Aus der Klinik für Neurologie
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DISSERTATION

Das Mikrobiom und die Rolle antineuronaler Autoantikörper als Risikofaktoren neuropsychiatrischer Symptome

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Abkürzungen und Begriffe

AIE	Autoimmune Enzephalitiden
AK	Antikörper
NMDAR	N-Methyl D-Aspartat Rezeptor
NMDAR-E	N-Methyl D-Aspartat Rezeptor Enzephalitis
VGKC	Voltage Gated Potassium Channel
NMOSD	Neuromyelitis-Optica-Spektrum-Erkrankung
LGI1	Leucine-rich Glioma Inactivated Protein 1
CASPR2	Contactin- assoziiertes- Protein 2
mGluR5	Metabotroper Glutamatrezeptor 5
GAD-AK	Glutamatdecarboxylase Antikörper
NR1	Untereinheit des NMDAR
HEK-Zellen	Human Embryonic Kidney - Zellen
OTU	Operational taxonomic unit
MDS	Multidimensionale Skalierung
MCPD	mothers of children with psychiatric disorders

Das Mikrobiom und die Rolle antineuronaler Autoantikörper als Risikofaktoren neuropsychiatrischer Symptome

ABSTRACT

Autoimmune Enzephalitiden gewinnen in der Neuropsychiatrie zunehmend an Bedeutung. Dennoch sind antineuronale Antikörper nicht ausreichend im differenzialdiagnostischen Denken innerhalb des klinischen Alltags berücksichtigt. In wie weit sie einen Risikofaktor für die Entstehung neuropsychiatrischer Symptome darstellen und solche eventuell sogar bei Kindern durch den Einfluss auf die fetale Hirnentwicklung in der Schwangerschaft auslösen, soll in dieser Dissertation beleuchtet werden. Außerdem soll untersucht werden, ob eine spezifische Mikrobiom-Zusammensetzung einen Risikofaktor für die Entstehung eben dieser antineuronaler Antikörper darstellt.

Im Zentrum dieser Arbeit stehen daher drei Studienansätze.
In einer retrospektiven Kohortenstudie mit N=100 PatientInnen mit verschiedenen Formen einer autoimmunen Enzephalitis stellten antineuronale Antikörper einen Risikofaktor für die Entwicklung neuropsychiatrischer Symptome dar. Zwei Drittel aller Patienten (N=60, 60%) wiesen psychiatrische Auffälligkeiten auf und ein erheblicher Anteil (N=31, 31%) wurde initial psychiatrisch hospitalisiert. Es wurden Symptome und klinische Hinweise identifiziert, die bei psychotischer Symptomatik als Warnhinweise für das zu Grunde liegende autoimmune Geschehen dienen können. Mit der Etablierung dieser „red“ und „yellow flags“ konnte bei einer Patientengruppe hypothetisch eine Reduktion der Zeit bis zur Diagnosestellung von 74 auf 31 Tage (um 58%) erzielt werden. Um herauszufinden, ob antineuronale Antikörper ebenfalls einen Risikofaktor für die Entwicklung neuropsychiatrischer Symptome bei Kindern via diaplazentarer Übertragung in der Schwangerschaft darstellen, wurden in einer prospektiven randomisierten kontrollierten Studie Seren N= 120 gesunder Mütter von Kindern mit psychiatrischen Erkrankungen auf das Vorhandensein von antineuronalen Antikörpern getestet. Dieser Studienansatz war Teil einer grundlagenwissenschaftlichen Arbeit, gemäß der antineuronale Antikörper die fetale Hirnentwicklung in der Schwangerschaft beeinflussen können. Durch die Übertragung von monoklonalen humanen NMDAR-Antikörpern auf schwangere Mäuse konnten beim Nachwuchs verschiedene neuropsychiatrische Symptome ausgelöst werden. Passend dazu ließen sich in den Seren von Müttern mit psychisch erkrankten Kindern leicht erhöhte NR1-IgG-Titer finden. Im dritten Studienansatz wurden in einer retrospektiven Fall-Kontroll Studie Stuhlproben von N=28 PatientInnen mit NMDA-Rezeptor Enzephalitis mittels 16S rDNA Sequenzierung analysiert. Dass eine veränderte Mikrobiom-Zusammensetzung in einem Zusammenhang zum Auftreten der NR1-Antikörper stehen könnte, konnte nicht belegt werden.

Die Zusammensetzung des Mikrobioms von Patienten war vergleichbar mit gesunden Kontrollen.

Schlussfolgernd könnten antineuronale Antikörper sowohl einen Risikofaktor für die Entwicklung neuropsychiatrischer Symptome bei Erwachsenen als auch für die kindliche Hirnentwicklung in der Schwangerschaft darstellen. Ob neuropsychiatrische Erkrankungen wie ADHS oder Schizophrenie dadurch beeinflusst werden, sollte mittels weiterer Studien umfassender erforscht werden. Um eine Verbesserung der Früherkennung autoimmuner Enzephalitiden zu erreichen, könnten die erarbeiteten klinischen Warnzeichen einer AIE („red und yellow flags“) hilfreich sein.

ABSTRACT
Autoimmune encephalitis is becoming increasingly important in neuropsychiatry. Nevertheless, antineuronal antibodies are not sufficiently taken into account in the differential diagnostic thinking within everyday clinical practice. This dissertation will examine the extent to which they represent a risk factor for the development of neuropsychiatric symptoms and possibly even trigger them in children through their influence on fetal brain development during pregnancy. In addition, it will investigate whether a specific microbiome composition represents a risk factor for the development of antineuronal antibodies.

The focus of this work is therefore on three study approaches.
In a retrospective cohort study with N = 100 patients with various forms of autoimmune encephalitis, antineuronal antibodies were a risk factor for the development of neuropsychiatric symptoms. Two thirds of all patients (N = 60, 60%) exhibited psychiatric abnormalities and a significant proportion (N = 31, 31%) were initially hospitalized for psychiatry. Symptoms and clinical indications were identified which, in the case of psychotic symptoms, can raise suspicion for the underlying autoimmune events. With the establishment of these “red” and “yellow flags”, it was possible to hypothetically reduce the time to diagnosis from 74 to 31 days (by 58%) in one patient group. To find out whether antineuronal antibodies also represent a risk factor for the development of neuropsychiatric symptoms in children via diaplacental transmission during pregnancy, sera from 120 healthy mothers of children with psychiatric disorders were tested for the presence of antineuronal antibodies in a prospective randomized controlled trial. This study approach was part of a basic scientific work according to which antineuronal antibodies can influence fetal brain development during pregnancy. By transferring monoclonal human NMDAR antibodies to pregnant mice, various neuropsychiatric symptoms could be triggered in the offspring. In line with this, slightly increased NR1 IgG titers were found in the sera of mothers with mentally ill children.

In the third study approach, stool samples from N = 28 patients with NMDA receptor encephalitis were analyzed using 16S rDNA sequencing in a retrospective case-control study. It could not be proven that an altered microbiome composition could be asscoiated with the development of the NR1 antibodies. The composition of the patient's microbiome was comparable to that of healthy controls.

In conclusion, antineuronal antibodies could represent a risk factor for the development of neuropsychiatric symptoms in adults as well as for brain development in children during pregnancy. Whether neuropsychiatric diseases such as ADHD or schizophrenia are influenced by this should be researched more comprehensively through further studies. In order to improve the early detection of autoimmune encephalitis, the developed clinical warning signs of an AIE ("red and yellow flags") could be helpful.

1. EINLEITUNG

Autoimmune Enzephalitiden (AIE) und deren zugrunde liegenden anti-neuronalen Antikörper (AK) haben in der Neurologie und Psychiatrie seit ihrer Entdeckung rasant an Bedeutung gewonnen (1) (2). Eine kürzlich veröffentlichte Studie zeigte sogar, dass die Prävalenz und Inzidenz der AIE der infektiösen Enzephalitis auf Bevölkerungsebene gleicht (3). Im Dezember 2019 wurden sie in die neuste deutsche S3-Leitlinie für Schizophrenie als Differenzialdiagnose aufgenommen (4). AIE bilden eine heterogene Gruppe neuro-immunologischer Erkrankungen, deren Gemeinsamkeit der Nachweis von Autoantikörpern gegen neuronale Bestandteile sowie das Ansprechen auf immunsuppressive Therapie ist. Die Antikörper können dabei gegen intrazelluläre (v.a. onkoneuronale Antikörper) oder extrazelluläre Antigene gerichtet sein (5). Erstere gehen meist mit einem Tumor einher (paraneoplastisches Syndrom) (6). Letztere sind aufgrund ihrer direkt pathogenen Wirkung häufig mit psychiatrischen Veränderungen assoziiert (7). Am häufigsten sind AIE mit Antikörpern gegen in der Zellmembran lokalisierte N-Methyl D-Aspartat Rezeptoren (NMDAR) oder den voltage gated potassium channel (VGKC) -Komplex. Die NMDAR Enzephalitis (NMDAR-E) wurde erstmalig im Jahr 2007 beschrieben. Dadurch konnte zum ersten Mal ein spezifisches autoimmunes Geschehen mit der Entstehung einer akuten Hirnentzündung in Zusammenhang gebracht werden, die sich vor allem durch schnell fortschreitende psychiatrische Symptome, kognitive Beeinträchtigung und epileptische Anfälle zeigt (8). Die Diagnosestellung erfolgt mittels Nachweis von IgG -Autoantikörpern im Liquor gegen die NR1- Untereinheit des NMDAR. Aufgrund der vielfältigen Erscheinungsformen und des uneinheitlichen klinischen Bildes werden AIE oft deutlich verzögert diagnostiziert. Ihrer frühzeitigen Erkennung kommt jedoch große Bedeutung zu, da durch eine Immuntherapie, vor

allem im Falle des Vorliegens extrazellulär bindender Antikörper, eine kausale und häufig erfolgreiche Behandlungsmöglichkeit besteht (9) (10).

Anti-neuronale AK scheinen nicht nur Einflüsse auf das adulte Hirn zu haben, sondern auch die kindliche Hirnentwicklung in der Schwangerschaft zu beeinflussen, wie auch durch andere autoimmune Erkrankungen gezeigt werden konnte. Zum Beispiel führen bei der Myasthenia Gravis die Antikörper gegen embryonale Acetylcholinrezeptoren nach diaplazentarer Übertragung zu schweren Entwicklungsstörungen, wie dem fetalen Acetylcholinrezeptor-Inaktivierungssyndrom (FARIS) oder einer Arthrogryposis multiplex congenita (11). Im Rahmen der passiven Immunität schütz die Mutter ihr Kind durch den Transfer von Immunglobulinen (IgG). IgG ist dabei der einzige AK, der die Plazentaschranke in signifikanter Menge überqueren kann. Dieser Mechanismus beginnt in der 13. SSW. Zu diesem Zeitpunkt ist die Blut-Hirn-Schranke des Feten noch nicht vollständig ausgebildet und somit auch für potentiell schädliche Antikörper durchlässig (12). Die Auswirkung von NMDAR-AK in der Schwangerschaft auf das Neugeborene wurde in Fallstudien beschrieben und reicht von vollkommener Gesundheit bis hin zum Tod (13) (14). In einem systematischen Review von 13 schwangeren Patientinnen mit NMDAR-E zeigten drei der Neugeborenen neurologische Defizite, sieben waren gesund und 3 verstarben (15). NMDA-Rezeptoren sind während der kindlichen Hirnentwicklung für das axonale und dendritische Wachstum, sowie den Erhalt von Nervenzellen unabdingbar. Auf Grund der extrazellulären Lokalisation (Zellmembran) des Antigens bei der NMDAR-E können die Antikörper direkt an das Protein binden und sowohl die Anzahl der NMDA Rezeptoren minimieren, als auch ihre Funktion stören (16). Die Antikörper sind damit direkt pathogen und stellen womöglich ein besonders hohes Risiko dar. Da es sich bei den Proteinen um Ionenkanäle handelt, kommt es zu elektrophysiologischen Veränderungen, sowie Störungen der synaptischen Übertragung und der neuronalen Plastizität (17) (18). In verschiedenen Studien konnte eine hohe NMDAR-AK-Seroprävalenz bei Gesunden festgestellt werden (19) (20), was mutmaßlich auch für asymptomatische Schwangeren gelten dürfte, wenngleich entsprechende Studien noch fehlen. Dies lässt vermuten, dass asymptomatische Mütter diese Antikörper möglicherweise diaplazentar übertragen und so eine mögliche Pathologie beim Kind verursacht werden könnte.

Die der AIE - und der NMDAR-E im Speziellen - zugrunde liegenden pathophysiologischen Mechanismen sind bis dato nur teils verstanden. Primärer Auslöser für die AK-Bildung scheint bei einem Teil der Patienten mit NMDAR-E ein Ovarialteratom zu sein, das das Ziel-Antigen exprimiert und präsentiert (18) (21). Eine Assoziation mit anderen Tumoren scheint im Falle der NMDAR-E eher selten vorzuliegen. Kann kein Ovarialtumor nachgewiesen werden, herrscht

bisher Unklarheit über weitere Auslöser. Möglich sind Virusinfektionen wie v.a. HSV-1 (22) (23) (24), eine genetische Prädisposition (25) (26) oder Impfungen (18) (27).
In einer kürzlich veröffentlichten Studie konnte eine Überrepräsentation des Darmbakteriums Clostridium perfringens bei Patienten mit einer Neuromyelitis-optica-Spektrum-Erkrankung (NMOSD) festgestellt werden, was einen möglichen pathophysiologischen Zusammenhang vermuten lässt (28). Ähnlich wie die NMDAR-E sind die NMOSD eine Gruppe neuro-autoimmuner Erkrankungen, bei denen sich Autoantikörper gegen Proteine im Gehirn und Rückenmark richten. In diesem Fall gegen das Wasserkanalprotein Aquaporin 4 in Astrozyten. Für die Autoimmunantwort spielen im Falle der NMOSD T-Zellen offenbar eine wichtige Rolle (29). Laut Cree el. al ist eine Kreuzreaktion zwischen diesen T-Zellen und einem ABC Transporter des Darm-Bakteriums Clostridium perfringens möglicherweise für die Entwicklung der Antikörper mit verantwortlich. Aufgrund der bisher wenig bekannten Risikofaktoren für die Entwicklung einer NMDAR-E ist die potentielle Rolle des Mikrobioms in der Genese daher besonders interessant und bisher noch nicht erforscht.

