Encefalopatie autoimmuni con epilessia: un nuovo approccio sperimentale e possibili target terapeutici

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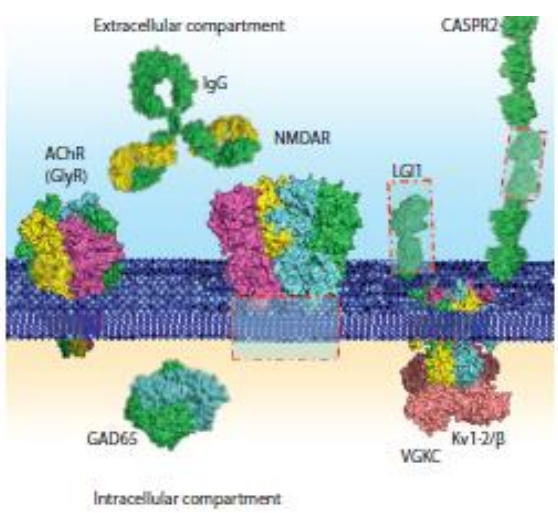
Parole chiave: Encefalopatie Autoimmuni con epilessia, Permeabilita' di Barriera Emato-Encefalica, Danno cerebrovascolare, Infiammazione

Sinossi

L'encefalite è una patologia neurologica causata da un processo infiammatorio che interessa il cervello. In passato, le infezioni erano la più frequente causa riconosciuta di encefalite, ma negli ultimi dieci anni sono state identificate diverse forme di encefaliti non-infettive, per lo più autoimmuni, cui può corrispondere un quadro clinico specifico associato ad anticorpi diretti contro antigeni neuronali di superficie o antigeni intracellulari (Prüss 2010; Bien 2017). Dati sperimentali, su modelli sia *in vitro* che *in vivo*, hanno dimostrato la diretta patogenicità di alcuni di questi anticorpi mediante la modificazione della funzione delle strutture legate all'antigene (Lai 2009; Mikasova 2012, Petit-Pedrol 2018). Comprendere sempre meglio il ruolo degli anticorpi nel meccanismo patogenetico della malattia è fondamentale sia per identificare e sviluppare terapie più efficaci e meno invasive, sia per prevenire la progressione della malattia.
Tra le encefaliti autoimmune clinicamente definite troviamo anticorpi diretti contro i recettori NMDA, AMPA, GABA A e GABA B; anticorpi diretti contro il complesso del canale del potassio voltaggio-dipendente e in particolare LGI-1 e Caspr2 (Vincent, 2011; Bien 2017).
Il malfunzionamento sia in senso inibitorio che in senso eccitatorio dei recettori o dei canali può essere responsabile dello sviluppo di crisi epilettiche e delle altre manifestazioni cliniche.
Nei pazienti con encefalopatie epilettiche (Vezzani and Granata 2005; Levite and Ganor 2008; Irani 2011) e anche in pazienti con altre forme di epilessia focale (Brenner 2013; Toledano 2014), sono state riscontrate alterazioni del sistema immunologico adattativo (linfociti B, T, NK) e livelli anormali di autoanticorpi contro antigeni neuronali di superficie, su siero e liquor. Il rapido aumento delle encefaliti autoimmuni identificate nell'ultimo decennio fa supporre che il numero salirà ancora.
Attualmente l'approccio terapeutico si basa *in primis* sull'uso di alte dosi di steroide associato ad infusione di immunoglobuline o plasmaferesi; in caso di mancata risposta o remissione incompleta si passa ai farmaci di seconda linea, immunosoppressori/immunomodulanti. Il non ottimale profilo di tollerabilità di tali farmaci, assunti in acuto e in cronico, è ben noto (Dalmau, 2018). Identificare terapie più mirate e meglio tollerate, tempestivamente somministrate, è requisito fondamentale per migliorare la prognosi. Benché in parte già noto (Seebohom 2015), a tale scopo riteniamo fondamentale comprendere in maggiore dettaglio sia il ruolo degli autoanticorpi nell'alterare la trasmissione sinaptica, sia il meccanismo di passaggio degli autoanticorpi nel sistema nervoso centrale. Come hanno ampiamente dimostrato studi sperimentali e clinici (Marchi, 2014), per studiare la patogenesi di queste malattie, dal "neurone" dobbiamo allargare lo sguardo sia al sistema cerebrovascolare sia all'intero sistema immunitario. L'infiammazione può causare alterazione della barriera emato-encefalica (BEE) e permettere l'ingresso di anticorpi nel sistema nervoso centrale (Ludwig, 2017; Brimberg, 2015), inoltre studi recenti hanno ipotizzato un contributo del complemento, delle cellule CD3 attivate, delle cellule citotossiche e dei segnali di trasduzione.



Proponiamo uno studio preclinico per analizzare la sequenza di eventi indotta dalla somministrazione di autoanticorpi isolati da pazienti con encefalite autoimmune nel modello sperimentale di cervello isolato di cavia. La dimostrazione di un diretto coinvolgimento delle cellule del sistema immunitario, del complemento e dell'endotelio nel danno, mediato dagli anticorpi, alle cellule neuronali può contribuire a sviluppare nuove strategie terapeutiche per contrastare le encefaliti autoimmuni (Mader, 2017).

Figura 1. *Proteine della superfice cellulare che possono diventare target antigenici nelle patologie neurologiche autoimmuni (Modificata da Vincent et al., Lancet Neurol 2011)*

Razionale

Diversi modelli sperimentali hanno già dimostrato che l'interazione patologica tra endotelio e leucociti, favorita da un danno della BEE, favorisce lo sviluppo di crisi epilettiche (Fabene 2008; Librizzi 2005, 2007 e 2012), in particolare promuovendo l'ipereccitabilità neuronale (Seiffert 2004; Van Vliet 2007). Inoltre, l'inibizione del danno di barriera bloccando l'interazione tra leucociti e vasi suggerisce un possibile ruolo delle cellule del sistema immunitario nella generazione delle crisi (Fabene 2008).

Il ruolo diagnostico della presenza di autoanticorpi a livello di siero e liquor è ben riconosciuto, in più il miglioramento clinico correla con la diminuzione del titolo anticorpale (Lancaster 2011). Si ipotizza che una BEE alterata sia responsabile del processo che conduce alla produzione di autoanticorpi contro antigeni del sistema nervoso centrale ma come gli autoanticorpi e/o i leucociti giungano nel parenchima non è ancora del tutto chiarito.

Partendo dai dati disponibili in letteratura e dai nostri risultati preliminari, andremo ad analizzare l'impatto fisiopatologico sull'eccitabilità neuronale e sul complesso cerebro-vascolare dell'infusione di mononucleati (PBMCs - Peripheral Blood Mononuclear Cells) e autoanticorpi derivati da pazienti nel modello sperimentale di cervello isolato di cavia mantenuto in vitro tramite perfusione arteriosa. Andremo a definire come le cellule del sistema immunitario e altre componenti del siero (albumina) contribuiscano agli effetti **acuti** neuro-vascolari e pro-infiammatori mediati da autoanticorpi diretti contro antigeni neuronali di superficie derivati dai pazienti. Inoltre, andremo a definire la via di accumulo degli autoanticorpi nel parenchima a livello cellulare e intracellulare.

Obiettivi

Testeremo l'ipotesi che gli autoanticorpi modifichino l'eccitabilità neuronale interagendo con il complesso neuro-vascolare, tramite processi eccitotossici e infiammatori. Verificheremo l'ipotesi corollaria che la presenza di un preesistente danno della BEE faciliti l'entrata e l'azione patologica degli autoanticorpi, dei mononucleati e delle proteine sieriche nel parenchima.

In questo progetto, studieremo la patogenicità degli autoanticorpi derivati da pazienti con encefalite autoimmune. Ci proponiamo di:

- Reclutare pazienti con encefalite associata ad autoanticorpi contro antigeni di superficie, conservarne i campioni di plasma derivante dalla plasmaferesi e il liquor, isolare cellule mononucleate (PBMCs);
- Purificare autoanticorpi derivati dai campioni di plasma di pazienti con positività per VGKC (n=1), LGI-1 (n=2), NMDAR (n=1) conservati presso il nostro istituto;
- Valutare l'effettodell'ingresso di autoanticorpi sul sistema neuro-vascolare e sulla permeabilità della BEE nel preparato del cervello di cavia. Valuteremo la capacità degli autoanticorpi di attuivare endoteliale e danno alla BEE (P2, vedi figura 3);

- Valutare se gli autoanticorpi da soli sono in grado di indurre attività elettrica patologica (comparsa di spikes, insorgenza spontanea di crisi) e fenomeni infiammatori (accumulo cellulare di IgG, attivazione gliale; P2-3, Fig. 3).
- Valutare il ruolo dei mononucleati (PBMCs) e dell'albumina nella generazione di attività epilettica e nelle malattie anticorpo-mediate (P4-5-6, Fig. 3)

