentivirus pLKO.1 puro vector, to transduce the patient's IPSCSs-derived NPCs.

iv) Generation of IPSCS-derived neurons from patients' fibroblast with loss-of-function (LOF) mutations in STXBP1.

We have successfully generated IPSCs lines and differentiated neurons from fibroblasts of a patient with *STXBP1* truncating mutation and three controls. We will reprogram fibroblasts of two additional patients carrying LOF mutations from our cohort.

We will obtain at least 15 independent IPSCSs lines from each fibroblasts culture and we will process at least three clones for each individual for neural differentiation. IPSCSs will be differentiated into Neural Rosettes-like structure, through embryoid bodies step (Fig. 4).

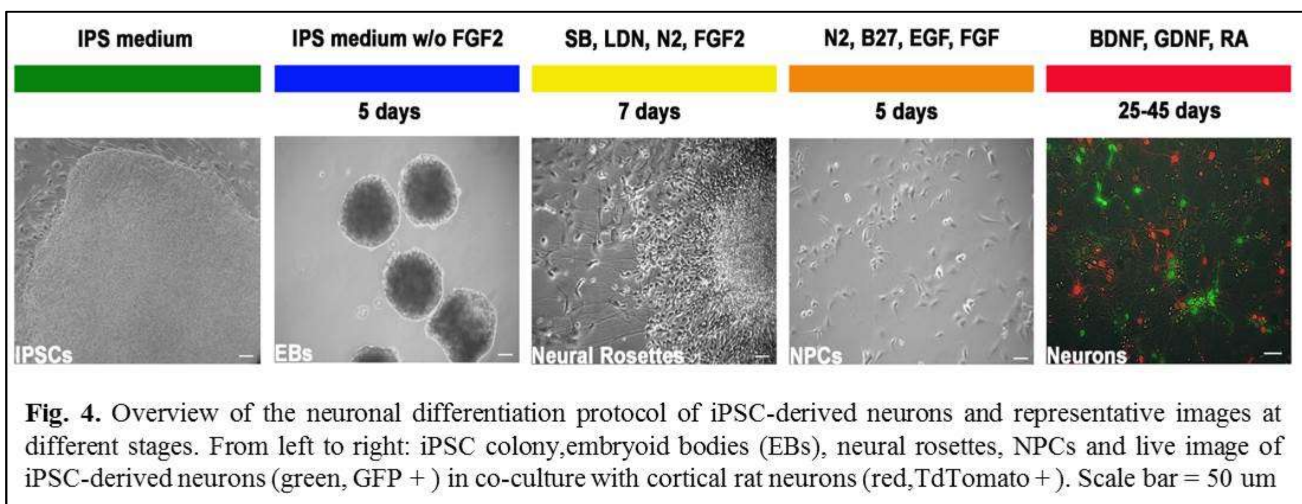

Fig. 4. Overview of the neuronal differentiation protocol of iPSC-derived neurons and representative images at different stages. From left to right: iPSC colony,embryoid bodies (EBs), neural rosettes, NPCs and live image of iPSC-derived neurons (green, GFP +) in co-culture with cortical rat neurons (red,TdTomato +). Scale bar = 50 um

The rosettes will be grown to obtain a homogeneous neural precursor cells (NPCs) culture. NPCs will be then grown in neuronal differentiation medium for at least 35 days to obtain mature neurons (Fig.5).

At every step of the protocol, the cells will be characterized for markers of pluripotency and of each neural differentiation step, using qRT-PCR, immuno-fluorescence and Western blot.

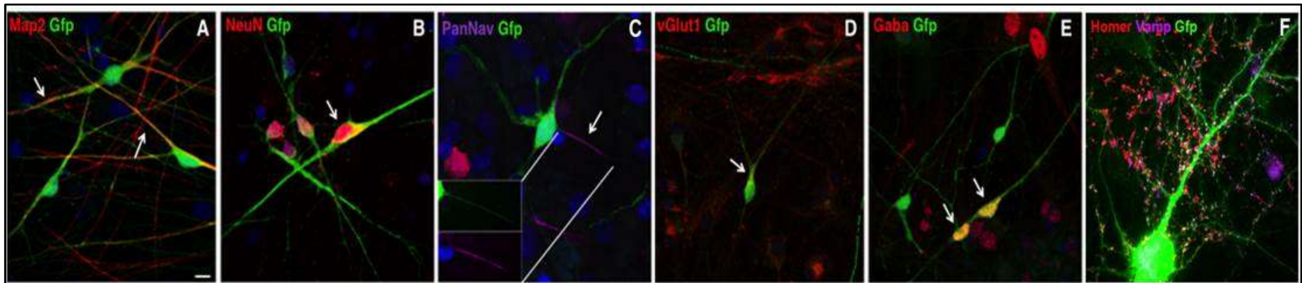

Fig. 5. Representative immunofluorescence images of the mature neuronal markers in iPSC-derived neurons after 4 weeks of differentiation: MAP2(**A**) and NEUN , voltage-gated Na+ channels (**C**).The inset in C shows co-localization of GFP and PanNav at the AIS. Nuclei were stained with DAPI. In **D** is shown VGLUT-1 as a glutamatergic pre-synaptic neuron marker; In **E** immunostaing of gabaergic neuron marker Gabaa nd in **F** co-immunostainig of presynaptic marker Vamp2 and post-synaptic neuron marker Homer.

v) Evaluation of the functional rescue elicited by SINEUPs in patients' cells.