2. FRAGESTELLUNG

Im Rahmen meiner Dissertation möchte ich daher die folgenden Hypothesen prüfen:
Hypothese 1: Das Vorhandensein anti-neuronaler Antikörper ist ein Risikofaktor für die Entwicklung neuropsychiatrischer Symptome. Weil das nicht ausreichend bekannt ist, könnte es zur verzögerten Diagnosestellung kommen, vor allem in der Psychiatrie.
Hypothese 2: Antineuronale AK stellen nicht nur einen Risikofaktor für die Entwicklung neuropsychiatrischer Symptome bei Erwachsenen dar, sondern beeinflussen auch die fetale Hirnentwicklung in der Schwangerschaft.
Hypothese 3: Die Zusammensetzung des Mikrobioms steht in einem pathophysiologischen Zusammenhang zum Auftreten der anti-neuronalen AK bei der NMDAR- E.

Im Spektrum dieser Arbeit stehen somit drei zentrale Fragestellungen, welche im Rahmen der zugrunde liegenden drei Publikationen erörtert wurden:

1) **Analog Studie I: retrospektive Kohortenstudie (Erstautor)**
   Gibt es typische neuropsychiatrische Symptome und klinische Erkennungsmerkmale bei Erwachsenen, die mit dem Vorhandensein anti-neuronaler AK einhergehen oder auf sie zurückzuführen sind?
2) **Analog Studie II (Teilarm einer größeren grundlagenwissenschaftlichen Studie): prospektive randomisierte kontrollierte Studie (Co-Autor)**
   Finden sich häufiger antineuronale Antikörper im Blut von Müttern, die ein Kind mit einer neuropsychiatrischen Erkrankung haben?

3) **Analog Studie III: retrospektive Fall-Kontroll Studie (Erstautor)**
   Ist eine veränderte Mikrobiom-Zusammensetzung ein Risikofaktor für die Entstehung antineuronaler Antikörper bei der NMDAR-E und kann somit zu neuropsychiatrischen Symptomen führen?

3. METHODIK

3.1 Patientenrekrutierung und Datensammlung der Studie I

Der nachfolgende Text entspricht zu Teilen dem Methodikteil der Originalpublikation. Übersetzung durch die Autorin.

In der Zeit von Mai bis Oktober 2016 wurden N=100 Patienten mit verschiedenen Formen der AIE im Zentrum für Autoimmune Encephalitis der Charité rekrutiert und in folgende Gruppen unterteilt:

1) Anti NMDAR Enzephalitis (N=53). Definiert durch das typische klinische Bild und den Nachweis von IgG-NMDAR AK im Liquor.
2) Extrazelluläre non-NMDAR AK (N=24): Patienten mit AK gegen LGI1 (N=14), CASPR2 (N=4), mGluR5 (N=2), Glycin-Rezeptor (N=1), ein unbekanntes Epitop (N=3).
3) Intrazelluläre AK (N=23): Patienten mit GAD-AK (N=9) oder onkoneuronalen AK (N=14).

Vor definitivem Studieneinschluss unterzeichneten alle Teilnehmer eine Einverständniserklärung. Die meisten Patienten waren im Rahmen des Krankheitsverlaufes in der Abteilung für Neurologie der Charité hospitalisiert. Ich analysierte ihre Krankengeschichten retrospektiv und erfragte zusätzliche klinische Daten telefonisch oder per E-Mail. Die folgenden Informationen wurden systematisch aus den Krankheitsgeschichten herausgearbeitet: Alter, Geschlecht, Krankheitsbeginn, neurologische und psychiatrische Symptome zum Zeitpunkt des Krankheitsbeginns und im Verlauf, Details der psychiatrischen Hospitalisierung, die zu AK-Suche führenden Symptome, Datum der Diagnosestellung und die Zeit zwischen dem Auftreten erster Symptome und Diagnose. Zusätzlich fügte ich alle Patientendaten nach entsprechender Einverständniserklärung systematisch in die Datenbank des Netzwerkes „GENERATE“ ein. Das „German Network for Research on Autoimmune Encephalitis“ ist ein nationales Patientenregister, das dem Datenaustausch zwischen verschiedenen spezialisierten Zentren dient – und somit als Basis klinischer Forschung fungiert.

3.2 Studiendesign der Studie II

Bei der gesamten publizierten Studie handelt es sich um einen Tierversuch. Der von Katharina Lang und mir durchgeführte Teilansatz, der zur Überprüfung einer der Hypothesen dient, erfolgte als prospektive, randomisierte, kontrollierte Studie. Dazu wurde Serum von 120

gesunden Müttern von Kindern mit psychiatrischen Erkrankungen, die in der Kinderpsychiatrie des Vivantes Klinikum in Berlin Friedrichshain hospitalisiert waren, gesammelt. Als Kontrollgruppe galten die Seren von 105 Müttern mit gesunden Kindern aus dem familiären und befreundeten Umkreis.

Die IgG-Konzentration der monoklonalen humanen Antikörper wurde mittels ELISA (Mabtech) im Labor des Zentrums für Autoimmune Encephalitis der Charité bestimmt.

Im Rahmen der gesamten Tierstudie wurden 8-10 Wochen alten Mäusen am Schwangerschaftstag E13 und E17 entweder 240 ug menschliche monoklonale AK intraperitoneal injiziert oder sie wurden nicht behandelt (N=6). Bei den AK handelte es sich entweder um einen hoch affinen NR1- IgG AK (N= 47; NRI) oder um nicht reaktive Klone (N= 40; CTL). Die rekombinanten monoklonalen NR1-reaktiven IgG AK waren zuvor aus Immunzellen von 2 Patientinnen mit aktiver NMDAR-E gewonnen worden (17). Bei allen AK handelte es sich um IgG1 AK, die die Plazentaschranke am besten überqueren. Die neugeborenen Mäuse wurden in gemischten Gruppen von 2-5 Mäusen beider Geschlechter aufgezogen. Eine Blutentnahme erfolgt am postnatalen Tag (P) 0, P7, P10 und P14, die AK-Titer wurden mittels ELISA bestimmt.

3.3 Studienpopulation und Rekrutierung der Studie III

Der nachfolgende Text entspricht zu Teilen dem Methodikteil der Originalpublikation. Übersetzung durch die Autorin.

Im Frühling 2017 wurden 28 Patienten mit NMDAR-E im Zentrum für Autoimmune Encephalitis der Charité rekrutiert. 5 Patienten und 3 Kontrollprobanden mussten auf Grund einer antibiotischen Therapie innerhalb der letzten 6 Wochen vor dem Rekrutierungszeitpunkt aus der Studie ausgeschlossen werden. 2 (9%) der verbleibenden Patienten befanden sich in einer akuten Krankheitsphase, 6 (26%) in Teilremission und 15 (65%) waren vollständig genesen. Fast alle Patienten waren weiblich (N=21, 91%), Geschlecht galt jedoch nicht als Kovariable. Bei 3 (13%) der Patienten war im Zuge der Diagnostik ein Ovarialteratom nachgewiesen und zum Studienzeitpunkt bereits entfernt worden. Alle Patienten hatte eine immunsuppressive Therapie erhalten. 5 (22%) waren zum Zeitpunkt der Stuhlentnahme noch unter Therapie. Die Kontrollgruppe bestand aus fallbezogenen, gesunden Probanden bestmöglich aus demselben Haushalt stammend, mit ähnlichen Lebensgewohnheiten und vergleichbarer Diät.
Studienprotokoll: Alle Studienteilnehmer erhielten postalisch je ein Paket mit den notwendigen Utensilien und einem standardisierten Fragebogen. Stuhlproben wurden zu Hause von den Patienten und Kontrollpersonen gesammelt und innerhalb von 24 Stunden bei

Zimmertemperatur an das Institut für klinische Molekularbiologie (IKMB) der Christian- Albrechts Universität Kiel verschickt. Dort wurden sie bei einer Temperatur von -80° gelagert.
Alle Studienteilnehmer füllten einen standardisierten Fragebogen aus, um eine gute Vergleichbarkeit der Probanden zu gewährleisten. Der Fragebogen beinhaltete umfassenden Angaben zu demografischen Daten, einem Defäkationsprotokoll, Diätgewohnheiten, Vorerkrankungen, aktueller Medikation, aktuellem Gesundheitsstatus, antibiotischer Therapie innerhalb der letzten 12 Monate, Informationen zu Rauchgewohnheiten und dem generellen Aktivitätslevel.
Probenaufarbeitung und statistische Analyse: Die DNA-Extraktion und Sequenzierung erfolgte im Institut für klinische Molekularbiologie (IKMB) der Christian- Albrechts Universität Kiel. Gleiches galt für die statistische Analyse, bei die Alpha- (intra-individuell) und Beta-Diversität (inter-individuell) bestimmt wurde. Die Sequenzierung erfolgte mittels 16S rDNA Sequenzierung, die Klassifikation in operational taxonomic units (OTU). Alpha-Diversität Indices beinhalteten den Shannon-Index (entsprechend der Artenanzahl und Abundanz) und dem Chao1 Index (den Artenreichtum wiederspiegelnd). Die Beta-Diversität wurde mittels multidimensionaler Skalierung (MDS) dargestellt, wodurch die Un-/Ähnlichkeit aller Probanden in Bezug auf die inter-individuelle Mikrobiom- Vielfalt gezeigt wurde.

4. ERGEBNISSE

4.1. Studie 1: **Klinische Erkennungsmerkmale für eine frühzeitige Diagnosestellung autoimmuner Enzephalitiden in der Psychiatrie**

4.1.1 *Erstsymptome autoimmuner Enzephalitiden*

Die Patienten der drei definierten Subgruppen zeigten alle neuro-psychiatrische Symptome zum Zeitpunkt des Krankheitsbeginns. Insgesamt 2/3 aller Patienten (N=60, 60%) wiesen psychiatrische Symptome auf und ein erheblicher Anteil (N=31, 31%) wurde initial in der Psychiatrie hospitalisiert. Die Art der Symptome unterschied sich in den untersuchten Gruppen deutlich. Alle Patienten mit NMDAR-AK (N=53) fielen durch psychotische Symptome auf und zeigten zusätzlich neurologische Auffälligkeiten oder aber entwickelten diese im Verlauf. Zu den initial psychiatrischen Symptomen zählten primär akute Verhaltensänderungen (N=46; 87%), Halluzinationen (N=23, 48%), paranoider Wahn (N=13, 26%) und Gedächtnisverlust (N=11, 21%). Neurologische Symptome waren in erster Linie epileptische Anfälle (N=10, 19), Sprachdefizite (N=10, 19%), Dyskinesien (N=7, 13%) und Kopfschmerzen (N=9, 17%).
Patienten der non-NMDAR Gruppe präsentierten ähnliche psychiatrische Symptome, wiesen oftmals aber auch psychosomatische Auffälligkeiten auf. Die neurologische Symptomatik war eher von faziobrachialen dystonen Anfällen (N=7, 29%) oder sensomotorischen Ausfällen (N=7,

29%) gekennzeichnet. Patienten mit dem Nachweis intrazellulär bindender AK präsentierten deutlich seltener psychiatrische Symptome. Der überwiegende Teil der Symptome war neurologischer Natur und manifestierte sich in Form von sensomotorischen Defiziten (N=13, 13%), cerebellären Ataxien (N=7, 30%), Bewegungsstörungen (N=3, 13%) und generalisierten tonisch-klonischen Anfällen (N=3, 13%).

Tabelle 1: Initiale klinische Symptome der 100 Patienten

TABLE 2 | Presenting clinical symptoms in all 100 patients.

Initial signs and symptoms	All patients (100)	NMDAR (53)	Non-NMDAR (24)	Intracellular antigens (23)
Psychiatric				
Acute behavioral changes	56 (56%)	46 (87%)	7 (29%)	3 (13%)
Hallucinations (visual, auditory)	25 (25%)	23 (43%)	1 (4%)	
Memory deficits (retro- and anterograde amnesia)	22 (22%)	11 (21%)	8 (33%)	4 (17%)
Confusion/aggression	18 (18%)	11 (21%)	6 (25%)	1 (4%)
Paranoid delusions	17 (17%)	13 (26%)	2 (8%)	1 (4%)
Depressed mood	13 (13%)	10 (19%)	4 (16%)	1 (4%)
Catatonia	10 (10%)	10 (19%)		
Mutism	8 (8%)	8 (15%)		
Anorexia	1 (1%)	1 (2%)		
Any of the above symptoms	65 (65%)	53 (100%)	14 (58%)	7 (30%)
Neurological				
Sensorimotor deficits	30 (30%)	8 (15%)	7 (29%)	13 (57%)
Seizures		10 (19%)	2 (8%)	5
Generalized tonic-clonic	13 (13%)	9 (17%)	1 (4%)	3 (13%)
Focal	4 (4%)	1 (2%)	1 (4%)	2 (9%)
Faciobrachial dystonic seizures	7 (7%)		7 (29%)	
Speech dysfunction (pressured speech, verbal reduction)	15 (15%)	10 (19%)	4 (16%)	
Movement disorders	11 (11%)	7 (13%)	1 (4%)	3 (13%)
Headache	12 (12%)	9 (17%)	1 (4%)	2 (9%)
Reduced levels of consciousness	7 (7%)	5 (9%)	2 (8%)	
Paralysis	7 (7%)	4 (8%)	1 (4%)	2 (9%)
Cerebellar ataxia	10 (10%)	1 (2%)	3 (12%)	7 (30%)
Diplopia	7 (7%)	3 (6%)		4 (17%)
Any of the above symptoms	67 (67%)	39 (74%)	20 (83%)	20 (87%)

Veröffentlicht in: Herken J and Prüss H (2017) Red Flags: Clinical Signs for Identifying Autoimmune Encephalitis in Psychiatric Patients. *Front. Psychiatry* 8:25. DOI: 10.3389/fpsyt.2017.00025

4.1.2 *Warnsignale für das Vorliegen autoimmuner Antikörper*

Als Nächstes untersuchte ich, welche Befunde bei den in der Psychiatrie hospitalisierten Patienten ein autoimmunes Geschehen vermuten ließen, sodass eine AK-Suche im Liquor veranlasst wurde. Tatsächlich konnten bestimmte klinische Konstellationen herausgearbeitet werden, die primär zu einer Antikörpersuche führten. Diese wurden daraufhin in „yellow flags“ und „red flags“ unterteilt und anhand ihrer Wichtigkeit als Warnhinweis eingeordnet (Tabelle 3). In der Gruppe der NMDAR-E wurde ein autoimmunes Geschehen oftmals vermutet, nachdem eine Liquorpleozytose (für gewöhnlich > 5-100 per mm3) im Rahmen der Diagnostik bei ursprünglichem Verdacht auf eine infektiöse Enzephalitis festgestellt wurde, jedoch kein Erregernachweis gelang (N=12, 27%). In allen 3 Gruppen führten epileptische Anfälle zu einer Liquorpunktion mit AK-Diagnostik (N=14, 14%). Gleiches galt für auffällige EEG- (z.B. Spikes, rhythmische Verlangsamung, fokale Pathologien oder extreme delta brush) oder MRT Befunde (z.B. mesiotemporale Hyperintensität). Im Fall der NMDAR-E mit initial primär psychotischen Symptomen führten zusätzliche neurologische Auffälligkeiten, wie z.B. fokal neurologische Defizite, (N=4, 7%), Aphasien (N=4, 7%) oder Dysarthrien (N=3, 6%) zu Antikörperdiagnostik.