Metodi

Tutti gli esperimenti saranno eseguiti nel prearato di cervello di cavia isolato mantenuto in vitro mediante perfusione arteriosa. In questa singolare preparazione i compartimenti vascolari e neuronali e la BEE sono preservati fino a 5 ore sia dal punto di vista morfologico che funzionale (de Curtis 1998; Librizzi 2000, 2001, 2006). Il cervello isolato rappresenta il preparato ideale per questo progetto di ricerca poiché consente di perfondere separatamente o simultaneamente anticorpi, citochine, proteine sieriche e cellule del sangue nel flusso arterioso attraverso il sistema vascolare. Dopo anestesia barbiturica intraperitoneale (125 mg/ kg), le cavie sono sottoposte a perfusione cardiaca con una soluzione fredda (15 ° C) e ossigenata (95% O_2 / 5% CO_2) composta da 126 mM NaCl, 3 mM KCl, 1.2 mM KH2PO4, 1.3 mM MgSO4, CaCl2 2,4 mM, NaHCO3 26 mM, glucosio 15 mM, HEPES 2,1 mM e destrano 3% (70.000 Da, pH 7,3). Dopo la decapitazione, il cervello viene rapidamente estratto, trasferito in una cameretta di registrazione e perfuso tramite una pompa peristaltica attraverso l'arteria basilare con la suddetta soluzione (7 ml/ min). La temperatura del cervello viene portata a 32 ° C per effettuare le registrazioni elettrofisiologiche. Dopo aver instaurato le condizioni in vitro, verranno eseguite le registrazioni di potenziali extracellulari che avverranno in continuo e inizieranno prima della perfusione di LPS / PBMCs / albumina/ auto-Abs (Fig. 2). I PBMC verranno isolati dal sangue intero di donatori sani utilizzando un gradiente di densità discontinua (Lymphoprep, Nycomed). I PBMC saranno successivamente messi in coltura in piastre da 24 pozzetti a una densità di 2 x 10 ^ 6 cellule / pozzetto in 1 ml RPMI 1640 (EuroClone) integrato con L-glutammina (2 mM), sodio piruvato (1 mM), amminoacidi non essenziali (0,1 mM), penicillina (100 U / ml), streptomicina (0,1 mg / ml), tampone HEPES (0,01 M) e FBS al 10%. I PBMC saranni trattati con concanavalina (ConA, 2 microg / ml) per indurre attivazione policlonale o non subiranno alcun trattamento per 48 ore. In ogni esperimento verranno iniettati tra i 2.500.000 e 3.000.000 PBMC.

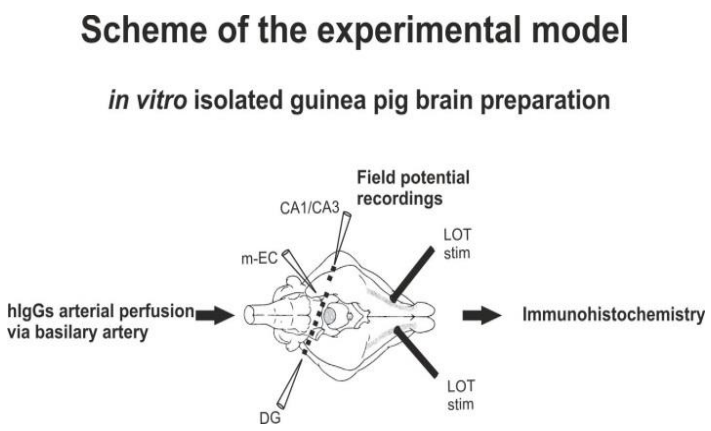
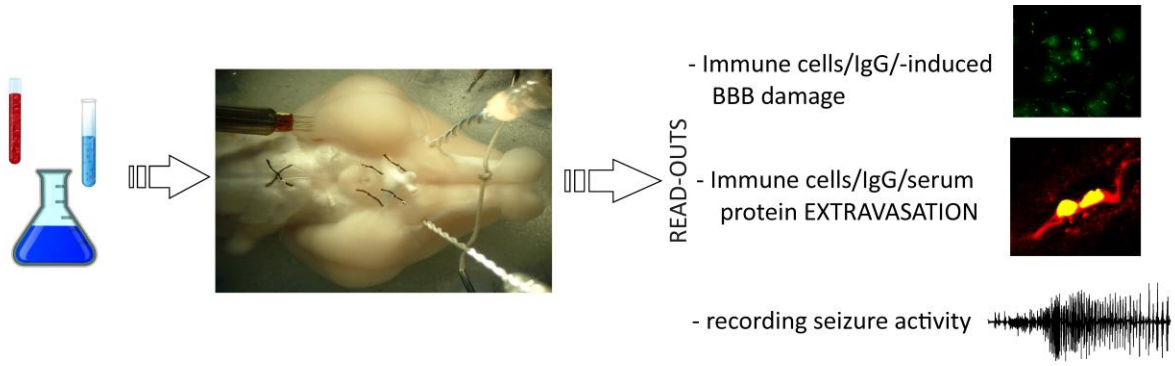


***Figure 2**: Schema sperimentale del preparato di cervello isolato di cavia (vedi testo).*

La purificazione degli auto-Abs verra' eseguita su campioni di plasma utilizzando una colonna di matrice di proteina A-Sepharose, con elevata capacità di legame per hIgGs. Il rilevamento e la purificazione di Abs anti-VGKC verra'eseguita mediante dosaggio radioimmunologico con dendrotoxin125I, mentre quello per il recettore anti-NMDA, attraverso un kit commerciale Euroimmun su tessuto neuronale di ratto e cellule HEK 293 trasfettate con la subunità NR1 del recettore NMDA. Il test per LGI1 utilizza un kit commerciale (Euroimmun) su cellule HEK 293 transfettate con LGI1 che esprimono un'elevata concentrazione dell'antigene sulla superficie. Gli auto-Abs purificati verranno marcati utilizzando un fluoroforo (FITC o Cy5) che consente la visualizzazione diretta mediante immunoistochimica (accumulo extracellulare, membrana o intracellulare). Un kit di coniugazione anticorpale è disponibile in commercio (Abcam) e il suo uso è stato ottimizzato su IgG di derivazione umana. Le registrazioni elettrofisiologiche verranno eseguite con elettrodi posizionati in diverse strutture cerebrali (ippocampo e nella corteccia entorinale mediale) per registrare

potenziali evocati spontanei e evocati. La stimolazione verrà eseguita attraverso un elettrodo bipolare posizionato sul tratto olfattivo laterale (LOT, Figura 2). Dopo ogni esperimento, i cervelli saranno divisi in due emisferi. Una metà sarà fissata per immersione in paraformaldeide al 4% in soluzione salina tamponata addizionata con fosfato (PBS, 0,1 M, pH 7,4) e sottoposta a reazioni di immunoistochimica per valutare i) infiammazione cerebrale (con Abs anti-GFAP, -IBA1, -IL-1beta; ICAM1), ii) danno cerebrovascolare (distribuzione di ZO-1) e iii) localizzazione cellulare o assorbimento di PBMCs e auto-Abs mediante markers fluorescenti. Sezioni coronali (50 µm) saranno tagliate al vibratomo (VT 1000S Leica) per tutta l'estensione del setto dalla corteccia piriforme (tavole A9.0-A13.0, Rapisarda) all'ippocampo (tavole A5.4- A7.4). Le sezioni raccolte su vetrini gelatinati verranno quindi montate in Fluorosave (Calbiochem) e coperte. Le sezioni verranno esaminate utilizzando un microscopio confocale a scansione laser D-Eclipse C1 (Nikon), dotato di quattro linee laser, montato su un microscopio ottico Eclipse TE2000-E (Nikon). L'altro emisfero verrà congelato per effettuare analisi di WB per la localizzazione subcellulare di PBMCs e auto-Abs. Questi campioni verranno utilizzati per ottenere frazioni citosoliche e di membrana grezze (Noé 2016). Le bande saranno visualizzate e quantificate utilizzando il sistema di imaging a infrarossi Odyssey (LI-COR Biosciences). Il segnale sarà normalizzato rispetto a quello dell'actina e confrontato tra i gruppi. Tutti i dati saranno analizzati usando ANOVA seguito da un appropriato test post-hoc. Un valore $p \leq 0,05$ sarà considerato significativo. Le procedure che coinvolgono gli animali saranno condotte in conformità con le linee guida istituzionali in conformità con le leggi e le politiche nazionali e internazionali.



EXPERIMENTAL PROTOCOLS

Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5	Protocol 6
LPS		LPS	LPS	LPS	LPS
Albumin			Albumin		Albumin
PBMCs				PBMCs	PBMCs
	auto-Abs	auto-Abs	auto-Abs	auto-Abs	auto-Abs
Effect on -BBB damage -brain inflamm. - excitability	Effect of autoAbs on NVU and BBB	Effect of auto-Abs on excitability and brain inflammation	Effect of BBB damage on auto-Abs-induced electrographic activity and inflammation		

***Figure 3**: Schema generale degli esperimenti nel cervello della cavia isolato (pannello superiore - vedi testo). Nel pannello inferiore, vengono illustrati i diversi protocolli sperimentali (colonne); gli elementi perfusi nel cervello isolato per ciascun protocollo sono identificati a sinistra e l'effetto atteso è identificato nella parte inferiore del pannello.*

Risultati preliminari

Risultati preliminari ottenuti mediante perfusione di IgG umane di controllo (hIgG; 5mg/g) attraverso l'arteria basilare nel cervello di cavia mantenuto in vitro, hanno mostrato extravasazione di IgG nel parenchima cerebrale (Fig. 4). Le hIgG si accumulavano esclusivamente nelle cellule microgliali attivate la cui reattività per la molecola Iba1 appariva sovraespressa. Inoltre, le cellule microgliali mutavano la loro forma da ramificata a fagocitica e quindi attiva. (Fig. 4).