Once we have assessed the efficacy in SH-SY5Y cell line, the SINEUPs, cloned into lentiviral vectors, will be tested by infection in neurons differentiated from IPSCSs, derived from fibroblasts of mutant patients.

Endogenous stxbp1 protein after SINEUPs transduction will be evaluated by Western blotting. Whole-cell lysates will be extracted with RIPA lysis buffer, electrophoresed separated in SDS– polyacrylamide gel and transferred to nitrocellulose membrane. Protein will be quantified by Uvitec software (Cambrige, Uk). Cells transduced with SINEUPD-□BD and a SINEUP-Scramble will be used as negative controls. GAPDH will be used as loading control protein for normalization.

We already developed a functional test to assess neuronal function, evaluating the dynamics of exocytosis isand endocytosis of synaptic vesicles (SV) in IPSCs derived neurons. This assay is based on the fluorescent probe synaptophysin-pHluorin, a pH-sensitive fluorescent probe which allows the monitoring of SV cycling at presynaptic site (6). To test the efficacy of this functional test on human IPSCS-derived neurons, we already transduced neuroprecursors with SypHy, and recorded at 35 DIV Human IPSCs-derived neurons. We found that derived-neurons showed the ability to induce the synaptic vesicle cycle and the recycling pool as WT mouse and rat cortical neurons (Figure 6).