Im Gegensatz dazu zeigten die Patienten der nicht-NMDAR-AK Gruppe vor allem faziobrachial betonte Anfälle (N=3, 12%), sensorische Missempfindungen (n=2, 8%) oder eine unerklärbare, nicht medikamentös verursachte Hyponatriämie (N=2, 8%). In weiteren Fällen führte eine erfolglose antipsychotische (N= 5, 5%) oder antiepileptische Therapie (N= 2, 2%) zur Verdachtsdiagnose eines autoimmunen Geschehens.

Tabelle 2: Klinische Symptome, die eine zu Grunde liegende NMDAR-E vermuten ließen.

TABLE 3 | Clinical symptoms and constellations that led to the determination of anti-neuronal antibodies in all 100 patients.

Symptoms	All patients (100)	NMDAR (53)	Non-NMDAR (24)	Intracellular antigens (23)
Epileptic seizures	14 (14%)	10 (19%)	2 (8%)	2 (8%)
Cerebrospinal fluid (CSF) abnormalities[a] and absent evidence for infectious encephalitis	13 (13%)	12 (27%)	1 (4%)	
Abnormal postures or movements	4 (4%)	4 (7%)		
Reduced levels of consciousness	4 (4%)	4 (7%)		
Aphasia or dysarthria	3 (3%)	3 (6%)		
Lack of improvement with antipsychotics	5 (5%)	4 (7%)	1 (4%)	
Autonomic instability	2 (2%)	2 (4%)		
Suspicious MRI or EEG findings	10 (10%)	3 (6%)	5 (20%)	2 (8%)
Steroid-responsive autoimmune thyroiditis	3 (3%)	2 (4%)		1 (4%)
Lack of improvement with antiepileptic medication	2 (2%)	1 (2%)	1 (4%)	
Focal neurological deficits	3 (3%)	1 (2%)	1 (4%)	1 (4%)
Sensory deficits	3 (3%)	1 (2%)	2 (8%)	
Rapidly progressing psychosis	4 (4%)	1 (2%)	2 (8%)	1 (4%)
Suggested by patients or families	3 (3%)	3 (6%)		
Positive effect of *ex juvantibus* immunotherapy	2 (2%)		1 (4%)	1 (4%)
Faciobrachial dystonic seizures	3 (3%)		3 (12%)	
Neuromyotonia	1 (1%)		1 (4%)	
Cerebellar ataxia	8 (8%)		2 (8%)	6 (26%)
Hyponatremia	2 (2%)		2 (8%)	
Paresthesia or malignant tumor[b]	7 (7%)			7 (30%)

[a]*Increased white blood cell count or CSF-specific oligoclonal bands.*
[b]*Small-cell lung cancer, testicular seminoma.*

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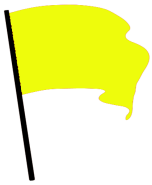
Tabelle 3: Warnzeichen, die auf eine autoimmune Genese bei Newonset-Psychosen hinweisen

Yellow Flags 	• Bewusstseinsbeeinträchtigung • Abnormale Bewegungen oder Körperhaltungen (orofaziale oder ExtremitätenDyskinesien) • Autonome Instabilität • Fokal neurologische Defizite • Aphasie oder Dysarthrie • Rasch voranschreitende Psychose (trotz Therapie) • Hyponatriämie • Katatonie • Kopfschmerz • Andere autoimmune Erkrankungen (z.B. Thyreoiditis)
Red Flags 	• Liquorpleozytose oder Oligoklonale Banden im Liquor ohne Hinweis für eine Infektion • Epileptische Anfälle • FBDS (faziobrachiale dystone Anfälle) • V.a. malignes neuroleptisches Syndrom • MRT Auffälligkeiten (mesiotemporale Hyperintensität) • EEG Auffälligkeiten (fokale oder diffuse Verlangsamung oder disorganized activity, epileptische Aktivität oder extreme delta brush)

Durch die Autorin übersetzt und veröffentlicht in: Herken J and Prüss H (2017) Red Flags: Clinical Signs for Identifying Autoimmune Encephalitis in Psychiatric Patients. *Front. Psychiatry* 8:25. DOI: 10.3389/fpsyt.2017.00025

4.1.3 *Analyse der Zeit zwischen Symptombeginn und Erstdiagnose*

Da ein früher Therapiebeginn im Fall der AIE mit Krankheitsremission assoziiert ist, analysierte ich als Nächstes die Zeit zwischen Symptombeginn und Diagnosezeitpunkt, um einen eventuellen Zeitverlust nachzuweisen. Eingeschlossen wurden hierfür die initial in der Psychiatrie hospitalisierten Patienten (N=31). Zwischen 2007 und 2012 betrug die Tagesanzahl bis zur Diagnosestellung im Mittel 483 Tage. Zwischen den Jahren 2013 und 2016 dagegen 74 Tage. Der große Unterschied kann vermutlich auf den wachsenden Bekanntheitsgrad der AIE zurückzuführen sein.

4.1.4 *Frühere Diagnosestellung bei Berücksichtigung definierter Warnhinweise*

Als Nächstes versuchte ich die erarbeiteten „yellow flags“ und „red flags“ retrospektiv in der Krankengeschichte der zuvor eingeschlossenen Patienten zu suchen. Einige Warnsignale ließen sich lange vor Zeitpunkt der Diagnosestellung auffinden. Im Anschluss wurde die potentielle Zeitersparnis berechnet, hätte das erstmalige Auftreten eines dieser Warnhinweise zu einer AK-Suche im Liquor geführt. Dabei ließ sich für die Patientengruppe mit der Erstdiagnose einer AIE zwischen 2013 und 2016 exemplarisch eine Reduktion von 74 auf 31 Tage (um 58%) nachweisen.

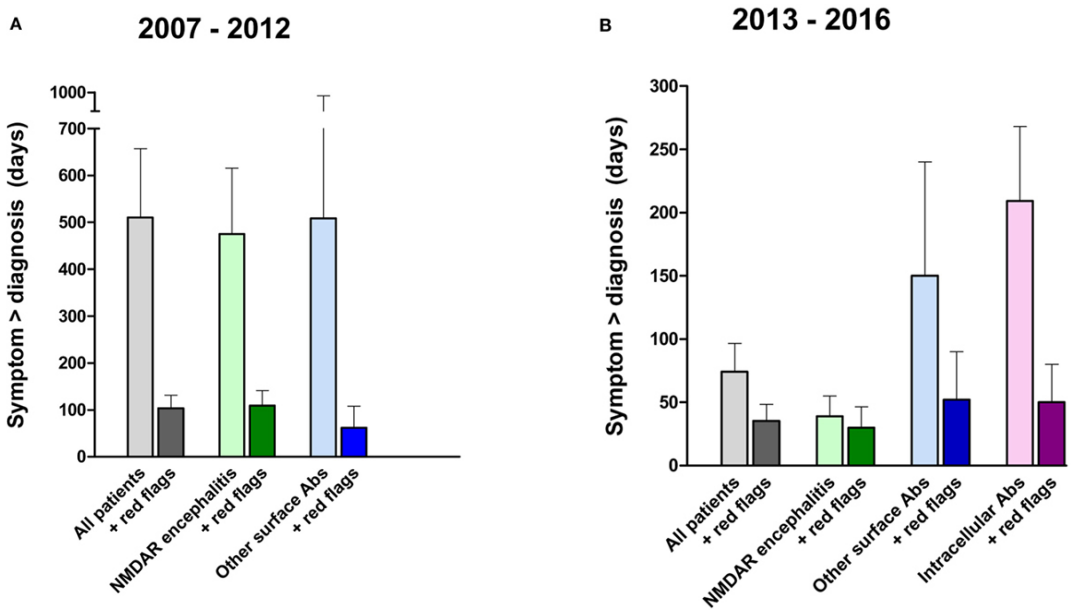


Abbildung 1: Zeit zwischen dem Beginn erster klinischer Symptome und der Diagnosestellung einer autoimmunen Enzephalitis. Verglichen wurden PatientInnen mit Krankheitsmanifestation zwischen 2007 bis 2012 (A) und 2012 bis 2016 (B). Die zeitliche Verzögerung von Symptombeginn bis zur Diagnosestellung (helle Farben) konnte in den letzten Jahren vermutlich durch einen wachsenden Bekanntheitsgrad der Erkrankung reduziert werden (man beachte die verschiedenen y-Achsen). Mittels Suche nach „red flag“ Kriterien in den Krankengeschichten der gleichen PatientInnen konnte eine hypothetische Reduktion der Zeit bis zur Antikörpersuche gezeigt werden (dunkle Farben).
Veröffentlicht in: Herken J and Prüss H (2017) Red Flags: Clinical Signs for Identifying Autoimmune Encephalitis in Psychiatric Patients. Front. Psychiatry 8:25. DOI: 10.3389/fpsyt.2017.00025

TABLE 2. Cohort Characteristics of Matched Mothers of Children with Psychiatric Disorders (n = 120, Mean Age = 42.3 ± 7.8 Years) and Healthy Control Mothers of Unaffected Children (n = 105, Mean Age = 43.6 ± 9.7 Years)

ICD-10	Disorder Classification	Cases	M	F
F1	Mental and behavioral disorders due to psychoactive substance use	2	1	1
F2	Schizophrenia, schizotypal, and delusional disorders	2	1	1
F3	Mood (affective) disorders	22	6	16
F4	Neurotic, stress-related, and somatoform disorders	19	11	8
F5	Behavioral syndromes associated with physiological disturbances and physical factors	1	0	1
F6	Disorders of adult personality and behavior	2	0	2
F7	Mental retardation	1	1	0
F84	Pervasive developmental disorders	8	8	0
F90	Attention-deficit hyperactivity disorders	32	24	8
F91; F92	Conduct disorders; mixed disorders of conduct and emotions	20	11	9
F93; F94; F98	Emotional disorders with onset specific to childhood; disorders of social functioning with onset specific to childhood and adolescence; other behavioral and emotional disorders with onset usually occurring in childhood and adolescence	12	9	3

Diagnoses of psychiatric disorders were coded according to the German modification of the ICD-10 as provided within health insurance data.
F = female; ICD-10 = International Classification of Diseases and Related Health Problems, 10th Revision; M = male.

Abbildung 2: veröffentlicht in:
Jurek, B., Chayka, M., Kreye, J., Lang, K., Kraus, L., Fidzinski, P., Kornau, H. C., Dao, L. M., Herken, J.,Wenke, N. K., & Long, M. (2019). Human gestational N-methyl-d-aspartate receptor autoantibodies impair neonatal murine brain function. *Annals of Neurology, 86*, 656–670. DOI: (1) Mit Genehmigung von „John Wiley and Sons".

4.2 Studie 2: **Diaplazentare Übertragung von NMDAR-Auto-AK führt potentiell zu kindlichen Gehirnentwicklungsstörungen**

4.2.1 Diaplazentare Übertragung und synaptische Bindung humaner NR1 AK im Mausmodell [1]

Humane monoklonale NR1 IgG1 AK und nicht-reaktive CTL AK wurden trächtigen Mäusen an Gestationstag E13 und E17 intraperitoneal injiziert (je 240 ug). Anschließend wurden AK-verursachte Effekte an den Neugeborenen in verschiedenen Entwicklungsphasen beobachtet.
Mittels ELISA Serum-Quantifizierung konnte bestätigt werden, dass die humanen Immunglobuline diaplazentar übertragen worden waren und sich im Serum der Neugeborenen angereichert hatten. Injektionen, die erst nach der Geburt durchgeführt wurden, zeigten zudem eine Übertragung über die Muttermilch. Die monoklonalen humanen NR1 AK zeigten das charakteristische Bindungsverhalten an bestimmten Hirnstrukturen, wie zum Beispiel den Körnerzellen im Cerebellum. Eine solche Bindung konnte bei den Kontroll-AK wie erwartet nicht gezeigt werden. Zudem lies sich mittels immunhistochemischer Färbung des fetalen Maus-Hirngewebes die Bindung der NR1-AK an den NMDAR nachweisen.

4.2.2 Serum Anti-NR1-AK Titer bei Müttern psychisch erkrankten Kindern

Um herauszufinden, ob Mütter psychisch erkrankter Kinder (MCPD) im Vergleich zu Müttern mit gesunden Kindern (CTLM) höhere AK Titer im Serum aufwiesen, wurden Serumproben von 120 MCPD mit derer 105 CTLM verglichen. Die Kinder der MCPD litten an verschiedenen psychischen Erkrankungen, zu diesen zählten vor allem ADHS (N =32), affektive Störungen (N= 22), neurotische und somatoforme Störungen (N = 19) und emotionale Störungen (N =12) (Abbildung 2). Die Probenkollektion

erfolgte 4-20 Jahre nach der Schwangerschaft. Mittels Durchflusszytometrie konnten leicht erhöhte NR1 IgG Titer bei der Gruppe der MCPD gefunden werden (p= 0.038). Das Serum von Patienten mit manifester NMDAR-Enzephalitis diente als Kontrolle (N=2). Die höchsten Titer gingen dabei mit keiner spezifischen Krankheit der Kinder einher.
Als nächstes wurde untersucht, ob eine maternale Injektion der humanen NR1 AK unter der Nachweisgrenze trotzdem zu erhöhten IgG Titern in den Seren der Neugeborenen führen kann.[1] Dazu wurden den Müttern die humanen IgG AK in schrittweiser Titration injiziert. Und tatsächlich zeigte sich in einer Gruppe eine Diskrepanz in der Seronegativität/-positivität zwischen Mutter und Nachkommen. Dieses Ergebnis lässt vermuten, dass eine Seronegativität einer schwangeren Frau die Anreicherung von NR1 AK im fetalen Kreislauf nicht ausschließt.