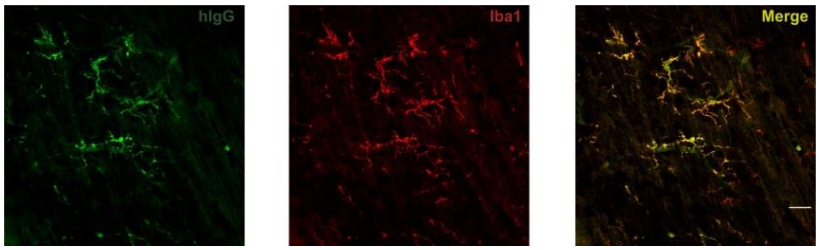


Figure 4*: HIgG immunofluorescenti (verde); microglia (Iba1; rosso) e co-localizzazione (segnale giallo). Scala della barra 50µm. hIgG e Iba1 colocalizzano nelle cellule microgliali parenchimali.*

Analisi di WB eseguite su omogenati di ippocampo e corteccia ottenuti da cervelli isolati di cavia trattati con hIgG vs cervelli isolati di cavia non trattati, hanno confermato i dati immunoistochimici (Fig. 5).

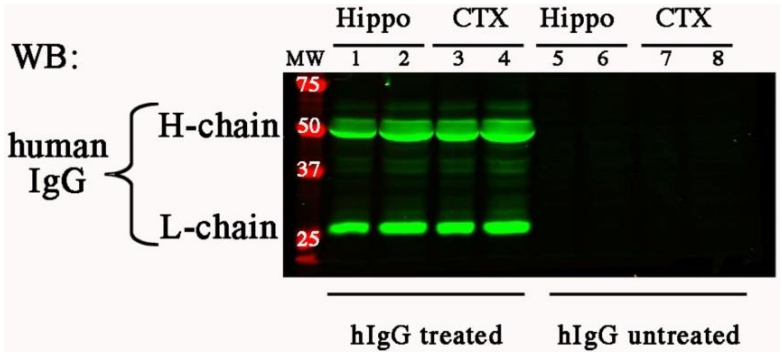


Figura 5. *WB rappresentativo di hIgG in omogenati ippocampali / corticali interi ottenuti da cavie trattate con hIgGs (corsie 1-4) e hIgGs non trattate (corsie 5-8). Due bande corrispondenti a catene pesanti (H) e leggere (L) hIgG sono state trovate solo negli animali trattati con IgG (corsie 1-4 vs 5-8).*

Abbiamo preliminarmente utilizzato il cervello di cavia isolato in vitro per esplorare se il trattamento acuto con hPBMC pretrattato con Con A (2,5-3 x 10 ^{6}) e albumina ricombinante umana (1 mg / ml) fosse in grado di indurre attività elettroencefalografica anormale e processi infiammatori in una condizione di lieve alterazione della BBB indotta da una precedente perfusione con lipopolisaccaride (LPS, 100 ng / ml). Il trattamento ha generato un'attività epilettiforme nella regione CA1 dell'ippocampo e nella corteccia entorinale mediale (Fig 6). L'analisi immunoistochimica ha rivelato l'adesione di cellule CD4 + (Fig. 4) e CD8 + (non mostrata) all'endotelio cerebrale e la loro trasmigrazione nel parenchima cerebrale.

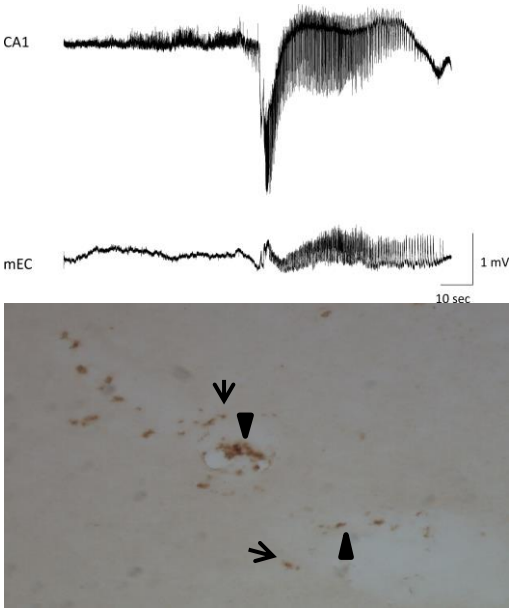


Figura 6*. Pannello in alto: attività epilettiforme registrata in CA1 e mEC nel cervello isolato di cavia dopo perfusione arteriosa con LPS (100 ng / ml), albumina ricombinante umana (1 mg / ml) e hPMC pretrattati con ConA (2, 5-3 x106). Pannelloin basso: Immagine a basso ingrandimento (10x) di una sezione corticale dello stesso cervello. Nota i linfociti in stretta associazione con vasi (punte di freccia) e nel parenchima cerebrale (frecce), che dimostra la trasmigrazione di cellule CD4 + attraverso la BEE registrate in CA1 e mEC della preparazione in vitro di cavia isolata dopo perfusione arteriosa con LPS (100 ng / ml), albumina ricombinante h (1 mg / ml) e hPMC pretrattati con ConA (2,5-3 x106).*

Risultati attesi

I risultati di questo studio forniranno nuovi spunti sulle alterazioni neuro-vascolari innescate dagli auto-Abs all'interno del parenchima cerebrale. Attendiamo i seguenti risultati:

• hPBMCs e proteine del siero (albumina) inducono cambiamenti riproducibili nell'attività elettroencefalografica basale in presenza di una BEE compromessa; studi precedenti hanno confermato l'incapacità dei PBMCs attivati di indurre un danno di barriera e di alterare l'eccitabilità del cervello (Librizzi et al., 2006);

• Gli Auto-Abs non sono in grado di alterare la BEE e l'eccitabilità neuronale, ma possono esacerbare i processi pro-infiammatori a livello cerebrale e l'eccitazione neuronale sostenuta da hPBMCs e proteine del siero (albumina), favorendo l'attivazione delle cellule gliali.

Il nostro studio contribuirà a colmare una significativa lacuna esistente nel campo delle patologie autoimmuni del cervello, con rilevanza diretta per quelle con fenotipo epilettico. Proponiamo un approccio di ricerca traslazionale in cui la patogenicità dei PBMC e degli auto-Abs dei pazienti vengano testati utilizzando un modello sperimentale in vitro, aprendo la strada a ulteriori studi utilizzando modelli in vivo disponibili presso il nostro Istituto (Carriero et al., 2012). Il nostro studio potrebbe aprire la strada a nuove strategie terapeutiche per contrastare l'ingresso e l'accumulo di auto-Abs nel parenchima cerebrale.

Analisi del rischio, possibili problemi e soluzioni

La possibilità che l'ingresso, in presenza di un danno di BEE, nel parenchima cerebrale di auto-Abs derivati dai pazienti, PBMC e di albumina (P6, vedi Fig 1), non sia sufficiente ad innescare significative e riproducibili alterazioni dell'attività neuronale e processi infiammatori, deve essere considerata. Come strategia alternativa, proponiamo:

i) il potenziamento della compromissione della BEE utilizzando TNF-alfa (10 μ? / ml) e IFNalfa (20 μ? / ml);

ii) di studiare l'effetto degli auto-Abs e dei PBMC dei pazienti e delle proteine del siero (perfusi per via arteriosa secondo il protocollo sperimentale di Fig. 3) sulla suscettibilità delle crisi evocate attraverso l'uso del proconvulsivante BIC. L'attività epilettiforme e l'aumentata permeabilità del BEE saranno indotte dalla perfusione arteriosa con BIC (50μM) per 3 minuti (Librizzi 2012). Il tempo trascorso in attività epilettiforme, l'ulteriore aumento del danno di BEE e l'attivazione di processi infiammatori saranno valutati nelle diverse condizioni sperimentali (trattati vs controlli).

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Gruppo di ricerca

Abbiamo creato un team multidisciplinare di ricercatori che comprende epilettologi, immunologi e scienziati di base che lavorano presso l'Unità di Epilettologia clinica e sperimentale della Fondazione IRCCS Istituto Neurologico "C Besta". La Dr.ssa Chiara Pastori (PI) è neurologa e ricercatrice specializzata in epilessia. La Dr.ssa Pastori ha una pluriennale esperienza di laboratorio dove ha acquisito le tecniche di registrazione elettrofisiologiche sul preparato in vitro di cervello isolato di cavia. Nel suo studio è supportata da un team di neurologi e scienziati di base. La Dr.ssa Laura Librizzi è una ricercatrice esperta nell'interazione tra compartimenti neuronali, extracellulari e vascolari durante l'ictogenesi. Il suo lavoro ha dimostrato che l'attività convulsiva di per sé induce mediatori dell'infiammazione nelle cellule gliali parenchimali / perivascolari e promuove l'indebolimento della BEE. Il dott. Flavio Villani è un neurologo e ricercatore clinico con comprovata esperienza nella diagnosi e nel trattamento delle epilessie focali farmacoresistenti e delle encefalopatie epilettiche autoimmuni. È inoltre coordinatore del Gruppo di Studio sulle epilessie disimmuni della LICE. La struttura da lui diretta rappresenta uno dei centri di riferimento in Italia per lo studio e la cura delle encefalopatie epilettiche autoimmuni.