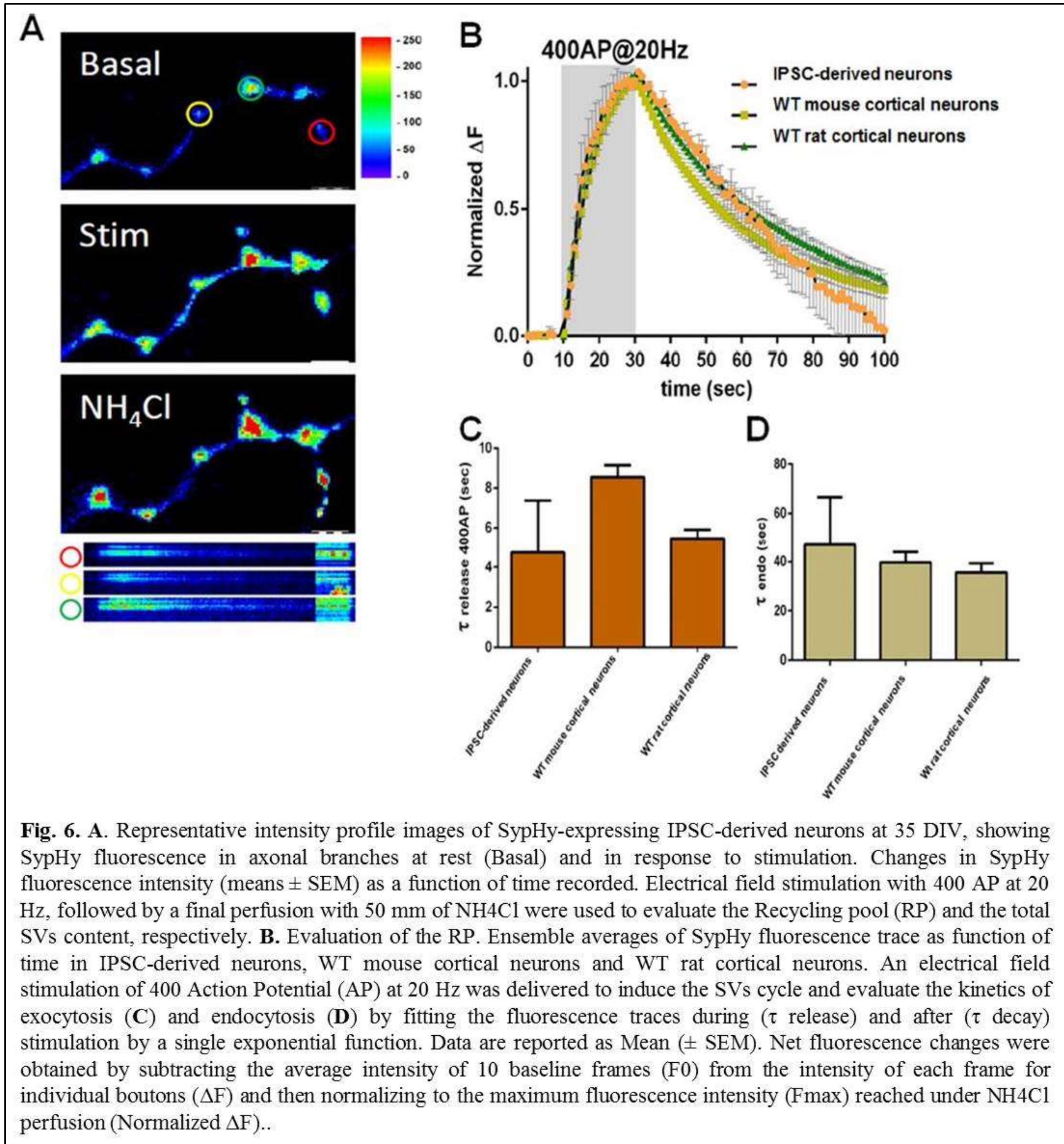

Fig. 6. A. Representative intensity profile images of SypHy-expressing IPSC-derived neurons at 35 DIV, showing SypHy fluorescence in axonal branches at rest (Basal) and in response to stimulation. Changes in SypHy fluorescence intensity (means ± SEM) as a function of time recorded. Electrical field stimulation with 400 AP at 20 Hz, followed by a final perfusion with 50 mm of NH4Cl were used to evaluate the Recycling pool (RP) and the total SVs content, respectively. **B.** Evaluation of the RP. Ensemble averages of SypHy fluorescence trace as function of time in IPSC-derived neurons, WT mouse cortical neurons and WT rat cortical neurons. An electrical field stimulation of 400 Action Potential (AP) at 20 Hz was delivered to induce the SVs cycle and evaluate the kinetics of exocytosis (**C**) and endocytosis (**D**) by fitting the fluorescence traces during (τ release) and after (τ decay) stimulation by a single exponential function. Data are reported as Mean (± SEM). Net fluorescence changes were obtained by subtracting the average intensity of 10 baseline frames (F0) from the intensity of each frame for individual boutons (ΔF) and then normalizing to the maximum fluorescence intensity (Fmax) reached under NH4Cl perfusion (Normalized ΔF)..

To evaluate the rescue of *STXBP1* function in patients' cells induced by SINEUP-STXBP1, we will transduce puromycine-selected-SINEUP-STXBP1-NPCs, SINEUPΔBD, SINEUP-ScrambleNPCs, and NPCs from healthy controls with lentiviral vectors expressing pHluorin-based probes, SypHy.SINEUP-STXBP1treated cells will be compared with SINEUP□BD and SINEUP-Scramble-treated cells and with IPSCs-derived neurons from healthy individuals used as positive control.

The functional assays on synaptic vesicle release and recycling will be carried in collaboration with Prof. A. Fassio (Univeristy of Genoa) and Prof. M. Verhage (Vrije Universiteit, Amsterdam).

EXPECTED RESULTS

We aim to develop a new potential therapeutic tool that embraces the precision medicine paradigm.
First, we expect to identify at least one SINEUP which up-regulates Stxbp1protein translation from the endogenous mRNA in SH-SY5Y cell line and then in patients' IPSCs-derived neurons. The increment of stxbp1 expression is expected to lead to a functional rescue which will be evaluated through a comparative analysis between patient and control fibroblast-derived neurons.
The effective compounds may be further tested in an existing heterozygous animal model which is available at the Laboratory of prof. Verhage and, in the future, in humans.
Because many of these patients suffer from drug-resistant epilepsy and manifest motor and/or neuropsychological deterioration, novel therapeutic tools are urgently needed to achieve seizure control and improve the outcome of these devastating disorders.
Genetic discoveries hold promise for the development of individualized treatments, which may prevent the initiation of specific physio-pathological processes triggered by genetic mutations and underlying the many different forms of epileptic encephalopathies. In this scenario, the modular architecture of SINEUPs allows to redirect translation enhancement activity to any target mRNA, by swapping its BD with the appropriate antisense sequence.
Our study is expected to significantly impact the field, by providing a valuable proof-of-concept of the use of RNA-technology SINEUPs for functional recovering of loss-of-function mutations in a large group of intractable neurological conditions of children.

ECONOMIC PLAN OF THE CURRENT PROJECT:

- 8.000 euro Research Contracts
- 7.000 euro Consumables
- 15.000 Total requested budget

COLLABORATIONS

• Department of Experimental Medicine, University of Genoa, Genoa (Prof. Anna Fassio): functional study of recirculation of synaptic vesicles

• Neuroscience Campus Amsterdam, Vrije Universiteit, Amsterdam, The Netherlands (Prof. Matthijs Verhage): functional study of recirculation of synaptic vesicles

• In collaboration with: LICE collaborative group on STXBP1

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