4.2.3 Erhöhte Mortalität und Verhaltensänderungen bei NR1-AK ausgesetzten Neugeborenen [1]

Die Mäuse, die während der embryonalen Entwicklung NR1-AK ausgesetzt waren, zeigten signifikant reduzierte Überlebensraten innerhalb der ersten 3 postnatalen Tage (66.7%), als die, die mit CTL-AK in Kontakt gekommen waren (100%). Zudem zeigten sich physiologische Auffälligkeiten, wie ein erhöhter arterieller pH und ein reduziertes Körpergewicht. Die mittels MRT verglichenen Hirnvolumina zeigten unmittelbar postnatal keine Unterschiede. 8 Wochen bis 10 Monate nach Geburt fiel jedoch eine signifikante Volumenreduktion v.a. des Cerebellums, des Hirnstamms und des Mittelhirns bei den NR1-AK-behandeltenMäusen auf. Zusätzlich zeigten die Mäuse der NR1 Gruppe Verhaltensänderungen in Form von beeinträchtigten postnatalen Reflexen und einer deutlich reduzierten Angstschwelle.

[1] An diesem Teilbereich von Studie II war ich nicht direkt beteiligt, das Ergebnis stützt jedoch meine eingangs formulierte Hypothese, weshalb es an dieser Stelle aufgeführt ist.

4.3 Studie 3: Rolle des Mikrobioms in der Pathogenese anti-neuronaler AK bei autoimmunen Enzephalitiden

4.3.1 Studienpopulation:

23 Patienten mit NMDAR-Enzephalitis und 24 Kontrollprobanden wurden im Zentrum für autoimmune Enzephalitiden der Charité rekrutiert. 43 von 47 Probanden waren weiblich. Es gab keine Unterschiede zwischen den Gruppen bezüglich Nikotinabusus, Übergewicht, Wohnort, Stuhlgang zum Zeitpunkt der Stuhlentnahme (Frequenz pro Woche und Konsistenz), Auftreten von Hämatochezie, gelegentlichen Bauchschmerzen, Ernährung oder der Reiseanamnese.
5 (22%) der NMDAR Enzephalitis Patienten mit Krankheitsaktivität waren zum Zeitpunkt der Stuhlprobengewinnung unter immunsuppressiver oder neuroleptischer Therapie. 2 Patienten und drei Kontrollen nahmen Probiotika zu sich.

4.3.2 Ähnliche Mikrobiomprofile bei NMDAR-E Patienten und Kontrollen

Die Zusammensetzung des Mikrobioms von Patienten mit NMDAR-E verglichen mit den gesunden Kontrollen zeigte keine Unterschiede (Abbildung 4). Dies gilt zum einen für die Alpha-Diversität (den intraindividuellen Unterschieden entsprechend), repräsentiert durch den Shannon-Index (entsprechend der Artenanzahl und Abundanz) und dem Chao1 Index (den Artenreichtum widerspiegelnd) (Abbildung 5). Zum anderen auch für die Beta-Diversität (der inter-individuellen Unterschiede entsprechend).

4.3.3 Alpha- und Beta-Diversität bei NMDAR-E Patienten mit einem Teratom

Bei 3 der 23 NMDAR-E Patienten war im Zuge der Krankheitsgeschichte ein Teratom nachgewiesen worden, wodurch sie eine Subgruppe mit potentiell anderen immunologischen Mechanismen darstellen. Die Patienten wurden mit passenden gesunden Kontrollen verglichen. Die Kontrollen stammten aus demselben Haushalt und waren vergleichbar in Alter und Geschlecht. Auch hier zeigen sich keine Unterschiede in der Mikrobiom-Zusammensetzung zwischen den Patienten mit NMDAR-E nach der Teratomentfernung und den Probanden.

4.3.4 Alpha- und Beta-Diversität bei NMDAR-E Patienten mit Krankheitsaktivität

Als letztes wurden die Patienten, die zum Zeitpunkt der Stuhlanalyse noch akute Krankheitssymptome aufwiesen (N=8), gesondert untersucht. 7 (88%) waren weiblich und 5 (63%) erhielten eine immunsuppressive Therapie zum Zeitpunkt der Stuhlprobengewinnung. Diese Patienten wurden mit 15 PatientInnen nach Genesung verglichen. 14 (93%) waren davon weiblich und 2 (23%) erhielten noch eine immunsuppressive Therapie. Auch hier zeigten sich keine Unterschiede im Shannon-Index. Allerdings konnten Unterschiede in der Anzahl der Gattungen von Clostridium XVIII, Clostridium IV, Oscillibacter, Prevotella und Blautia nachgewiesen werden (Abbildung 6), die nach Bonferroni Korrektur jedoch nicht signifikant waren. Die beta-Diversität zeigte erneut keine Unterschiede der Gattungen ($p = 0.082$) und der OTU Level ($p = 0.15$).

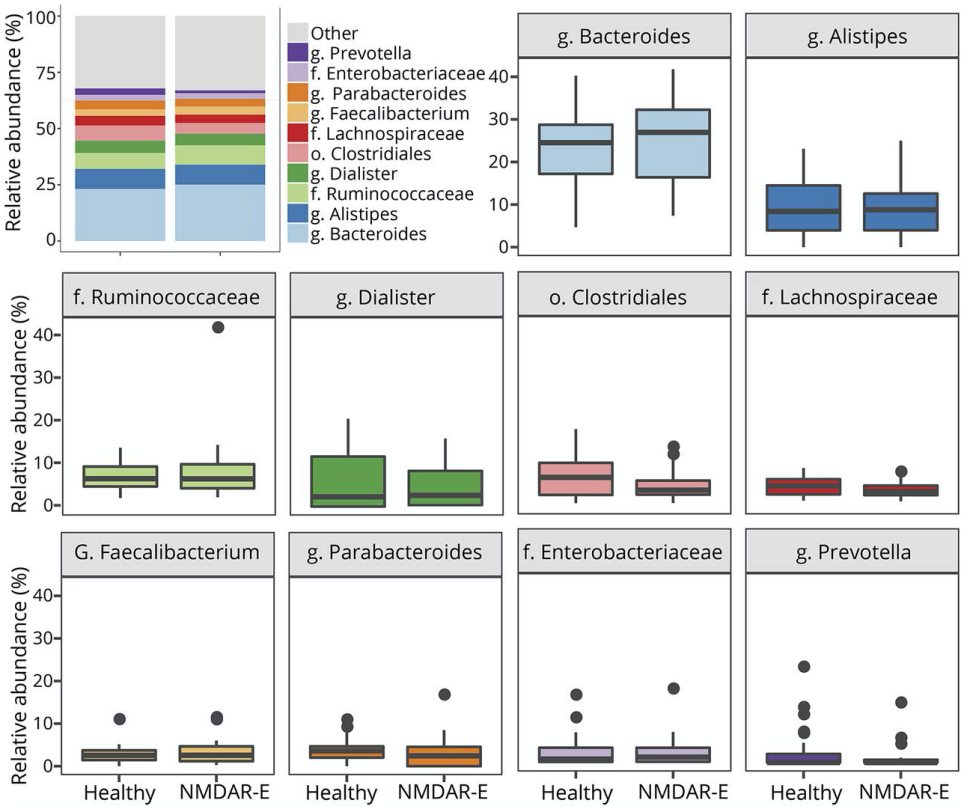


Abbildung 4: Mikrobiomprofile von Patienten mit NMDAR-Encephalitis und gesunden Kontrollen. Gezeigt werden alle Familien und Gattungen mit einer mittleren Abundanz von mehr als 25%. Die übrigenGattungen sind in der Gruppe „other" zusammengefasst. **Aus:** Herken, J., Bang, C., Rühlemann, M., Finke, C., Klag, J., Franke, A., Prüss, H. (2019). Normal gutmicrobiome in NMDA receptor encephalitis. Neurol Neuroimmunol Neuroinflamm Nov 2019, 6 (6) e632; DOI:10.1212/NXI.0000000000000632. Mit Genehmigung von „John Wiley and Sons".

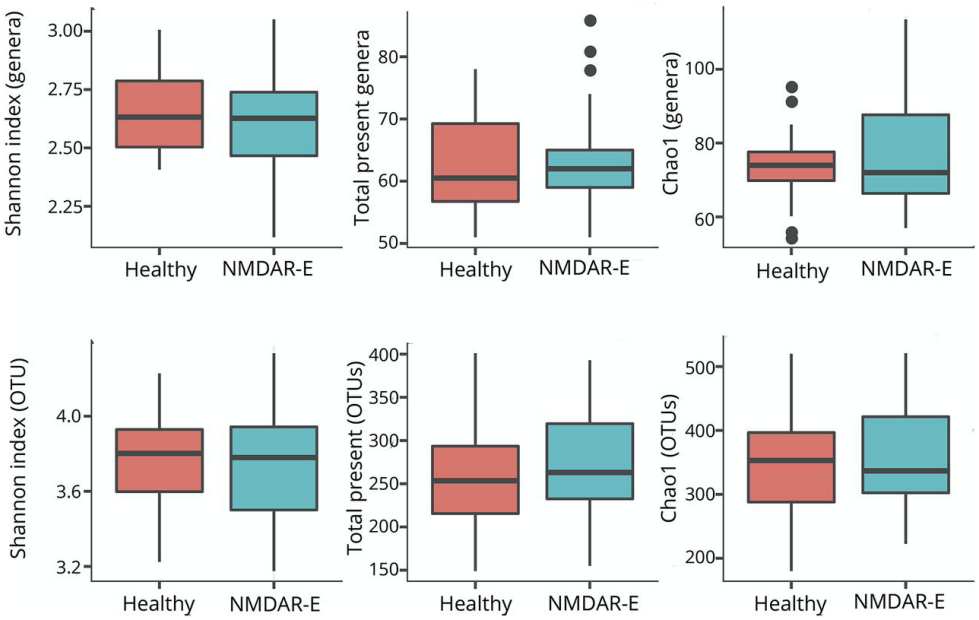


Abbildung 5: Boxplots der Alpha-Diversität. Es zeigten sich keine signifikanten Unterschiedein der Diversität (Shannon Index) sowie in der Anzahl/ Dichte (Chao1) der Mikrobiom-Gattungen und Spezies innerhalb der Gattungen (OTUs) zwischen den Patienten mit NMDAR-Enzephalitis und gesunden Kontrollen. **Aus:** Herken, J., Bang, C., Rühlemann, M., Finke, C., Klag, J., Franke, A., Prüss, H. (2019). Normal gut microbiome in NMDA receptor encephalitis. Neurol Neuroimmunol Neuroinflamm Nov 2019, 6 (6) e632; DOI:10.1212/NXI.0000000000000632. Mit Genehmigung von „John Wiley and Sons".

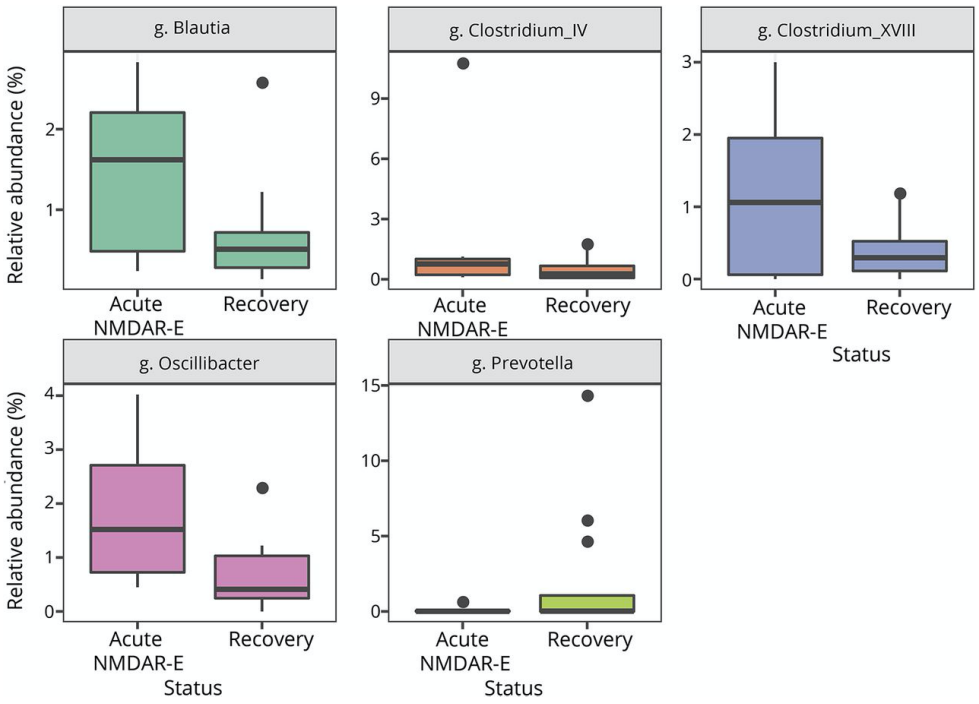


Abbildung 6: Überrepräsentation von Bakterienspezies bei Patienten mit Krankheitsaktivität einer NMDAR-Encephalitis. Fünf Arten zeigten hierbei eine Überrepräsentation. Allerdings ging die statistische Signifikanz nach Korrektur für multiples Testen verloren. **Aus:** Herken, J., Bang, C., Rühlemann, M., Finke, C., Klag, J., Franke, A., Prüss, H. (2019). Normal gut microbiome in NMDA receptor encephalitis. Neurol Neuroimmunol Neuroinflamm Nov 2019, 6 (6) e632; DOI:10.1212/NXI.0000000000000632. Mit Genehmigung von „John Wiley and Sons".

5. DISKUSSION

Basierend auf der Analyse klinischer Erstmanifestationen autoimmuner Enzephalitiden im Zentrum für Autoimmune Encephalitis der Charité konnte gezeigt werden, dass das Vorhandensein anti-neuronaler AK ein Risikofaktor für die Entwicklung neuropsychiatrischer Symptome darstellt (Tabelle 1). Je nach Untergruppe der AK und Zielantigen stehen dabei typische Symptomkonstellationen im Vordergrund. Analog aktueller Veröffentlichungen (30) (31) präsentierten sich AIE auch in meinem Patientenkollektiv initial oftmals mit psychiatrischen Symptomen. Kürzlich wurde für ein Syndrom mit dominierenden psychotischen Symptomen und wahrscheinlicher AK Pathophysiologie sogar der neue Begriff „autoimmune Psychose" (AP) eingeführt (32) (33) (34). Ein wichtiges Ergebnis dieser Arbeit ist daher die deutlich verzögerte Diagnosestellung der AIE in meinem Patientenkollektiv und somit bestehende Gefahr der Fehldiagnose als primär psychiatrische Erkrankung.