Budget Proposto

COSTI	**COSTO TOTALE**	**COFIN BESTA**	**FONDAZIONE LICE**
1. Personale strutturato	8.000	8.000	0
2. Contratti di ricerca	40.000	30.000	10.000
3. Attrezzature	0	0	0
4. Softwares	5.000	5.000	
5. Consumabili	25.000	22.500	2.500
6. Animali	2.000	2.000	0
7.Congressi, pubblicazioni e disseminazione	1.500	0	1.500
7. Overheads	8.500	7.500	1.000
Totale	90.000 €	75.000 €	15.000 €

Budget Totale

Stipendio del personale

Personale permanente:

Dott. Flavio Villani: neurologo, 8.000 euro (1mpp);

Contratti di ricerca:

Dott.ssa Laura Librizzi: ricercatrice senior, 20.000 euro (6mpp); Finanziato da MoH

Dott.ssa Chiara Pastori: neurologa, 10.000 euro (4mpp); Finanziato da MoH

Da reclutare: contratto di ricerca, 10.000 euro (5mpp); Finanziato dalla Fondazione LICE

Attrezzatura*:* nessuna

Softwares: Labview, Coreldraw and Origin 7.5 per analisi dati elettrofisiologici e statistica.

Consumabili:

Reagenti per analisi radiochimiche e biochimiche e analisi di laboratorio (rilevamento e purificazione di hIgG da pazienti): 12.000 euro

Anticorpi primari e secondari (GFAP; IBA-1; ZO-1, ICAM1): 4.000 euro;

Farmaci per il trattamento degli animali (anestetico; bicucullina methiodide; LPS): 2.000 euro;

Materiali di consumo di laboratorio (routine di reagenti chimici e laboratorio usa e getta): 4.000 euro

Reagenti per analisi WB: 1.000 euro;

Costi relativi alle registrazioni elettrofisiologiche: (capillari di vetro, connettori oro, fili): 2.000 euro

Animali (costi di acquisto e spedizione di animali): 2.000 euro
Partecipazione a congressi, spese di pubblicazione e disseminazione risultati: contributo per la partecipazione al prossimo incontro LICE per 2 persone coinvolte nel progetto e sottomissione di un manoscritto scientifico: 1.500 euro

Overhead: spese generali dovute alla gestione del progetto: 8.500 euro

Costo totale del progetto: 90.000 euro

Budget richiesto alla Fondazione LICE: 15.000 euro

Stipendi del personale: 10.000 euro (5mpp)

Consumabili: 2.500 euro

Costi per partecipazione a congressi, pubblicazioni scientifiche e disseminazione: 1.500 euro

Altri supporti finanziari
Fondo 5x1000 Fondazione IRCCS Istituto Neurologico C. Besta
ASSOCIAZIONE PAOLO ZORZI per le Neuroscienze ONLUS

Understand auto-antibodies mediated epilepsy: a new experimental approach to identify therapeutic targets.

Applicant: Dr. Chiara Pastori, MD

Clinical and Experimental Epilepsy Unit. Neurological Institute Foundation IRCCS "Carlo Besta", via Celoria 11, 20133 Milano.

Key words: Autoimmune encephalopathies with epilepsy, Blood Brain Barrier permeability, Cerebro-vascular damage, Inflammation

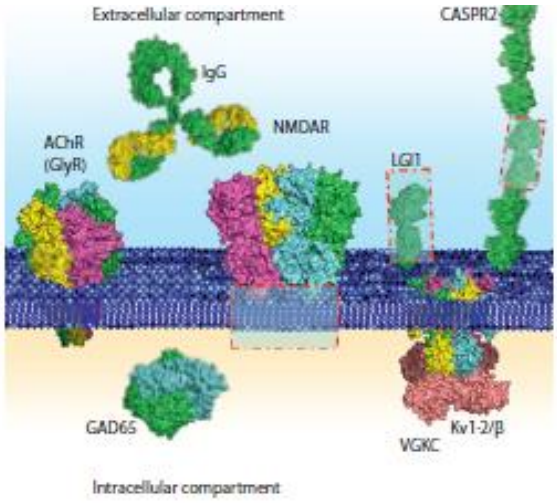
Abstract

Encephalitis is a neurological disorder caused by brain inflammation. In the past, the most frequently recognized causes of encephalitis were infectious, but in the last 10 years an increasing number of non-infectious, mostly autoimmune, encephalitis cases have been identified and represent specific clinical syndromes associated with antibodies (auto-Abs) that target either neuronal cell-surface or intracellular antigens (Prüss 2010; Bien 2017). *In vitro* and *in vivo* experimental evidences have demonstrated that some of these cell surface-targeted auto-Abs are pathogenic and modify the structure and function of their target neuronal proteins (Lai 2009; Mikasova 2012; Petit-Pedrol 2018). Understanding the contribution of auto-Abs to the pathogenesis of autoimmune encephalitis is essential for the development of efficient and less invasive treatment options and for disease prevention.

Clinically defined auto-Abs are directed against NMDARs, AMPARs, GABA type A and B receptors, voltage-gated potassium channel complexes (VGKCs) including LGI1 and Caspr2 (Vincent, 2011; Bien 2017). Dysfunction of both excitatory and inhibitory receptors and/or voltage-gated potassium channels may lead to epileptiform activity (a common comorbidity in autoimmune encephalitis) and to diverse symptoms in patients.

Immunological alterations characterized by cells of adaptive immunity such as T and B cells or NK cells as well as by abnormal levels of auto-Abs against neuronal surface antigens are found in both serum and CSF of patients affected by epileptogenic encephalopathies (Vezzani and Granata 2005; Levite and Ganor 2008; Irani 2011) and in a number of subjects suffering from other forms of focal epilepsies (Brenner 2013; Toledano 2014). The rapid increase in the number of syndromes and auto-Abs identified in the last ten years suggests that other autoimmune encephalitis with seizures as prominent symptom have to be discovered.

The current approach for these diseases includes therapies with glucocorticoids and intravenous immunoglobulin (IgG), or plasma exchange and, in absence of a clinical response, immunomodulant/ immunosuppressive drugs. The adverse effects of acute and prolonged treatments with these drugs are well known (Dalmau, 2018). Focused therapies should be considered as important therapeutic aims to improve the functional outcome after autoimmune encephalitis. Although the pathogenicity of most auto-Abs is well established (Seebohom 2015), their contribution in altering synaptic transmission and brain excitability, together with the circumstances associated with their entrance into the brain, remains unexplored and unknown. The neuro-centric view of epilepsy in autoimmune encephalitis has been challenged by clinical and experimental compelling evidences suggesting a role of the immune/inflammatory cerebrovascular systems in the pathogenesis of seizure disorders (Marchi, 2014). Inflammation and dysfunction of microvascular endothelial cells can result in disruption of blood-brain barrier integrity (BBB), which in turn allows the influx of auto-Abs from the periphery into the brain (Ludwig, 2017; Brimberg, 2015). Recently, the concept that auto-Abs can mediate brain pathology by varied mechanisms including complement activation, CD3 cells activation, cell cytotoxicity, signal transduction has been hypothesized. We propose pre-clinical study to analyze the pathogenic sequence of events promoted by the administration of auto-Abs isolated from



patients with autoimmune encephalitis in an isolated guinea pig brain preparation. The demonstration of a direct involvement of the activation of immune system cells, complement and brain endothelial cells in antibody-mediated neuronal cell dysfunction may reveal therapeutic strategies to counteract neurological pathologies associated with autoimmune encephalitis (Mader, 2017).

Figure 1. *Cell-surface proteins can become antigenic targets in autoimmune neurological diseases (Modified from Vincent et al., Lancet Neurol 2011)*

Rationale

The pathogenic link between leukocyte and endothelial interaction, blood-brain barrier (BBB) damage and seizure generation has been extensively demonstrated in experimental models (Fabene 2008; Librizzi 2005, 2007 and 2012). BBB impairment elicits neuronal hyperactivity (Seiffert 2004; Van Vliet 2007) and inhibition of BBB damage by blockade of leucocyte-vascular interaction suggests a possible role for immune system cells in seizure generation (Fabene 2008).

The diagnostic link between epileptogenic encephalitis and blood/CFS auto-Abs levels is recognized, including the demonstration of neurologic improvement during immunomodulation correlating with decreased serum and CSF auto-Abs titers (Lancaster 2011). A compromised BBB is suspected to be responsible for the process that leads to the production of peripheral antibodies against central nervous system epitopes. How do the leucocytes or auto-Abs access the brain parenchyma is still unresolved.

Building from available evidence and our preliminary results, we will analyze the pathophysiological impact of specific patients-derived Peripheral Blood Mononuclear Cells (PBMCs) and auto-Abs on neuro-vascular cells and neuronal excitability in the whole brain preparation of the guinea pig maintained in vitro by arterial perfusion. We will define how immune system cells and other component of serum (e.g. albumin), contribute to the ***acute*** neurovascular and proinflammatory effects triggered by patient-derived auto-Abs directed against neuronal cell-surface antigens. Then, we will define the route of auto-Abs accumulation in the brain at the cellular and sub-cellular levels.

Aims

We will test the **hypothesis** that auto-Abs modify brain excitability by direct interference with neuronal-vascular integrity and through excitotoxic and pro-inflammatory processes. We will test the **corollary hypothesis** that pre-existing BBB alteration facilitates peripheral Abs targeting of exposed neuronal epitopes and immune cells and serum proteins entrance into brain parenchyma.