Auf Grund des heterogenen Erscheinungsbilds und dieser oftmals verzögerten Diagnosestellung bedarf es Hilfsmittel zur Anwendung im klinischen Alltag. Durch die retrospektive Analyse der Krankengeschichten gelang es, bestimmte Symptom- oder

Laborkonstellationen herauszuarbeiten, die den Verdacht auf ein autoimmunes Geschehen lenken sollten. Diese „red flags“ und „yellow flags“ (Tabelle 3) wurden anhand ihres Vorhersagewerts gewichtet und können in das differentialdiagnostische Denken und die Diagnostik im psychiatrischen Alltag intergiert werden. Dabei sollten „red flag“-Kriterien auf dem Boden einer neu aufgetretenen Psychose stets zu direkter Autoantikörpersuche im Liquor führen. „Yellow flag“-Kriterien sollten den Verdacht auf ein zu Grunde liegendes autoimmunes Geschehen lenken. Resultat der Anwendung könnte, gestützt durch die vorliegenden Daten, eine frühzeitige Identifikation einer AIE bei führend psychotischer Symptomatik sein.
Die Diagnose einer autoimmunen Enzephalitis ist in den letzten Jahren (2013-2016) schneller und häufiger gestellt worden als in der Zeitspanne zwischen 2007 und 2012. Dies ist vermutlich auf die beginnende Integration dieser neue Krankheitsgruppe in das differenzialdiagnostische Denken zurückzuführen. Ein weiterhin wachsendes Bewusstsein ist notwendig, um die Diagnosestellung in Zukunft zusätzlich zu beschleunigen. Da die Krankheitsgruppe der AIE dennoch recht neu ist, fehlen systematisch kontrollierte Studien und umfassende Reviews. Aus diesem Grund kann die Fall-Analyse, die meinen Ergebnissen zu Grunde liegt, nur mit einer Evidenzstärke des Levels 4 bewertet werden.

Die Hypothese, dass anti-neuronale AK nicht nur als Risikofaktor für die Entwicklung neuropsychiatrischer Erkrankungen bei Erwachsenen gelten, sondern auch die fetale Hirnentwicklung beeinflussen, konnte im Tierversuch belegt werden. Die Ergebnisse weisen darauf hin, dass ein transitorischer Kontakt mit antineuronalen AK im fetalen Zustand potentiell lebenslängliche neuropsychiatrische Auswirkungen für das Kind haben kann. Die Beobachtung, dass sich die NR1-AK diaplazentar übertragen lassen und im fetalen Hirn anreichern ist von immenser Bedeutung. Nach entsprechendem Kontakt mit NR1-AK zeigten die kindlichen Mäuse eine reduzierte NMDAR-Dichte, eine erhöhte Mortalität und eine reduzierte Hirnentwicklung. Vor allem aber zeigten sich bis in die Adoleszenz anhaltende, milde Verhaltensstörungen.
Auf dem Boden dieser Ergebnisse liegt die Vermutung nahe, dass diaplazentar übertragene anti-neuronale AK (speziell NR1 AK) potentiell neuropsychiatrische Erkrankungen bei Kindern auslösen können. Um dies zu untermauern, untersuchte wir die Serum-AK- Titer von Müttern mit psychisch erkrankten Kindern (MCPD). Die Kinder wiesen keine spezifische Erkrankung auf, viel mehr zeigten sie ein breites Spektrum psychiatrischer Syndrome, von ADHS über Schizophrenie bis hin zur bipolaren Störung (Abbildung 2). In der Serum-Analyse ließen sich leicht erhöhte NR1-AK- Titer in der Gruppe der MCPD nachweisen. Der verhältnismäßig kleine Effekt dürfte durch die lange Zeitspanne zwischen Geburt und Testung (4-20 Jahre) beeinflusst sein, da die Titer-Höhe mit der Zeit abnimmt. Diese Hypothese muss jedoch durch weitere größere Studien abgesichert werden. Die Kinetik von AK-Titern über eine gewisse Zeitspanne müsste untersucht werden und eine Testung unmittelbar nach Geburt könnte eventuell zu

einem weiter reichenden Ergebnis führen. So könnte der AK-Titer während der Schwangerschaft und nach der Geburt ursprünglich deutlich höher gewesen sein, mit der Zeit gesunken und zum Zeitpunkt der Bestimmung im Rahmen der vorliegenden Studie bereits wieder an einem Tiefpunkt gelegen haben. Zusätzlich scheinen AK-Titer lediglich teilweise mit dem klinischen Bild zu korrelieren, so dass davon ausgegangen werden kann, dass andere Faktoren wie beispielswiese die AK-Affinität eine Rolle spielen (21). Als Letztes könnte die Pathogenität der Auto AK in niedrigen Titern eventuell auch durch andere bisher nicht beachtete Faktoren (wie z.B. Infektion, genetische Veranlagung, etc.) potenziert werden und so dennoch zu schädlichen Einflüssen führen.

Eine Verbindung zwischen sowohl Infektionen (24) (22), als auch Genetik (25) (18) in Bezug auf die Pathogenese der AIE wird angenommen. Die Hypothese, dass die Zusammensetzung des Mikrobioms in einem pathophysiologischen Zusammenhang mit dem Auftreten der NMDAR-E stünde, konnte nicht belegt werden. Anders als bei den NMOSD zeigten sich keine Unterschiede in der Mikrobiom-Zusammensetzung, auch in derer der Untergruppen nicht. Probanden und Kontrollen zeigten je ein „normales“ Mikrobiom. Wahrscheinlich basiert die Pathophysiologie der NMSOD trotz der nahen Verwandtschaft zu den AIE auf unterschiedlichen Mechanismen. Während die Immunantwort in NMOSD stärker durch T-Zellen beeinflusst ist (29), geht man im Fall der autoimmunen Enzephalitis mit NMDAR-AK vor allem von einer zu Grunde liegenden humoralen Immunantwort aus (21) (36). Die Ergebnisse basieren auf einer sehr kleinen Studienteilnehmerzahl. Zusätzlich befand sich der überwiegende Teil der Patientinnen nicht mehr in der Akutphase der Erkrankungen, sondern teilweise sogar in Remission. Eine größere Probandenzahl, die vornehmlich Patientin mit hoher Krankheitsaktivität umfasst, würde eventuell einen Unterschied aufzeigen. Tatsächlich zeigte die Subgruppe der Patienten mit akuter Enzephalitis eine Überrepräsentation von fünf Gattungen, auch wenn die statistische Signifikanz nach Korrektur für multiples Testen verloren ging. Weiterhin hätte es hilfreich sein können eine positive Kontrollgruppe einzugliedern, die bekanntermaßen Unterschiede in der Mikrobiom-Zusammensetzung aufweist, z.B. solche Patienten mit NMOSD. Fünf der in die Studie eingeschlossenen Patienten erhielten eine immunsuppressive Therapie zum Zeitpunkt der Stuhlprobe. Eventuell könnte der Einfluss dieser Therapie auf das Mikrobiom einen Unterschied kaschieren. Im Fall der NMOSD hatte eine Therapie mit Rituximab als Immunsuppressivum jedoch keinen Effekt gezeigt (28).

6. SYNOPSIS

Mit der Erforschung autoimmuner Enzephalitiden scheint eine Subgruppe neuropsychiatrischer Erkrankungen entdeckt zu werden, die auf die Präsenz bestimmter AK zurückzuführen ist und oftmals gut therapiert werden kann. Seit ihrer erstmaligen Beschreibung gewinnen AIE kontinuierlich an Bedeutung. Dennoch ist davon auszugehen, dass einige Patienten mit neuropsychiatrischen Symptomen nach wie vor nicht korrekt diagnostiziert werden. Zudem ist die Pathophysiologie sowie Entstehung autoimmuner Enzephalitiden nur teilweise erforscht.

Um eine Verbesserung der Früherkennung zu erreichen, bedarf es einer allgemeinen Kenntnis der klinischen Warnzeichen einer AIE („red und yellow flags“) seitens des behandelnden Arztes. Zudem sollte gestützt durch die wachsende Inzidenz der AIE (3) eine Lumbalpunktionen klinische Routine in der Diagnostik von de novo Psychosen werden. Die reine Serumdiagnostik ist hierbei nicht ausreichend, da AK bei der NMDAR-E zum Diagnosezeitpunkt im Serum fehlen können, während sie im Liquor immer vorhanden sind (37). Zudem hängt die klinische Bedeutung von Serum-AK wahrscheinlich mit einer vorhergegangenen oder gegenwärtigen Störung der Blut-Hirn Schranke zusammen (20).
Die Studienergebnisse der Mikrobiom- Analyse lassen trotz der bestehenden Limitationen davon ausgehen, dass das Mikrobiom in der Pathophysiologie der NMDAR-E keine relevante Rolle spielt. Viel mehr erinnern die Ergebnisse der Arbeit erneut daran, wie wenig bisher über die Ätiologie der AIE und im Speziellen der NMDAR-E bekannt ist.
Damit ein wachsendes Verständnis der pathophysiologischen Mechanismen sowie die Entwicklung neuartiger Therapieansätze gewährleistet werden kann, benötigt es breit gefächerte Forschungsansätze und internationale Zusammenarbeit. Zusätzlich scheint ein interdisziplinäres Vorgehen immer mehr an Bedeutung zu gewinnen, da AIE nicht nur den Fachbereich der Neurologie und Psychiatrie betreffen, sondern wohlmöglich auch Bekanntheit in der Pädiatrie, Gynäkologie oder Gastroenterologie erlangen müssen.
So scheint das Vorhandensein antineuronaler AK nicht nur bei Erwachsenen neuropsychiatrische Symptome zu verursachen, sondern auch die kindliche Hirnentwicklung in der Schwangerschaft potentiell maßgeblich zu beeinflussen. Ob neuropsychiatrische Erkrankungen wie ADHS oder Schizophrenie sogar teilweise durch diesen Einfluss entstehen, sollte mittels weiterer Studien umfassender erforscht werden. Sollten zukünftige Forschungsergebnisse die These unterstützen, könnte ein AK-Screening in die Schwangerschaftsvorsorge aufgenommen werden. Auf diesem Wege könnten die gesundheitlichen Auswirkungen auf das Kind durch entsprechende Behandlung vermieden werden.

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8. EIDESSTATTLICHE VERSICHERUNG

„Ich, Julia Herken, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: **Das Mikrobiom und die Rolle antineuronaler Autoantikörper als Risikofaktoren neuropsychiatrischer Symptome** selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe. Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet. Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.
Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.
Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.
Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

9. ANTEILSERKLÄRUNG AN DEN ERFOLGTEN PUBLIKATIONEN

Julia Herken hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Herken J and Prüss H (2017) Red Flags: Clinical Signs for Identifying Autoimmune Encephalitis in Psychiatric Patients. *Front. Psychiatry* 8:25. DOI: 10.3389/fpsyt.2017.00025

- Mitentwicklung des Studiendesigns
- Co-Autorin des Projektantrags und des Ethikkommissionsantrag
- Rekrutierung der Studienteilnehmer
- Eintrag der Patienteninformationen in die Datenbank „GENERATE“
- Umfassende Analyse der elektronischen Krankengeschichte sowie Ergänzung durch Interviews
- Dateneingabe und Auswertung, wodurch sämtliche Tabellen des Papers entstanden sind (Tabelle 1-4, Figur 2)
- Schreiben des Manuskripts und substanzielle Mitwirkung an der Anfertigung der Publikation in der vorliegenden Form

Publikation 2: Herken, J., Bang, C., Rühlemann, M., Finke, C., Klag, J., Franke, A., Prüss, H. (2019). Normal gut microbiome in NMDA receptor encephalitis**.** *Neurol Neuroimmunol Neuroinflamm Nov 2019,* 6 (6) e632; DOI:10.1212/NXI.0000000000000632

- Mitentwicklung des Studiendesigns
- Co-Autorin des Projektantrags und des Ethikkommissionsantrags
- Rekrutierung der Studienteilnehmer. Dies galt sowohl für die Erkrankten, als auch für die passenden Kontrollpersonen, die möglichst aus dem nahen Umfeld stammen sollten. Telefonisch wurden so anhand entsprechender Kriterien passende gesunde Probanden ausgewählt
- Verschicken und Empfangen der für die Studie benötigten Utensilien
- Klinische Untersuchung mittels Fragebögen, inklusive der Auswertung und statistischen Analyse
- Schreiben des Manuskripts und substanzielle Mitwirkung an der Anfertigung der Publikation in der vorliegenden Form

Publikation 3: Jurek, B., Chayka, M., Kreye, J., Lang, K., Kraus, L., Fidzinski, P., Kornau, H. C., Dao, L. M., Wenke, N., Long, M., Rivalan, M., Herken, J., Winter, Y., Leuber, J., Mayer, S., Mueller, S., Boehm-Sturm, P., Dirnagl, U., Schmitz, D., Kölch, M., Prüss, H. (2019). Human gestational N-methyl-d-aspartate receptor autoantibodies impair neonatal murine brain function. *Annals of Neurology, 86*, 656–670. DOI: (1)

- Rekrutierung der Studienteilnehmer der Pilotstudie gemeinsam mit Katharina Lang, jedoch zu einem kleineren Anteil als sie. Dazu führten wir über mehrere Monate in der Kinder-und-Jugend-Psychiatrie des Vivantes-Klinikums Friedrichshain die Befragung von Müttern von erkrankten Kindern durch. Bei Teilnahmewunsch an der Studie führten wir die Aufklärung und Blutentnahmen zur AK-Suche durch.

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Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

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Unterschrift des Doktoranden/der Doktorandin

frontiers in Psychiatry

ORIGINAL RESEARCH
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Red Flags: Clinical Signs for Identifying Autoimmune Encephalitis in Psychiatric Patients

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Autoimmune mechanisms causing diverse psychiatric symptoms are increasingly recognized and brought about a paradigm shift in neuropsychiatry. Identification of underlying antibodies against neuronal ion channels or receptors led to the speculation that a number of patients go misdiagnosed with a primary psychiatric disease. However, there is no clear consensus which clinical signs in psychiatric patients should prompt further investigations including measurement of anti-neuronal autoantibodies. We therefore aimed to analyze the presenting symptoms in patients with autoimmune encephalitis and the time between symptom onset and initiation of antibody diagnostics. For this, we recruited 100 patients from the Charité Center for Autoimmune Encephalitis between May and October 2016, including all types of autoimmune encephalitides. Psychiatric abnormalities were the most common clinical symptoms and were the presenting sign in 60%. One-third of patients were initially hospitalized in a psychiatric ward. All patients positive for antibodies against the *N*-methyl-D-aspartate receptor showed behavioral changes, hallucinations, memory deficits, catatonia, or delusions. Patients positive for antibodies against other cell surface or intracellular antigens were often hospitalized with a psychosomatic diagnosis. The time between occurrence of first symptoms and antibody testing was often alarmingly prolonged. In patients with symptom onset between 2013 and 2016, the mean delay was 74 days, in cases diagnosed between 2007 and 2012 even 483 days, suggesting though that increased awareness of this novel disease group helped to expedite proper diagnosis and treatment. By analyzing the medical records in detail, we identified clinical signs that may help to assist in earlier diagnosis, including seizures, catatonia, autonomic instability, or hyperkinesia. Indeed, reanalyzing the whole cohort using these "red flags" led to a 58% reduction of time between symptom onset and diagnosis. We conclude that the timely diagnosis of an autoimmune psychiatric disease can be facilitated by use of the described clinical warning signs, likely enabling earlier immunotherapy and better prognosis. Also, the threshold for cerebrospinal fluid analysis and autoantibody testing should be low.

Keywords: autoimmune encephalitis, schizophreniform syndrome, cerebrospinal fluid analysis, anti-neuronal autoantibodies, immunotherapy

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INTRODUCTION

The growing number of newly described autoimmune encephalitides has drawn a remarkable link between immunology and psychiatry within the last several years (1–3). Since the pioneering discovery of *N*-methyl-D-aspartate receptor (NMDAR) autoantibodies (4), various further antibodies against receptors and ion channels were identified in patients with psychiatric abnormalities, such as against AMPA, GABA, glycine receptors, metabotropic glutamate receptor 5 (mGluR5), and dopamine-D2 receptors (**Table 1**), not only in humans (5). Patients are often first hospitalized in psychiatric departments before being transferred to a neurology ward (6, 7), stimulating the intriguing question of whether a subset of patients may go misdiagnosed with a primary psychiatric disease (1, 2, 8, 9). Recently, a high prevalence of cerebrospinal fluid (CSF) abnormalities including the detection of anti-neuronal autoantibodies has been observed in 54.4% of psychotic patients (10), highlighting their potential role in psychiatry and underlining the need for increased clinical and scientific awareness in order to not overlook treatable etiologies.

Antibody-mediated encephalitides can be categorized based on the presence of anti-neuronal antibodies targeting (i) neuronal cell surface antigens and (ii) intracellular antigens (11, 12). Autoantibodies directed to cell surface proteins are more frequently found in patients with psychiatric abnormalities, likely due to a suspected direct pathogenic effect (12–14). The demonstration of specific effects of NMDAR antibody-containing CSF *in vivo* convincingly substantiates the link between autoantibodies and the schizophreniform syndrome seen in these patients (15). Most recent work using CSF-derived human monoclonal NMDAR antibodies showed that the antibody is sufficient to change NMDAR expression and electrophysiology (16). Thus, the presence of this antibody alone represents a risk factor for neuropsychiatric symptoms, supporting the need for sufficiently aggressive immunotherapy in affected patients.

Such a clear causative role of autoantibodies on psychiatric symptoms has yet to be shown for further surface-directed antibodies. Nonetheless, psychotic symptoms are common in numerous other autoimmune encephalitides (**Table 1**). For example, patients with antibodies against the voltage-gated potassium channel complex (VGKCc) often present with hallucinations, depression, and memory deficits (13, 14, 17). Neuropsychiatric symptoms were found in 44% of VGKCc antibody-positive patients, occasionally treated for primary psychiatric diagnoses (14). Less well known, patients with antibodies against intracellular targets can also present with psychiatric symptoms (18).

The prognosis of autoimmune encephalitides largely depends on the rapid initiation of immunotherapy. Any delay in diagnosis causes costs and morbidity, while early immunotherapy results in substantial recovery in 70–80% of the patients (6, 19–23). This is especially striking considering the often severe course of the disease, sometimes requiring prolonged episodes of intensive care unit treatment and mechanical ventilation (6). Delayed

TABLE 1 | Classification of encephalitis groups in the present study and commonly associated clinical features.

Encephalitis groups of the present study	Antibodies	Number of patients	Psychiatric symptoms	Additional symptoms	Typical patient
(A) NMDAR encephalitis (*n* = 53)	NMDA receptor	*n* = 53 (53%)	Psychosis, schizophreniform illness, catatonia, hallucinations, aggression	Epileptic seizures, dyskinesia, autonomic instability, speech dysfunction, decreased consciousness	Young women, association with ovarian teratomas
(B) Non-NMDAR cell surface antigens (*n* = 24)	Caspr2	*n* = 4 (4%)	Insomnia, panic attacks, schizophreniform illness, depression	Morvan syndrome, neuromyotonia, muscle spasms, fasciculations	Middle age or elderly patients, may be associated with thymoma
	LGI1	*n* = 14 (14%)	Amnesia, confusion, memory deficits, depression	Limbic encephalitis, faciobrachial dystonic seizures, hyponatremia	Middle age or elderly patients, male:female (2:1), may be associated with thymoma
	Metabotropic glutamate receptor 5	*n* = 2 (2%)	Behavioral changes, emotional instability, memory deficits	Limbic encephalitis, Ophelia syndrome	Young adults, may be associated with Hodgkin's lymphoma
	Glycine receptor	*n* = 1 (1%)	Behavioral changes, schizophreniform syndrome	Stiff-person syndrome (SPS) or progressive encephalomyelitis with rigidity and myoclonus, hyperekplexia	middle age or elderly patients, may be associated with thymomas and lymphomas
(C) Antibodies against intracellular antigens (*n* = 23)	Synaptic antigens: anti-GAD antibodies	*n* = 9 (9%)	Schizophreniform illness, autism, attention-deficit/ hyperactivity disorder	Limbic encephalitis, seizures, SPS, brainstem dysfunction, ataxia	Middle age or elderly patients, might be associated with small-cell lung cancer
	Onconeuronal antigens: anti-Yo, -Hu, -CV2, -Ri, -Ma2 antibodies	*n* = 14 (14%)	Behavioral changes	Limbic encephalitis, cerebellar degeneration, sensory neuropathy	Elderly patients, often with malignant tumors (small-cell lung carcinoma, Hu; testicular seminoma, Ma2)

NMDAR, N-methyl-D-aspartate-receptor; LGI1, leucine-rich glioma inactivated 1; Caspr2, contactin associated protein 2.

recognition of the disease can also result in inadequate use of neuroleptics, which in patients with NMDAR encephalitis frequently worsens the symptoms, leading to the working diagnosis of a neuroleptic malignant syndrome (7).

We therefore aimed to retrospectively ascertain the time and frequency of delayed diagnosis of autoimmune encephalitides and asked whether specific clinical signs can assist in earlier recognition, antibody testing, and proper diagnosis of the disease. Indeed, a number of warning signs ("red flags") can help to facilitate the timely diagnosis of an autoimmune psychiatric disease, likely enabling earlier immunotherapy and better prognosis.

MATERIALS AND METHODS

Patient Selection

$N = 100$ patients with different forms of autoimmune encephalitides were recruited in the Charité Centre for Autoimmune Encephalitis from May to October 2016. Patients were grouped in three categories (**Table 1**):

(A) Anti-NMDAR encephalitis ($n = 53$), defined by a compatible clinical picture and positive IgG-NMDAR antibodies in the CSF (**Figure 1A**).
(B) Non-NMDAR surface antibodies ($n = 24$), including patients with antibodies against the neuronal cell surface antigens LGI1 ($n = 14$), CASPR2 ($n = 4$), mGluR5 ($n = 2$, **Figure 1B**), glycine receptor ($n = 1$) and against an unknown epitope determined on brain section immunofluorescence testing ($n = 3$).
(C) Antibodies against intracellular epitopes ($n = 23$), including patients with GAD antibodies ($n = 9$) or onconeuronal antibodies, such as Yo, Hu, Ri, or CV2 ($n = 14$, **Figure 1C**).

Informed Consent

Written informed consent was received from participants at the Charité Department of Neurology or their representatives prior to inclusion in the study, and analyses were approved by the Charité University Hospital Institutional Review Board.

Clinical Data Collection

Most patients were hospitalized in the Charité Department of Neurology during the disease course. Medical charts were retrospectively analyzed, and clinical and para-clinical information was collected during follow-up visits in the outpatient clinic or *via* email/telephone interviews. The following information was systematically retrieved from medical records: age, sex, date of disease onset, neurological and psychiatric symptoms during initial clinical presentation, psychiatric and neurological signs during follow-up, department of initial hospitalization, details of psychiatric hospitalization, symptoms that led to determination of antibodies, date of diagnosis, and time from first symptoms to diagnosis.

RESULTS

Demographic Data

Median age in our cohort was 41 years (range 14–92 years) and 71% were female. Patients positive for NMDAR antibodies were younger (mean age 30 [14–57] years) and mainly women (91%). In contrast, patients with antibodies against non-NMDAR surface antigens were predominantly of male gender (67%) and older (mean age 53 [29–78] years). Patients positive for antibodies against intracellular proteins were predominantly female (65%), mean age was 56 (37–92) years.

Initial Hospitalization in a Psychiatric Department

In order to estimate the overlapping symptoms with primary psychiatric disorders, we analyzed the frequency of patients initially hospitalized in a psychiatric department and the frequency of psychotic symptoms at first evaluation and during follow-up. $N = 31$ patients (31%) were initially hospitalized on a Psychiatry ward, commonly for psychotic or suspected psychosomatic symptoms. Almost two-thirds of all patients ($n = 60$; 60%) showed psychotic symptoms at the beginning of the disease, even if hospitalization was not required, 7% presented with psychosomatic symptoms.

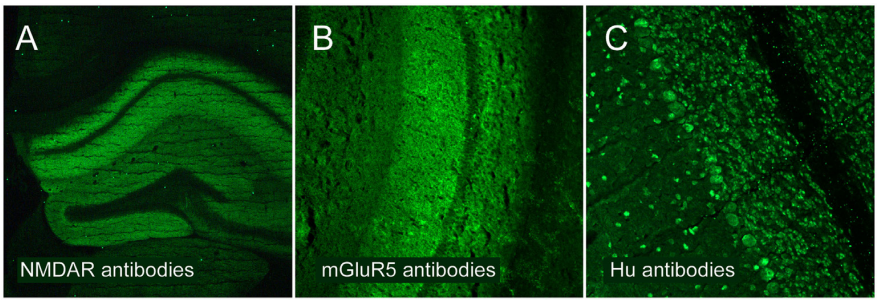

FIGURE 1 | **Classification of encephalitis groups analyzed in the present study**. Underlying autoantibodies show different patterns of brain binding using immunofluorescence testing. **(A)** Patients with NMDAR encephalitis and high-level autoantibodies against the NR1 subunit of the NMDAR. **(B)** Patients with non-NMDAR antibodies targeting neuronal surfaces, such as antibodies against the metabotropic glutamate receptor 5 (mGluR5). **(C)** Patients with antibodies targeting intracellular epitopes, such as anti-Hu antibodies.

Psychiatric symptoms were not equally distributed across the three encephalitis groups. All patients with NMDAR antibodies ($n = 53$) showed psychotic symptoms. In patients positive for antibodies against other neuronal surface or intracellular antigens, psychosomatic symptoms were common at presentation: 8/24 in the non-NMDAR group (33%), 5/23 in the intracellular antigens group (22%). However, psychotic symptoms did also occur: 6/24 in the non-NMDAR group (25%), 4/23 in the intracellular group (17%). Of the NMDAR antibody-positive patients, 21/53 (40%) were seen by a psychiatrist at first evaluation, while this was the case for only one patient positive for intracellular protein antibodies (4%).

Initial Symptoms

The frequency of first clinical signs was again not equally distributed between the encephalitis groups (**Table 2**). Patients positive for NMDAR antibodies typically presented with psychiatric symptoms and either developed a spectrum of neurological abnormalities, such as seizures, movement, or speech disorders, or already showed them at first evaluation. Their initial psychiatric symptoms were acute behavioral changes ($n = 46$; 87%), hallucinations ($n = 23$; 43%), paranoid delusions ($n = 13$; 26%), and memory deficits, especially short-term memory loss ($n = 11$; 21%). Also, mutism ($n = 8$; 15%), catatonia ($n = 10$; 19%), and depressive symptoms ($n = 10$; 19%) were commonly seen at presentation. One young woman got initially hospitalized with the clinical picture of anorexia. First symptoms in some patient were neurological, consisting of epileptic seizures ($n = 10$; 19%), speech dysfunction such as pressured speech and verbal reduction ($n = 10$; 19%), dyskinesia ($n = 7$; 13%), and headache ($n = 9$; 17%).

Patients of the non-NMDAR group presented also with psychiatric symptoms in most cases, such as acute behavioral changes ($n = 7$; 29%), aggression/confusion ($n = 6$; 25%), or memory deficits ($n = 8$; 33%). Hallucinations and paranoid delusions were also seen (**Table 2**). The neurological symptoms of this group were more characteristic and included faciobrachial dystonic seizures (FBDS, in patients with LGI1 antibodies) ($n = 7$; 29%) and sensorimotor deficits ($n = 7$; 29%).

Patients positive for intracellular epitope antibodies presented less frequently with psychiatric symptoms, including acute behavioral changes and memory deficits. The majority of symptoms in this group were neurological, such as sensorimotor deficits ($n = 13$; 57%), cerebellar ataxia ($n = 7$; 30%), movement disorders ($n = 3$; 13%), and generalized tonic-clonic seizures ($n = 3$; 13%).

In most patients of all three groups, both psychiatric and neurological symptoms occurred during the first month of disease. Interestingly, $n = 13$ (13%) of all patients presented with a depressed mood, in four cases leading to the diagnosis of major depression. Appearance of additional neurological symptoms led to reclassification of diagnosis.

Which Clinical Features Led to Examination of Autoantibodies?

We next determined which clinical symptoms, routine laboratory findings, or imaging abnormalities triggered the testing for autoantibodies in all 100 patients, the results of which finally allowed the firm diagnosis of autoimmune encephalitis (**Table 3**). Indeed, several clinical constellations of neurological and psychiatric symptoms were more common than others to stimulate antibody testing. We semi-quantitatively classified these constellations as

TABLE 2 | Presenting clinical symptoms in all 100 patients.