In this project, the pathogenicity of auto-Abs collected from patients suffering from autoimmune encephalopathies will be investigated. We will:

- Recruit patients with encephalopathies positive to auto-Abs against to neuronal surface antigen to collect serum from undergoing apheresis treatment, CSF samples and isolate PBMCs;
- Purify auto-Abs from patient's plasma samples positive to auto-Abs: VGKC (n=1), LGI1 (n=2), NMDAR (n=1), stored in our repository.
- Explore the impact of brain-directed auto-Abs on Neuro-Vascular Unit (NVU) and BBB permeability *in vitro* brain preparation. We will test the capability of auto-Abs to lead cerebral endothelial cells activation and BBB impairment (P2, see Fig 3).
- Investigate if auto-Abs by themselves are able to induce abnormal electrographic activity (spikes and/or seizure occurrence), and pro-inflammatory signs (IgG cellular accumulation, reactive glial cells; P2-3, Fig 3).
- Explore the contribution of immune cells (PBMCs) and albumin to the generation of epileptic discharges and to auto-Abs disease precipitation (P4-5-6, Fig 3).

Methods

All the experiments will be performed in the isolated guinea pig brain maintained *in vitro* by arterial perfusion. In this unique preparation the vascular and neuronal compartments and the blood-brain barrier (BBB) are morphological and functional preserved up to 5 hours (de Curtis 1998; Librizzi 2000, 2001, 2006). The *in vitro* isolated brain has remarkable potentials for this research project since it consents to separately or simultaneously perfuse Abs, cytokines, serum proteins and blood borne cells into the arterial stream under conditions of flow through the resident preserved vascular system.

Briefly, after intraperitoneal barbiturate anaesthesia (125mg/kg), guinea pigs are transcardially perfused with a cold (15°C) oxygenated (95% O_2/5% CO_2) solution composed of 126mM NaCl, 3mM KCl, 1.2mM KH_2PO_4, 1.3mM $MgSO_4$, 2.4mM $CaCl_2$, 26mM $NaHCO_3$, 15mM glucose, 2.1mM HEPES, and 3% dextran (molecular weight, 70,000Da; pH 7.3). Following decapitation, brains are rapidly dissected out, transferred to a recording chamber, and are perfused via a peristaltic pump through the basilar artery with the above solution (7ml/min). Brain temperature is slowly raised to 32°C for electrophysiological experiments. After the establishment of the *in vitro* condition, electrophysiological recordings are performed continuously during the experiments before and after LPS/PBMCs/h-recombinant albumin/auto-Abs perfusion (Fig 2).

PBMCs are isolated from anticoagulated whole blood of healthy donors by using a discontinuous density gradient (Lymphoprep, Nycomed, Oslo, Norway). PBMCs are then cultured in 24-well plates at a density of 2 x 10^6 cells/well in 1 ml RPMI 1640 (EuroClone) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), HEPES buffer (0.01 M), and 10% FBS. PBMCs are either treated with concanavalinA (ConA; 2 microg/ml) for polyclonal activation or are left unstimulated for 48h. Between 2,500,000 and 3,000,000 PBMCs were injected in each experiment.

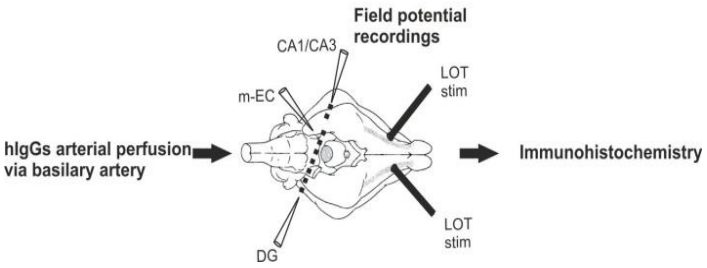


Figure 2*: Scheme of the in vitro isolated guinea pig brain preparation (see text).*

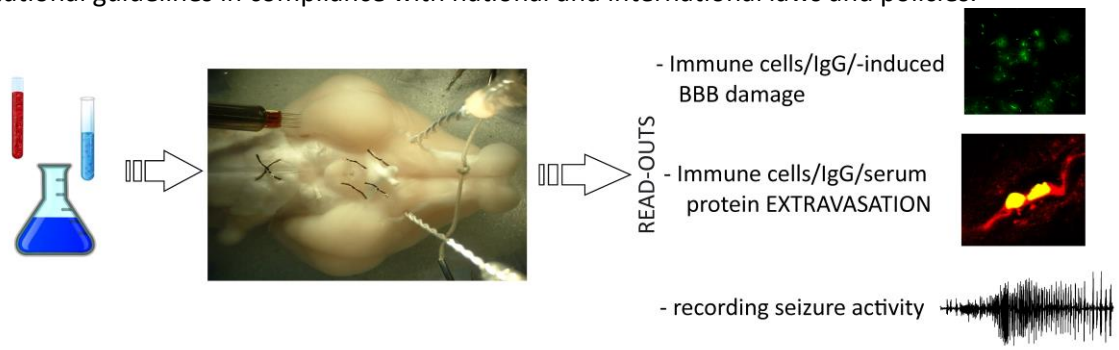
Auto-Abs purification is performed on plasma samples using a Protein A-Sepharose matrix column, with high binding capacity for hIgGs. The detection and purification of Abs anti-VGKC is performed by radioimmunoassay with dendrotoxin125I. Immunocytochemistry cell based assay for Abs anti-NMDAR is achieved using a commercial biochip Euroimmun kit on rat neuronal tissue and subunit NR1 NMDAR transfected HEK 293 cells. The cell based assay for LGI1 uses a commercial biochip kit (Euroimmun) on LGI1 transfected HEK 293 cells that express high concentration of the antigen on the surface: Ab-binding is detected with a fluorescent anti-hIgG. Purified auto-Abs will be tagged using a fluorophore (FITC or Cy5) allowing for their direct visualization using immunohistochemistry (extracellular, membrane or intra-cellular accumulation). An antibody conjugation kit is commercially available (Abcam) and its use is being optimized on human-derived IgG.

Electrophysiological recordings are performed with multiple electrodes in different brain structures to record spontaneous and stimulus-evoked potentials. Stimulation is delivered on the lateral olfactory tract (LOT) and recordings are performed in the hippocampus and in the entorhinal cortex (Figure 2).

At the end of each experiment, brains will be split into two hemispheres. One half will be fixed by immersion into 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1M, pH 7.4) and processed for immunohistochemistry to evaluate i) brain inflammation (with Abs anti-GFAP, -IBA1, -IL-1beta; ICAM1), ii) cerebrovascular damage (ZO-1 distribution) and iii) brain cellular localization or uptake of PBMCs and auto-Abs using tagged fluorescence. Coronal sections (50 µm) will be cut by vibratome (VT 1000S Leica) throughout the septal extension of the piriform cortex (plates A9.0–A13.0, according to Rapisarda atlas 22) and of the hippocampus (plates A5.4–A7.4). Sections collected on gelatin-coated slides will be then mounted in Fluorosave (Calbiochem) and cover-slipped. Slide-mounted sections will be examined using a laser scanning confocal microscope D-Eclipse C1 (Nikon), equipped with four laser lines, mounted on a light microscope Eclipse TE2000-E (Nikon).

The other hemisphere will be snap-frozen for WB analysis of sub-cellular localization of PBMCs and auto-Abs. These specimens will be used to obtain crude cytosolic and membrane fractions for WB (Noé 2016). Bands will be visualized and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences), normalized versus actin signal, and compared among groups.

All data will be analyzed using ANOVA followed by appropriate post-hoc test. A p-value ≤ 0.05 will be considered significant. Procedures involving animals and their care will be conducted in conformity with institutional guidelines in compliance with national and international laws and policies.



EXPERIMENTAL PROTOCOLS

Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5	Protocol 6
LPS		LPS	LPS	LPS	LPS
Albumin			Albumin		Albumin
PBMCs				PBMCs	PBMCs
	auto-Abs	auto-Abs	auto-Abs	auto-Abs	auto-Abs

Effect on -BBB damage -brain inflamm. - excitability

Effect of autoAbs on NVU and BBB

Effect of auto-Abs on excitability and brain inflammation

Effect of BBB damage on auto-Abs-induced electrographic activity and inflammation

***Figure 3**: General scheme of the experiments in the isolated guinea pig brain (upper panel - see text). In the lower panel, the different experimental protocols are illustrated (columns); the elements perfused in the isolated brain for each protocol are identified on the left and the expected effect is identified in the bottom part of the panel*

Preliminary results

Preliminary results obtained perfusing commercially available control human IgGs (hIgG; 5mg/g) via basilar artery into the *in vitro* guinea pig brain showed hIgGs extravasation in the brain parenchyma (Fig. 4).