Initial signs and symptoms	All patients (100)	NMDAR (53)	Non-NMDAR (24)	Intracellular antigens (23)
Psychiatric				
Acute behavioral changes	56 (56%)	46 (87%)	7 (29%)	3 (13%)
Hallucinations (visual, auditory)	25 (25%)	23 (43%)	1 (4%)	
Memory deficits (retro- and anterograde amnesia)	22 (22%)	11 (21%)	8 (33%)	4 (17%)
Confusion/aggression	18 (18%)	11 (21%)	6 (25%)	1 (4%)
Paranoid delusions	17 (17%)	13 (26%)	2 (8%)	1 (4%)
Depressed mood	13 (13%)	10 (19%)	4 (16%)	1 (4%)
Catatonia	10 (10%)	10 (19%)		
Mutism	8 (8%)	8 (15%)		
Anorexia	1 (1%)	1 (2%)		
Any of the above symptoms	65 (65%)	53 (100%)	14 (58%)	7 (30%)
Neurological				
Sensorimotor deficits	30 (30%)	8 (15%)	7 (29%)	13 (57%)
Seizures		10 (19%)	2 (8%)	5
Generalized tonic-clonic	13 (13%)	9 (17%)	1 (4%)	3 (13%)
Focal	4 (4%)	1 (2%)	1 (4%)	2 (9%)
Faciobrachial dystonic seizures	7 (7%)		7 (29%)	
Speech dysfunction (pressured speech, verbal reduction)	15 (15%)	10 (19%)	4 (16%)	
Movement disorders	11 (11%)	7 (13%)	1 (4%)	3 (13%)
Headache	12 (12%)	9 (17%)	1 (4%)	2 (9%)
Reduced levels of consciousness	7 (7%)	5 (9%)	2 (8%)	
Paralysis	7 (7%)	4 (8%)	1 (4%)	2 (9%)
Cerebellar ataxia	10 (10%)	1 (2%)	3 (12%)	7 (30%)
Diplopia	7 (7%)	3 (6%)		4 (17%)
Any of the above symptoms	67 (67%)	39 (74%)	20 (83%)	20 (87%)

TABLE 3 | Clinical symptoms and constellations that led to the determination of anti-neuronal antibodies in all 100 patients.

Symptoms	All patients (100)	NMDAR (53)	Non-NMDAR (24)	Intracellular antigens (23)
Epileptic seizures	14 (14%)	10 (19%)	2 (8%)	2 (8%)
Cerebrospinal fluid (CSF) abnormalities[a] and absent evidence for infectious encephalitis	13 (13%)	12 (27%)	1 (4%)	
Abnormal postures or movements	4 (4%)	4 (7%)		
Reduced levels of consciousness	4 (4%)	4 (7%)		
Aphasia or dysarthria	3 (3%)	3 (6%)		
Lack of improvement with antipsychotics	5 (5%)	4 (7%)	1 (4%)	
Autonomic instability	2 (2%)	2 (4%)		
Suspicious MRI or EEG findings	10 (10%)	3 (6%)	5 (20%)	2 (8%)
Steroid-responsive autoimmune thyroiditis	3 (3%)	2 (4%)		1 (4%)
Lack of improvement with antiepileptic medication	2 (2%)	1 (2%)	1 (4%)	
Focal neurological deficits	3 (3%)	1 (2%)	1 (4%)	1 (4%)
Sensory deficits	3 (3%)	1 (2%)	2 (8%)	
Rapidly progressing psychosis	4 (4%)	1 (2%)	2 (8%)	1 (4%)
Suggested by patients or families	3 (3%)	3 (6%)		
Positive effect of *ex juvantibus* immunotherapy	2 (2%)		1 (4%)	1 (4%)
Faciobrachial dystonic seizures	3 (3%)		3 (12%)	
Neuromyotonia	1 (1%)		1 (4%)	
Cerebellar ataxia	8 (8%)		2 (8%)	6 (26%)
Hyponatremia	2 (2%)		2 (8%)	
Paresthesia or malignant tumor[b]	7 (7%)			7 (30%)

[a]*Increased white blood cell count or CSF-specific oligoclonal bands.*
[b]*Small-cell lung cancer, testicular seminoma.*

"yellow flags" and "red flags," depending on their power to predict the presence of autoantibodies in such patients (**Table 4**).

In the NMDAR encephalitis group, viral encephalitis was a common working diagnosis, often suggested by the clinical picture, acute neurological changes, and CSF pleocytosis. NMDAR autoantibody testing was often initiated once the search for a viral or bacterial pathogen remained negative ($n = 12$; 27%). In all three groups, the occurrence of epileptic seizures frequently initiated CSF investigation including determination of antibodies ($n = 14$; 14%). Suspicious MRI and EEG were another reason for antibody testing, in particular in patients with non-NMDAR surface antibodies ($n = 5$; 20%), but much less in NMDAR antibody-positive patients ($n = 3$; 6%). Patients were frequently transferred from a psychiatric to a neurological ward at this point. Similarly, in patients hospitalized for a schizophreniform syndrome, detection of abnormal neurological signs resulted in antibody testing. These deficits included decreased levels of consciousness ($n = 4$; 7%), abnormal postures or movements ($n = 4$; 7%), and aphasia or dysarthria ($n = 3$; 6%) in patients of the NMDAR encephalitis group. Focal neurological signs were the trigger for antibody testing in one patient each of the NMDAR (2%), non-NMDAR surface antibody (4%), and intracellular epitope antibody (4%) groups (**Table 3**).

Non-NMDAR antibodies testing was performed in several cases because of the occurrence of FBDS ($n = 3$; 12%), sensory deficits ($n = 2$; 8%), or the detection of hyponatremia in the context of unexplained neuropsychiatric symptoms ($n = 2$; 8%). In two patients with non-NMDAR surface antibodies, antibody testing was initiated because of a rapidly progressing psychosis ($n = 2$; 8%). A common reason to test for antibodies against intracellular epitopes was the occurrence of paresthesia in the context of a malignant tumor ($n = 7$; 30%) or clinical deficits resulting from cerebellar symptoms ($n = 6$; 26%).

TABLE 4 | Warning signs pointing to an autoimmune etiology in new-onset psychosis.

Yellow flags

- Decreased levels of consciousness
- Abnormal postures or movements (orofacial, limb dyskinesia)
- Autonomic instability
- Focal neurological deficits
- Aphasia or dysarthria
- Rapid progression of psychosis (despite therapy)
- Hyponatremia
- Catatonia
- Headache
- Other autoimmune diseases (e.g., thyroiditis)

Red flags

- Cerebrospinal fluid (CSF) lymphocytic pleocytosis or CSF-specific oligoclonal bands without evidence for infection
- Epileptic seizures
- Faciobrachial dystonic seizures
- Suspected malignant neuroleptic syndrome
- MRI abnormalities (mesiotemporal hyperintensities, atrophy pattern)
- EEG abnormalities (slowing, epileptic activity or extreme delta brush)

"Red flag" criteria should always prompt determination of anti-neuronal autoantibodies in psychiatric patients. "Yellow flag" criteria should raise suspicion of an autoimmune etiology and include autoimmune encephalitis in the differential diagnoses, in either case if several findings are present.

We further identified seven cases in which the lack of clinical improvement after antipsychotic ($n = 5$; 5%) or antiepileptic therapy ($n = 2$; 2%) led to the suspicion of an autoimmune encephalitis. Another two patients with psychotic symptoms

and cognitive impairment had the working diagnosis of steroid-responsive encephalopathy with autoimmune thyroiditis (SREAT), which triggered antibody testing that resulted in positive NMDAR ($n = 2$; 4%) and onconeuronal ($n = 1$; 4%) antibodies (**Table 3**). Finally, in one case, the patient's family suggested the diagnosis of autoimmune encephalitis after internet research, prompting the testing of NMDAR antibodies which returned positive.

Taken together, several clinical symptoms and abnormalities repeatedly led to antibody testing, bringing about the correct diagnosis of autoimmune encephalitis. We consider these warning signs as "red flags" (**Table 4**) which might facilitate earlier diagnosis of autoimmunity in psychiatric symptoms.

Time from First Symptom to Diagnosis

Given that the prognosis in patients with autoimmune encephalitis depends on the rapid initiation of immunotherapy, we next analyzed the time between symptom onset and diagnosis. For this, patients who were treated primarily in a psychiatry department ($n = 35$) were divided in two groups. In the first group, symptoms started between 2007 and 2012. Here, the delay was very prolonged with a mean time of 483 days (**Figure 2A**). In the second group with symptom onset between 2013 and 2016, the mean time between disease onset and diagnosis was 74 days (**Figure 2B**). The reduction was obvious in both groups for which data were available, namely the NMDAR encephalitis (reduction from 475 to 40 days) and non-NMDAR antibody group (reduction from 509 to 150 days). It seems likely that increased awareness of this new disease group after 2012 and a lower threshold for antibody testing in clinical routine helped to markedly reduce the delay, even though there is an obvious need and opportunity for further improvement.

Earlier Diagnosis of Autoimmune Encephalitis in Psychiatric Patients Using the "Red Flags"

Having established warning signs ("yellow flags" and "red flags") that may guide clinicians in the indication for autoantibody testing in patients with different autoimmune encephalitides (**Table 4**), we then retrospectively applied these criteria to our cohort of encephalitis patients hospitalized in a psychiatric ward. In this way, we aimed to estimate the potential reduction in delay between symptom onset and diagnosis of autoimmune encephalitis. Indeed, reanalysis of the medical records showed that most patients had well-documented evidence of "yellow flag" and "red flag" criteria in their medical records, long before an autoimmune etiology and antibody testing was considered. As a typical example, a patient with a schizophreniform syndrome developed catatonia and autonomic instability (both are "yellow flags") 4 weeks after the symptom onset, but only an epileptic seizure 10 weeks after symptom onset prompted autoantibody testing and revealed positive NMDAR antibodies. We then calculated the time from symptom onset to diagnosis, hypothetically assuming that the first documentation of a "red flag" in the medical chart would have resulted in the determination of autoantibodies. In this example, using the "yellow flag" and "red

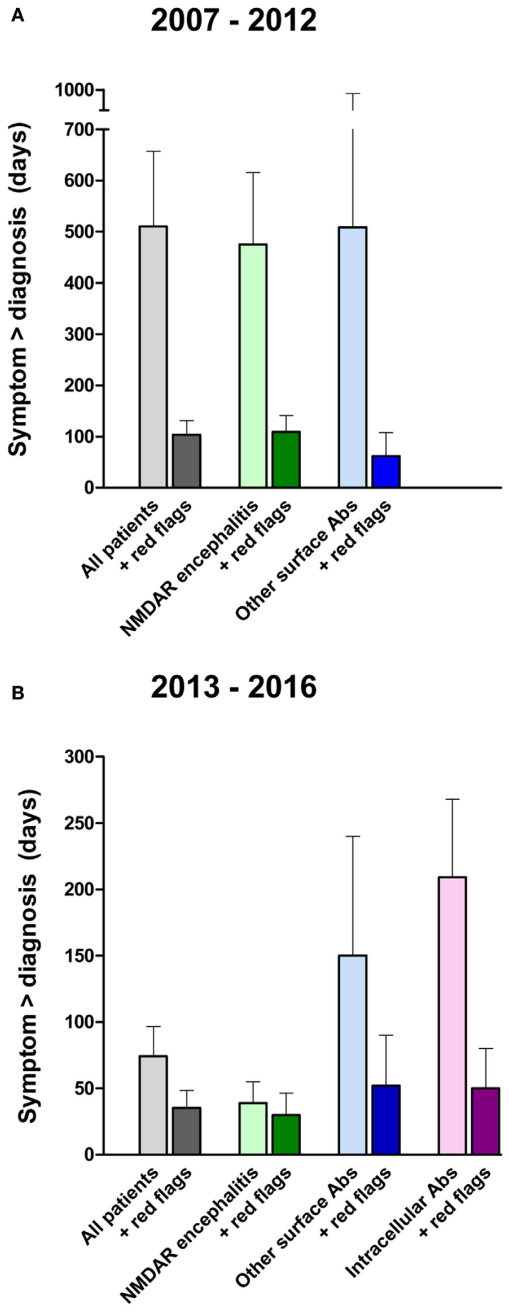

FIGURE 2 | Time between onset of clinical symptoms and diagnosis of antibody-associated encephalitis. Comparing patients with disease onset between 2007 and 2012 **(A)** versus 2013 to 2016 **(B)**, the delay from symptom onset to the diagnosis of autoimmune encephalitis (light colors) has been reduced within the last years, likely due to increased awareness (please note the different *y*-axes). Applying the "red flag" criteria to the same patients by reanalyzing medical records resulted in a marked hypothetical reduction of the delay until antibody testing and encephalitis diagnosis (dark colors).

flag" criteria reduced the delay from symptom onset to diagnosis from 10 to 4 weeks.

Indeed, the analysis of our cohort showed a marked reduction in the time until diagnosis (**Figure 2**). For the more recent patients with symptom onset between 2013 and 2016, a reduction of 58% from 74 to 31 days was detectable. In detail, time between appearance of first symptoms and final diagnosis was reduced from 40 to 10 days (75%) in patients with NMDAR encephalitis, 150 to 52 days (65%) in patients with non-NMDAR surface antibodies, and 209 to 50 days (76%) in patients with antibodies against intracellular epitopes (**Figure 2B**).

DISCUSSION

In accordance with recent publications (7, 8, 24), our results confirm that a broad spectrum of psychiatric symptoms frequently are the first complaints in patients with autoimmune encephalitis. While psychosis typically led to hospitalization of patients with NMDAR encephalitis, a psychosomatic disorder was often suspected in patients with surface non-NMDAR and intracellular epitope antibodies. The "psychosomatic" symptoms included, for example, FBDS in LGI1 antibody-positive patients, muscle spasms, and fasciculations in Caspr2 antibody-positive patients or sensory deficits in patients with onconeuronal antibodies. Interestingly, most patients in all three encephalitis groups showed additional neurological symptoms during the first month of disease.

Analysis of the present cohort of 100 encephalitis patients showed that several clinical symptoms or laboratory findings eventually led to the suspicion of an autoimmune etiology and the determination of autoantibodies. These "yellow flags" and "red flags" are summarized in **Table 4**, classified based on their predictive value to point to an underlying autoimmune encephalitis in the clinical workup of patients with psychiatric abnormalities. Given that systematic controlled trials and systematic reviews of cohort or case-control studies are lacking due to the novelty of this field and the relative rarity of autoimmune encephalitides, this case series analysis can only represent level 4 of evidence. Generally, some constellations are very typical for a given form of encephalitis, e.g., the presence of new-onset psychosis in young women with ovarian teratomas indicating NMDAR encephalitis or the combination of amnesia, hyponatremia, and the pathognomonic FBDS (brief repetitive stereotyped movements predominantly affecting the arm and ipsilateral face) indicating LGI1 antibody encephalitis. Clearly, typical features can be absent and delay the proper diagnosis (11, 25–27). Also, future work will likely add further or modify the proposed criteria.