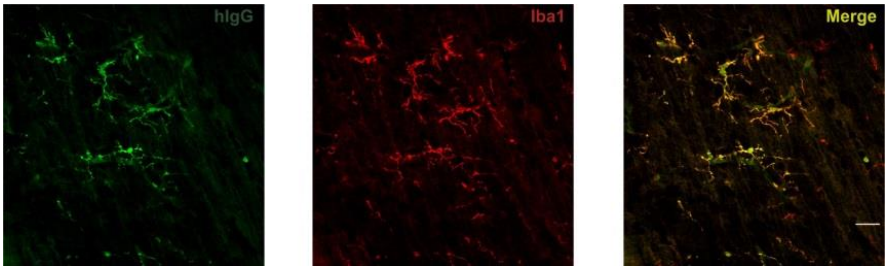


Figure 4: *Immunofluorescent hIgG (green); ionized calcium binding adaptor molecule 1 (Iba1; red), and their co-localization (yellow signal, merge). Scale bar 50µm. Note as hIgG and Iba1 staining fully co-localized in parenchymal microglial cells.*

HIgGs accumulated in activated microglial cells. Ionized calcium binding adaptor molecule 1 (Iba1) staining appeared up-regulated and microglial cells moved from their ramified to fully active phagocytic form. HIgGs signal co-localized exclusively with reactive microglial cells demonstrating a hIgGs- induced immunological response (Fig 4).

Western blot analysis performed on hippocampus and cortex homogenates obtained from control hIgGs treated vs untreated isolated brains, confirmed immunohistochemical data (Fig. 5). No signal was detected using an anti guinea pig-IgG in the same samples, indicating experimental specificity for human derived IgG (data not shown).

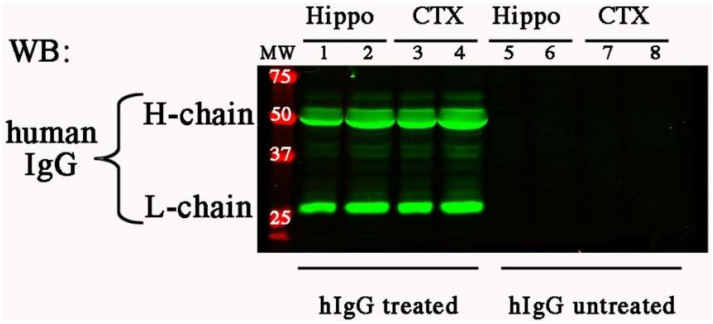


Figure 5. *Representative WB analysis of hIgG in whole hippocampal/cortical homogenates obtained from hIgGs-treated (lanes 1-4)* and hIgGs-untreated (lanes 5-8) guinea pigs. Two bands corresponding to heavy (H) and light (L) hIgG chains were found in hIgGs-treated animals only (lanes 1-4 vs 5-8).

We preliminarily utilized the *in vitro* isolated guinea-pig brain to explore if acute treatment with hPBMCs pretreated with Con A (2,5-3 x10 ^{6}) and h-recombinant albumin (1mg/ml) was able to induce abnormal electrographic activity and pro-inflammatory signs (adhesion molecules expression, reactive astrocytes and microglia) in a condition of mild BBB impairment induce by previous perfusion of lipopolysaccharide (LPS, 100ng/ml).The treatment generated epileptiform activity in CA1 region and entorhinal cortex (Fig 6).

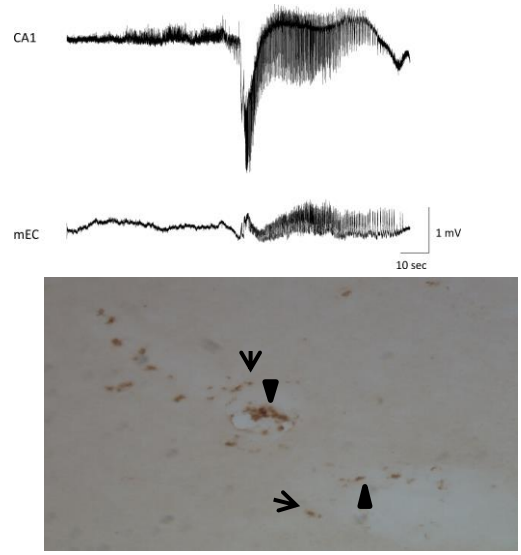


Figure 6. *Upper panel: representative focal seizures recorded in CA1 and mEC of the in vitro isolated Guinea pig brain preparation after arterial perfusion with LPS (100ng/ml), h-recombinant albumin (1mg/ml) and hPMCs pretreated with ConA (2,5-3 x10^{6}). Lower panel: Representative low-power (10x) IHC image of cortical section of stimulated brain (lymphocytes in close association with vessels (arrowheads) as well in brain parenchyma (arrows), demonstrating the transmigration of CD4+ cells through the BBB focal seizures recorded in CA1 and mEC of the in vitro isolated guinea pigbrain preparation after arterial perfusion with LPS (100ng/ml), h-recombinant albumin (1mg/ml) and hPMCs pretreated with ConA (2,5-3 x10^{6}).*

Immunohistochemical analysis revealed CD4+ (Fig 4) and CD8+ (not shown) cells adhesion to the endothelium and their transmigration into the brain parenchyma.

Expected outcomes

The results of this study will provide novel insights on the pathophysiological neuro-vascular modifications triggered by auto-Abs in the brain. We expect the following findings:

- hPBMCs and serum components (e.g. albumin) provoke reproducible changes in the basal electrographic activity if the BBB is impaired; previous studies confirmed the inability of activated PBMCs to induce BBB damage and to alter brain excitability (Librizzi et al., 2006;)
- Auto-Abs are not able to impair the BBB and to alter neuronal excitability, but they can exacerbate brain pro-inflammatory processes and neuronal excitation sustained by hPBMCs and serum components (e.g. albumin), favoring glial cells activation;

Our study will contribute to fill a significant knowledge-gap existing in the field of auto-immune brain disorders, with direct relevance to epileptogenic encephalitis characterized by the presence of circulating auto-Abs. We propose a translational research approach where patients' PBMCs and auto-Abs are tested using an experimental *in vitro* model, opening for further studies using *in vivo* models available at our

Institute (Carriero et al., 2012). We will outline the specificity between auto-Abs challenge and brain excitability changes, inflammatory damage and BBB impairment. Our study could pave the way to novel therapeutical strategies to counteract auto-Abs entrance and accumulation in brain parenchyma.

Risk analysis, possible problems and solutions: The possibility that patient-derived auto-Abs and PBMCs, in presence of an impaired BBB and albumin entrance into the isolated *in vitro* brain preparation (P6; see Fig 1), may not be sufficient to trigger significant and reproducible pro-inflammatory brain changes and neuronal activity should be considered. As alternative strategy we will:

i) potentiate BBB impairment by using a stronger endothelial cells activation by using TNF-alpha (10 u/ml) and IFNalpha (20 u/ml);

ii) investigate the effect of patients' PBMCs, human recombinant albumin, auto-Abs (arterially perfused according with the experimental protocols; see Fig 3) on seizure susceptibility evoked using BIC. Epileptiform activity and increased BBB permeability will be induced by arterial perfusion with BIC (50µM) for 3 min (Librizzi 2012). The time spent in seizure, the extent of seizure-dependent BBB increase in permeability and inflammation will be compared among the experimental conditions.

Research team

We have assembled a multidisciplinary team of investigators encompassing epileptologists, immunologists and basic scientists working at the Clinical and Experimental Epileptology Unit of the Fondazione IRCCS Istituto Neurologico "C Besta".

Dr Pastori (PI) Dr. Chiara Pastori (PI) is a neurologist and researcher specializing in epilepsy. Dr. Pastori has a multi-year laboratory experience where she has acquired the electrophysiological recording techniques on the in vitro isolated guinea pig brain preparation. In her study she is supported by a team of neurologists and basic scientists.

Dr Laura Librizzi is an expert in the interaction between neuronal, extracellular and vascular compartments during ictogenesis. Her work has proved that seizure activity per se induce inflammatory mediators in parenchymal/perivascular glial cell and promote BBB impairment.

Dr Flavio Villani is a staff neurologist and clinical researcher with expertise in the diagnosis and treatment of focal drug-resistant epilepsy and autoimmune epileptic encephalopathies. He is also the coordinator of LICE task force on dysimmune epilepsies. He is the Head of a structure which represents a reference center in Italy for the study and treatment of autoimmune epileptic encephalopathies.

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Proposed budget

COSTS	TOTAL COST	COFIN BESTA	FONDAZIONE LICE
1. Staff salary	8.000	8.000	0
2. Researcher Contracts	40.000	30.000	10.000
3. Equipment (leasing-rent)	0	0	0
4. Softwares	5.000	5.000	
5. Supplies	25.000	22.500	2.500
6. Animals	2.000	2.000	0
7.Meetings, pubblication costs, dissemination	1.500	0	1.500
7. Overheads	8.500	7.500	1.000
Total	90.000 €	75.000 €	15.000 €

Budget description (total cost)

Staff salary*:*
Dr Flavio Villani: neurologist, 8.000 euro (1mpp); Permanent staff

Researcher Contracts:
Dr Laura Librizzi: senior researcher, 20.000 euro (6mpp); Funded by MoH
Dr Chiara Pastori: neurologist, 10.000 euro (4mpp); Funded by MoH
To be recruited: research contract, 10.000 euro (5mpp); Funded by LICE Foundation

Equipment: none

Softwares*:* Labview, Coreldraw and Origin 7.5 for electrophysiological recordings analysis and statistic: 5.000 euro

Supplies **(including**):

Reagents for radiochemical and biochemical assays and laboratory analysis (detection and purification of hIgGs from patients): 12.000 euro

Primary and secondary antibodies (GFAP; IBA-1; ZO-1, ICAM1): 4.000 euro;

Drugs for animal treatment (Anhestetic; Bicuculline Methiodide; LPS): 2.000 euro;

Laboratory consumables (routines chemical reagents and lab disposable): 4.000 euro

Reagents for WB analysis: 1.000 euro;

Electrophysiological recordings related costs: (glass capillaries; gold connettors; wires): 2.000 euro

Animals (purchase and shipping costs of animals): 2.000 euro

Travel, publication and dissemination costs: contribution for attendance at next LICE meeting for 2 persons involved in the project and manuscript submission fees: 1500 euro

Overhead: general expenses due to the management of the project: 8.500 euro

Total cost of the project: 90.000 euro

Budget requested to Fondazione LICE: 15.000 euro

Salaries: 10.000 euro (5mpp)

Supplies: 2.500 euro

Travel, pubblications and dissemination costs: 1.500 euro

Overheads**:** 1.000 euro

Other Financial Support

5x1000 Fund by Neurological Institute Foundation Carlo Besta
ASSOCIAZIONE PAOLO ZORZI per le Neuroscienze ONLUS

Title of the project:

Study of GABAergic alterations in neurodevelopmental pathologies determining drug-resistant epilepsy.

Responsabile:

Gabriele Ruffolo MD, PhD

Scientific Guarantor:

Prof. Eleonora Palma

SYNOPSIS

The present project will be focused on the study of $GABA_A$ receptors ($GABA_ARs$) in neurodevelopmental pathologies characterized by the early onset of severe epilepsy. This topic encloses different unanswered questions about the relationship between epileptogenesis and central nervous sytem (CNS) onthogeny, in particular as the role of an altered neurotransmission in the pathologic development and its repercussions on the predisposition of the brain to generate seizures.

Specifically, we will use tissues from Dravet syndrome (DS), Tuberous sclerosis complex (TSC), Focal Cortical dysplasia (FCD), Rett syndrome (RS), Fragile X syndrome (FRAX) and Angelman syndrome (AS) (**Table 1**) to perform electrophysiology, molecular biology and immunohistochemistry experiments.

Of note, the functional study of neurotransmitter receptors in these rare pathologies is often difficult because of the low availability of fresh and viable tissues, suitable for cell culturing or to prepare slices for the electrophysiology. Here, we will overcome this limitation by taking advantage of the technique of membrane microtransplantation in *Xenopus* oocytes (*Eusebi et al., 2009*), that makes possible to record currents evoked from the neurotransmitter receptors of the patients starting from small quantities of brain tissue from autopsy or surgery. Furthermore, it has been previously demonstrated that the membrane proteins injected in the oocytes, still embedded in their lipidic *moyeau*, preserve their native function (*Palma et al., 2003*).

The main focus of this study will be $GABA_AR$ and its function in the various neuropathologies (**Table 1**). It has already been shown by the proponent's group and other Authors that $GABA_ARs$ demonstrate a certain degree of alteration in many conditions that imply an impairment of the normal brain development (*Talos et al., 2012; Tang et al., 2016; Ruffolo et al., 2016, 2018; Braat and Kooy, 2015*). It is not completely clear though, how this GABAergic dysfunction participates in the processes that eventually determine both epilepsy and cognitive dysfunctions in the patient (*Moore et al., 2017; Bozzi et al., 2018*).

Thus, in order to better clarify this last matters, different approaches will be used to study $GABA_ARs$ from different perspectives. The aforementioned electrophysiological study will be performed on patients of different "developmental periods" and compared with age-matched controls. In particular, this section of the study will investigate the GABA reversal potential (E_{GABA}) in the various pathologies, since this measure appears to correlate with the overall degree of development of the GABAergic system (*Ruffolo et al., 2016*).

To strengthen the electrophysiology results, we will perform also qRT-PCR and immunohistochemistry studies to quantify and determine the localization of the main proteins responsible of $GABA_ARs$ function. Specifically, we will quantify the mRNAs for the main $GABA_AR$ subunits in the different pathologies basing on previous

results obtained by us and other Authors (*Ruffolo et al., 2016, 2018; Brooks-Kayal and Pritchett, 1993, Cepeda et al., 2006*) with particular attention to the subunits of which the expression is more related to epilepsy and brain immaturity (*Fritschy et al., 1994; Succol et al.,2012*).

In the light of this last experiments, we will perform immunohistochemical analyses aimed to unravel specific site- or cell- dependant alterations.

The same qRT-PCR and immunohistochemistry experiments will be performed also to study cation chloride cotransporters (CCCs). These proteins, and in particular NKCC1 and KCC2, are the main factors in the control of chloride homeostasis in the brain and their altered expression and function severely impairs GABAergic transmission (*Kahle et al., 2006*).

In conclusion, all the data collected "on the lab bench" will be correlated with the clinical records of the patients, as an accurate neurological and neuropathological analysis will be performed by expert clinicians, in collaboration with the group of prof. Eleonora Aronica.

Table 1. ***Developmental diseases and their abbreviations***

Pathology	Abbreviation
Tuberous Sclerosis	TSC
Focal Cortical dysplasia	FCD
Rett syndrome	RS
Dravet syndrome	DS
Fragile-X syndrome	FRAX
Angelman syndrome	AS

RATIONALE

Here, different laboratory techniques and medical neuropathology analysis will be used to study neurodevelopmental epilepsies (Table 1) and how neurodevelopment and epileptogenesis interact to determine the epileptic phenotype in the patient. A particular focus will be devoted to the study of GABAergic function. The importance of GABAergic transmission in proven by the studies of various Authors (*Talos et al., 2012; Tang et al., 2016; Ruffolo et al., 2016, 2018; Braat and Kooy, 2015*) that led to the hypothesis that different neurodevelopmental syndromes could share the common hallmarks of brain immaturity. As an example, Talos and coll.(2016) demonstrated that $GABA_A$Rs are altered in TSC, and that E_{GABA} in the neurons isolated from these patients is more depolarized than in physiological conditions, and similar results were obtained by Ruffolo and coll. (2016), where a depolarized E_{GABA} was found in a cohort of TSC patients. Subsequently, similar alterations have been identified in RS and DS thus strengthening the hypothesis of GABAergic implication in developmental failures.

However, the study of neurodevelopmental pathologies is made difficult by the rarity of these syndromes (*e.g.* 1/6000 – 10.000, *Talos et al., 2012, Ruffolo et al., 2018*) and the fact that not all these patients undergo surgical interventions, hence making it difficult to obtain viable tissue to use for research purposes. As a matter of fact,

the aforementioned studies took advantage of animal models of disease or very complex and elaborated culturing techniques (*e.g.* induced pluripotent stem cells, iPSC)

The approaches here proposed (membrane microtransplantation for electrophysiological study, qRT-PCR, immunohistochemistry for selected proteins and clinical examination of the patients) will allow a complete characterization of the different aspects of GABAergic function in these neurodevelopmental disorders using human tissues obtained from patients of different age and disease progression stage, thus achieving a dynamic view of the GABAergic function across physiopathology and development.

With this translational approach, we expect to characterize the $GABA_A$Rs in the different neurodevelopmental pathologies included in the study, and better define the common GABAergic hallmarks that could play a fundamental role in the general physiopathologic mechanisms underlying developmental failures in these syndromes. This will lead both to a better comprehension of these conditions and to the perspective of new therapeutic strategies.

OBJECTIVES

The main objectives will aim to the study of GABAergic function in different neurodevelopmental pathologies:

1) The **electrophysiological study** of the cerebral cortex tissues from epilepsy patients (**Table 1**) suffering from pathologies involving a certain degree of neurodevelopmental alteration. This study will be carried out with the technique of membrane microtransplantation (*Eusebi et al., 2009*) and will examine $GABA_A$Rs function as the main aim, but will be extended to AMPA receptors (*Ruffolo et al., 2016*)

2) The **molecular study,** through **quantitative real time-PCR** and **immunohistochemistry**, of the same tissues will analyze the expression and perform the quantification of the relevant receptor subunits. This information can reveal if subunits typically associated with epilepsy and/or brain immaturity are differentially expressed in our cohort of patients.

3) The **neuropathological** and **clinical** study and its correlation with the results obtained in the two previous aims completes this framework and begins the translation of the "basic science" results to the clinical practice.

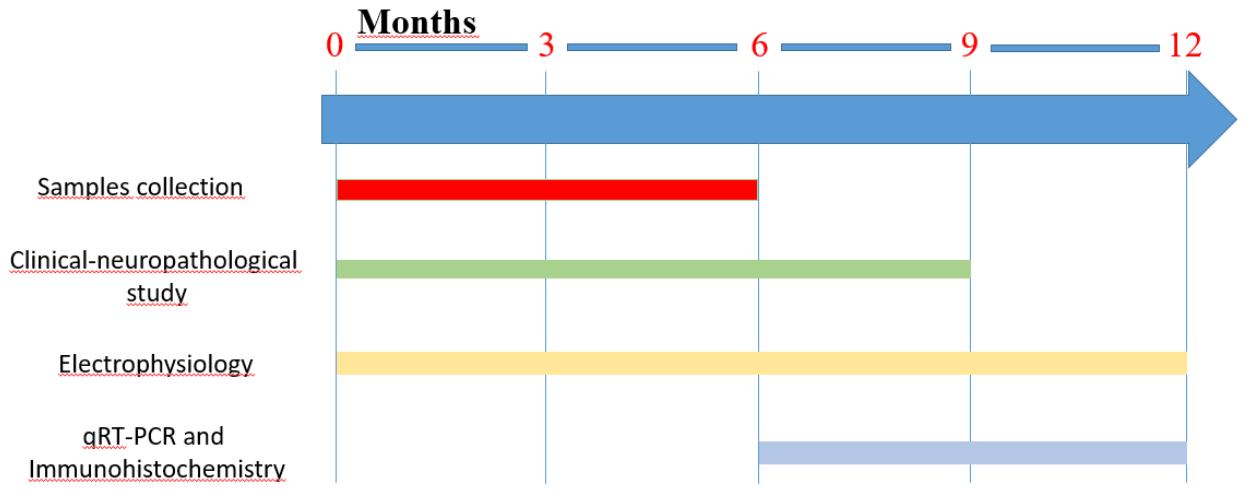

Figure 1. *Gannt chart: Some samples are already available in order to begin the electrophysiology*

experiments as soon as possible. The following molecular biology experiments will be based on the results obtained with the electrophysiological study.

KEYWORDS

Neurodevelopment, Epilepsy, $GABA_AR$, human brain tissues

METHODS

Clinical examination

The clinical cases and controls included in this study will be selected from the databases of Departments of Neuropathology of the Academic Medical Center (AMC, University of Amsterdam) and from NICHD Brain and Tissue Bank for Developmental Disorders.

The TSC cases included in this study will be obtained from the archives of the Departments of Neuropathology of the Academic Medical Center (AMC, University of Amsterdam) and University Medical Center Utrecht (UMCU, Utrecht). The patients will be included in the study only if classified by expert neurologists as "drug-resistant" (*Kwan et al., 2010*) Epilepsy duration will be calculated as the interval in years from the age at seizure onset to the age at tissue sampling. Cases will be included as controls only when there was no known history of epilepsy, normal cortical structure for the corresponding age and without significant brain pathology. Informed consent will be obtained for the use of brain tissue for research purposes. Tissue will be obtained and used in accordance with the Declaration of Helsinki and the AMC Research Code provided by the Medical Ethics Committee and approved by the science committee of the UMC Utrecht Biobank.

Membrane preparation and injection

Tissues will be homogenized using a Teflon glass homogenizer with 2 ml of assay buffer of the following composition (in mM): 200 glycine, 150 NaCl, 50 ethylene glycol tetraacetic acid (EGTA), 50 ethylenediaminetetraacetic acid (EDTA), 300 sucrose; 20 µl protease inhibitors (Sigma Aldrich Inc., St. Louis, MO, USA); pH 9 (adjusted using NaOH). The homogenate will be centrifuged for 15 min at 9,500 g. The supernatant will be collected and centrifuged for 2 h at 105 g at 4°C. The pellet was washed, re-suspended in 5 mM glycine and used directly, or aliquoted and stored at -80°C for later use. In a set of experiments intranuclear injection of human complementary DNA (cDNA) in *Xenopus* oocytes will be performed. For details see *Eusebi et al., 2009, Miledi et al., 2006,*

Electrophysiology in microtransplanted or cDNA injected oocytes

From 12 to 48 h after injections, we will record membrane currents from voltage-clamped Xenopus laevis oocytes using two microelectrodes filled with 3M KCl (*Conti et al., 2011*). The oocytes will be placed in a recording chamber (0.1 mL volume) and perfused continuously with oocyte Ringer solution (OR: NaCl 82.5 mM; KCl 2.5 mM; CaCl 2 2.5 mM; MgCl 2 1 mM; Hepes 5 mM, adjusted to pH 7.4 with NaOH) at room

temperature (20 – 22 C°). The perfusion will be controlled by computer (Biologique RSC-200; Claix, France) through a gravity driven system (9-10 ml/min).

GABA will be always freshly dissolved in OR and where otherwise indicated applied for 4 s to elicit inward currents (I_{GABA}).

When performing dose-response relationships we will test different concentrations of GABA, ranging from 1 μM to 2 mM, and these doses will be determined basing on the total absence of response at the lower dose tested and the presence of a plateau phase at the maximum dose in the curve. GABA pulses will be applied every 4 minutes to avoid receptor desensitization and to determine the half-maximal effect (EC_{50}) our data will be fitted to Hill equations, using least-square routines as previously described (*Palma et al., 2002*)

Unless otherwise stated the recordings will be performed at a holding potential (V_H) of -60 mV. When constructing current-voltage (I-V) relationships the V_H will be stepped for 2-4 minutes at the desired value before applying the neurotransmitter. For these experiments, electrodes will be filled with K-Acetate (3 M – *Conti et al., 2011*) to reduce the leakage of high concentration of Cl- from electrodes into the oocytes. The GABA reversal potential (E_{GABA}) will be determined by fitting the I-V relationships with a regression curve-fitting software (Sigmaplot 12 software). In some experiments GABA will be co-applied with other drugs after a short pre-incubation (L655-708, selective inverse agonist of $GABA_A$Rs containing the α5 subunit; THDOC and DS2, positive modulators of the "tonic" δ-containing $GABA_A$Rs (*Jensen et al., 2013*); Zn2+, which inhibits preferentially γ2-lacking $GABA_A$Rs (*Palma et al., 2007*). In order to test the effect of $GABA_A$R modulators (*i.e.* bendodiazepines) on current amplitude, the cells will be preincubated for 10 seconds with the drugs before the coapplication of 4 s of GABA. In these cases, when reporting data, we will express the percentage of increase or decrease of the GABA-evoked current relative to the first GABA application that preceded the exposition to the drug.

Real time qRT PCR

For RNA isolation, frozen material will be homogenized in Qiazol Lysis Reagent (Qiagen Benelux, Venlo, The Netherlands). Total RNA will be isolated using the miRNeasy Mini kit (Qiagen Benelux, Venlo, The Netherlands). Sample RNA quality control will be performed using an Agilent 2100 bioanalyzer. A RIN (RNA Integrity Number) value greater than 6 comparable in controls and epileptic patients, will be considered as indicator of RNA of good quality and not degraded. To evaluate the expression of $GABA_A$ (α1-5), NKCC1and KCC2 mRNA in control and pathological tissues, five micrograms of total RNA will be reverse-transcribed into cDNA using oligo dT primers (PCR primers, Eurogentec, Belgium), will be designed using the Universal Probe Library of Roche (https://www.roche-applied-science.com) on the basis of the reported mRNA sequences. qPCR and quantification of data will be performed using the computer program LinRegPCR in which linear regression on the Log(fluorescence) per cycle number data is applied to determine the amplification efficiency per sample.

Immunohistochemistry

Immunohistochemistry will be carried out as previously described (*Aronica et al., 2007*) using rabbit polyclonal antibodies. Sections, after incubation with the primary Ab combined will be incubated for 2 h at RT with Alexa Fluor ® 568-conjugated anti-rabbit IgG (1:100, Fisher Scientific, Landsmeer, The Netherlands). Sections will be then analyzed using Leica Confocal Microscope TCS SP8 X (Leica, Son, The Netherlands).

EXPECTED RESULTS

We expect to shed more light on the physiopathological mechanisms underlying GABAergic function in the normal and pathologic development. In particular, our investigation will obtain a characterization of $GABA_A$Rs in neurodevelopmental syndromes through means of electrophysiological, molecular and clinical studies. This integrated approach will grant the possibility of having a complete perspective on the studied phenomena and allow a quicker translation from basic science to the clinical practice. Thus, the results that will stem from this research could address different unsolved issues, namely:

- the relationship between GABAergic dysfunction and developmental failures
- the importance of precise $GABA_A$R-dependant phenomena in "developmental epileptogenesis"
- the possibility of taking advantage specific development-linked molecular targets to devise therapeutic interventions that could not only alleviate the symptoms of the patient, but also hypothetically drive cerebral development towards a more physiologic outcome.

Noteworthy, the knowledge of "developmental epileptogenesis" is still partial and more information about the common physiopathological mechanisms that interact to determine both predisposition to seizures and cognitive impairment are needed. Our study will shed more light on some basic aspects of neurotransmission in these neurodevelopmental diseases and will pave new roads to continue this line of investigation.

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BUDGET

Voce di spesa	**Allocazione fondi**	**Descrizione**
Contratti di ricerca	--	--
Attrezzature (**NOTA**: **Materiale di consumo da laboratorio**)	**10.000 €**	Sali per soluzioni, reagenti (neurotrasmettitori, farmaci, collagenasi).
Servizi informatici e Data-base	--	--
Viaggi;	**3000 €**	Partecipazione a congressi internazionali: AES, ILAE, iscrizione congresso LICE
Costi pubblicazioni *	**2000 €**	Pubblicazione dei prodotti della ricerca su riviste peer-reviewed internazionali
Disseminazione	--	--
TOTALE	**15.000 €**	--

*****The contribution of the Foundation will be mentioned in the acknowledgements each scientific publication that will stem from the project here described.

COLLABORATIONS

This project will involve research centres at “Sapienza” (Rome), in particular the electrophysiology laboratory of prof. Palma and at Academic Medisch Centrum (AMC, Amsterdam, The Netherlands), in particular at the neuropathology unit.

The group led by prof. Aronica (both neuropathologists and basic neuroscientists) will perform the molecular biology experiments and obtain the clinical and neuropathological data of the cases and controls included in this study.

CO-FUNDING

No personal co-funding