The most common triggers for autoantibody diagnostic were CSF abnormalities in the absence of an infectious disease. The symptom overlap with viral encephalitis is remarkable regarding neurological and psychiatric changes (28, 29), suggesting that autoantibodies should always be determined, at the latest if virus diagnostic (using PCR) remains negative. CSF is abnormal in almost all patients with NMDAR encephalitis during the disease course (11, 24), underlining the relevance of routine CSF testing in psychiatric patients. This is also valid for the other forms of encephalitis, although patients with LGI1 antibodies have a lower frequency of CSF pleocytosis (41%) or elevated protein (47%) and rarely have intrathecal LGI1 antibody synthesis (25).

The occurrence of epileptic seizures in a psychotic patient was another common reason to reassess the working diagnosis of a primary psychiatric disease and test for antibodies. EEG changes not explained by medication are almost always present in autoimmune encephalitis. The alterations are rarely specific, showing focal or diffuse slow activity frequently associated with one or several foci of epileptic activity, eventually revealing subclinical seizures (27). However, the pattern referred to as "extreme delta brush" in NMDAR encephalitis is quite disease-specific (30). Suspicious MRI findings led to the correct diagnosis in relatively few cases in the present cohort (10%), which is likely explained by the fact that brain MRIs are unremarkable in more than 50% of patients with NMDAR encephalitis (11, 23, 28). If present, however, MRI abnormalities should always prompt autoantibody investigation, even though other diseases might cause similar imaging changes, such as gliomas (25, 28, 31).

Lejuste et al. observed a very high rate of patients with NMDAR encephalitis in which intolerance to antipsychotic drugs led to transfer to a Neurology department or intensive care unit (7). In line with their findings, the combination of autonomic instability and increased creatine kinase levels after neuroleptic therapy in several cases led to the suspicion of a malignant neuroleptic syndrome. Therefore, we included progression under antipsychotic therapy, suspected malignant neuroleptic syndrome and autonomic instability to the "red flag" criteria (**Table 4**). Finally, the presence of an autoimmune thyroiditis together with psychotic symptoms and cognitive impairment resulted in antibody investigation in three cases in the present cohort. It was shown recently that serum thyroid antibodies were elevated in 24.7% of 180 psychotic patients (10). Beneficial effects from steroids suggest the less well-defined constellation of SREAT (32). However, occurrence of specific brain-directed antibodies in our cohort (e.g., NMDAR antibodies) support the idea that SREAT represents increased susceptibility to autoimmunity, rather than that antithyroid antibodies are directly pathogenic. Findings of elevated thyroid peroxidase and thyroglobulin antibodies in psychotic patients should nonetheless raise suspicion and guide autoantibody testing.

Apart from the clinical application of the here proposed criteria, the present study reinforces the recent discussion that autoantibodies may participate in the development of psychiatric disorders, such as schizophrenia, in greater extend than previously assumed. For example, the reduction of NMDAR-specific currents and consecutively impaired glutamatergic neurotransmission is well known under the NMDAR hypofunctionality hypothesis of schizophrenia (33). In parallel, synaptic and extrasynaptic reduction of NMDAR by autoantibodies in NMDAR encephalitis leads to the typical schizophreniform symptoms seen in these patients (34). While internalization of NMDAR after contact with autoantibodies has been established as an important disease mechanism (16, 35), further pathologies are likely to happen in parallel, such as chemokine transfer

from immune cells to NMDAR-bearing neurons *via* volume transmission (36). It seems that these novel synaptic and extrasynaptic autoimmune disorders have brought about a paradigm shift in neuropsychiatry, and further research is urgently needed to clarify the detailed mechanisms of how autoimmunity and inflammation cause or modify neuropsychiatric diseases.

An important finding of our study was the alarmingly long delay between first symptoms and the final diagnosis of autoimmune encephalitis in many cases. It is known from the literature that patients with autoimmune encephalitis have often been misdiagnosed with a sole psychiatric disease despite the presence of neurological comorbidities (7). We could show here that the identification of encephalitis patients occurred much faster in more recent cases (2013–2016) compared to earlier patients, likely due to increased awareness of this novel disease group. The data collectively suggest that continuing increase in disease awareness will lead to further shortening of the time until diagnosis. This is needed as early and sufficiently aggressive immunotherapy is required for a better prognosis (22, 23, 25, 26). Using the here proposed "yellow flag" and "red flag" criteria will likely facilitate the timely diagnosis of an autoimmune psychiatric disease, as demonstrated by the hypothetical reanalysis of our cohort for the presence of such clinical signs. Finally, we conclude that CSF analysis should become clinical routine in patients with new-onset psychosis for several reasons. First, CSF abnormalities were the major indicator for an autoimmune encephalitis in psychotic patients. Second, some antibodies including NMDAR antibodies can be present in CSF only and would therefore be overlooked in serum (37). Third, recent data suggest that the rate of CSF abnormalities can be >50%, thus being much higher than previously thought and an important step to identify patients with treatable etiologies (10). Taken together, the threshold for CSF analysis and autoantibody testing should be low, in particular, when "red flags" are present.

AUTHOR CONTRIBUTIONS

JH and HP initiated the study and conducted the data analyses, wrote the paper, performed the data collection, read and approved the final version of this manuscript.

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ARTICLE OPEN ACCESS

Normal gut microbiome in NMDA receptor encephalitis

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Abstract

Objective

To determine whether the gut microbiota shows overabundance of commensal bacteria species in patients with anti-NMDA receptor (NMDAR) encephalitis, similar to patients with MS or neuromyelitis optica where they potentially balance pro- and anti-inflammatory immune responses or participate in disease pathogenesis by molecular mimicry.

Methods

Intestinal microbiota was characterized in patients with NMDAR encephalitis (n = 23, mean age: 34 ± 12.7 years; 21 females) and age/sex/environment-matched healthy controls (n = 24, 40 ± 14.2 years; 22 females) using stool bacteria 16S rDNA sequencing and classification in operational taxonomic units (OTUs). Statistical analyses focused on intraindividual and interindividual bacterial diversity and identification of differentially abundant taxa.

Results

Patients with NMDAR encephalitis and controls had similar microbiome profiles of the gut microbiota regarding intraindividual bacterial diversity, OTU distribution, ratio between regional and local species diversity when testing all OTUs, and genera with a relative abundance greater than 0.5%. Similarly, the subgroup of NMDAR encephalitis patients with an ovarian teratoma (n = 3) showed no differences in microbiome variation compared with controls. Patients in the acute encephalitis stage (n = 8) showed significant differences in the numbers of *Clostridium XVIII*, *Clostridium IV*, *Oscillibacter*, *Prevotella*, and *Blautia*; however, significance was lost after correction for multiple testing.

Conclusion

Patients with NMDAR encephalitis and controls both had a normal gut microbiome. The lack of overabundance of certain bacterial species in patients suggests that microbiome changes are no major contributors to the pathogenesis, disease course, or prognosis in NMDAR encephalitis. Despite the small sample size and heterogeneous groups, findings indicate differences to other neuroimmunologic diseases.

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Go to Neurology.org/NN for full disclosures. Funding information is provided at the end of the article.

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Glossary

AQP = aquaporin-4; **EAE** = experimental autoimmune encephalomyelitis; **GLM** = generalized linear model; **MDS** = multidimensional scaling; **NMDAR** = NMDA receptor; **NMOSD** = neuromyelitis optica spectrum disorder; **OTU** = operational taxonomic unit.

The role of the gut microbiota has been increasingly recognized in neurologic diseases. Overabundance of commensal bacteria species was seen in Guillain-Barré syndrome, Parkinson disease, MS, and neuromyelitis optica spectrum disorder (NMOSD).[1–8] Experimental data in germ-free mice demonstrated increased susceptibility to experimental autoimmune encephalomyelitis after selective bacterial colonization, indicating a role of the gut microbiota for balancing proinflammatory and anti-inflammatory immune responses.[9,10] Intestinal microbiota may even be a novel therapeutic target for extraintestinal inflammatory diseases as shown for possible beneficial effects in patients with MS from nutritional administration of a probiotic[11] and potentially from fecal microbial transplantation.[12] Recently, overabundance of the bacterium *Clostridium perfringens*, a commensal bacterium of the gut microbiota, was demonstrated in 16 patients with NMOSD, indicating a potential role in the disease pathogenesis.[5]

Similar to NMOSD, anti-NMDA receptor (NMDAR) encephalitis is an antibody-mediated disease of the CNS, and there are even cases of overlap between the 2 entities.[13] Autoantibodies against the NR1 subunit of the NMDAR are directly pathogenic and underlie the clinical disease spectrum ranging from amnesia, psychosis, and epileptic seizures to dyskinesias, coma, and vegetative dysfunction.[14,15] The potential role of the gut microbiome for the development of NMDAR encephalitis is particularly interesting as only few disease triggers are known, such as ovarian teratomas,[16] viral infections of the brain,[17,18] and potentially seasonal factors.[19] We therefore aimed to investigate the intestinal microbiome of patients with NMDAR encephalitis and healthy controls.

Methods

Standard protocol approvals, registrations, and patient consents

The study was approved by the Charité ethics committee (EA1/274/16) and Charité data protection (#0626/16/ST3). All study participants gave their written informed consent.

Study population

Twenty-eight patients with NMDAR encephalitis were recruited from the Charité Centre for Autoimmune Encephalitis during spring 2017. Five patients and 3 controls received antibiotic treatment within the last 6 weeks before stool collection (for cystitis, pyelonephritis, tonsillitis, sinusitis, or bacterial vaginosis) and were therefore excluded from the analyses. Of the remaining 23 patients with NMDAR encephalitis, 2 (9%) had acute encephalitis, 6 (26%) were in the recovery phase with persisting clinical deficits, and 15 patients (65%) had recovered. Three patients (13%) had an ovarian teratoma removed during clinical workup. Almost all patients (n = 21; 91%) were female; thus, sex was not considered a covariate. Demographic and clinical characteristics are shown in table e-1 (links.lww.com/NXI/A156).

All patients had received immunotherapy according to current guidelines. These included the following:

1. Steroids in 18 patients (78%), 1,000 mg IV methylprednisolone for 5 days, and mean 2.0 cycles;
2. IV immunoglobulins in 11 (48%), 2 g/kg body weight, and mean 1.9 cycles;
3. Plasmapheresis in 15 (65%) and mean 3.6 series of 5–10 sessions each;
4. Rituximab in 13 (57%), 1,000 mg per cycle every 6 months, and mean 2.7 cycles (3 patients ongoing);
5. Azathioprin in 3 (13%), 250 mg/d for 4 months, 75 mg/d for 1.5 years, and 100 mg/d for 6 months, respectively;
6. Cyclophosphamide in 2 (9%), 750 mg/kg once in 1 patient, and 5 times in the second;
7. Methotrexate in 1 (4%), 10–15 mg/wk over 3.5 years;
8. Bortezomib in 1 patient (4%) and 2 cycles of 4 subcutaneous injections of 1.3 mg/m^2.

Five patients (22%) still received immunosuppression at the time of stool collection. In the others, the mean immunotherapy-free interval was 4.4 years.

The control group consisted of 24 case-matched healthy individuals. Matching was prioritized for similar living environment, lifestyle, and dietary habits. Most controls were friends of the same sex and age or family members (mostly siblings) who shared the apartment. Controls had no history of autoimmune or gastrointestinal disease or cancer.

Study protocol and clinical assessment

Patients and controls collected fecal samples at home in standard stool collection tubes. The samples were shipped immediately (within 24 hours) at room temperature and were stored at −80°C until processing.

A standardized survey was completed by all patients and controls including demographic data (birth, sex, body size and weight, and size of city of residence [metropolis, city, center city, small town, and village]), information on smoking history (never smoked, current smoker, and the number of pack-years), general activity level (type and extent of physical activity),

defecation history (general character of feces using Bristol stool scales, incidence of constipation or diarrhea within the last 3 months [never, sometimes 25%, often 50%, very often 75%, and always 100%], number of average defecation per week, rectal tenesmus, and presence of hematochezia and abdominal pain), dietary habits (nutritional style [omnivore, ovo-lacto-vegetarian, pesco-vegetarian, vegan diet, and others] and regular intake of probiotics), preexisting illnesses (including fever in the last 7 days), regular medication (beginning of intake, cause of medication, dosage, and antibiotics within the last 6 months), current general state of health (very good, impaired, bad, very bad, and unacceptable), need for current antibiotic treatment, and travel history (longer than 4 weeks within the last 12 months).

DNA extraction, 16S rDNA sequencing, and quality control

DNA was extracted using the QIAcube and the QIAamp DNA stool kit (Qiagen) and a prior beat-beating step. Variable regions v1-v2 of the 16S rRNA gene were amplified using the primer pair 27F/338R. PCR products were normalized using the SequalPrep Normalization Plate Kit (Life Technologies) and sequenced on an Illumina MiSeq (2 × 300 bp).

Raw data were obtained with exact agreement of the index sequences, demultiplexed with a dual-indexing approach, and subjected to quality control. This consisted of trimming low-quality sequence ends,[20] combining forward and backward amplicon reads into a single sequence (VSEARCH), quality control based on estimated error (VSEARCH), and sequence quality (FastX toolkit). Reference and DeNovo-based identification of chimeric sequences (VSEARCH) took place,[21] as well as a filtering step to exclude nonbacterial (and thus unspecific or erroneous) sequence data (Simple non-Bayesian taxonomy classifier [SINTAX]). The clean sequences were classified in operational taxonomic units (OTUs) (VSEARCH), normalized to 10,000 sequences per sample, and taxonomically annotated (SINTAX).[22] Based on this information OTU, an operational definition used to classify groups of closely related individuals, and taxon abundance tables were created for subsequent analysis.

Statistical analysis

Statistical analysis was performed on alpha- (intraindividual) and beta- (inter-individual) diversity measures (Bray-Curtis dissimilarity and UniFrac distance). For differences in alpha-diversity, the Wilcoxon rank-sum test was used when value distribution deviated from normality, otherwise the 2-sample *t* test. Alpha-diversity indices contained the Shannon diversity index (accounts for both abundance and evenness of the species present) and the Chao1 index (reflecting species richness).

Unconstrained multidimensional scaling (MDS) plots of beta-diversity measures were generated using the cmdscale function in R.[23] MDS plots generally visualize the level of similarity of individual cases of a data set. To test for differences in beta-diversity, permutational multivariate analysis of variance was performed using the adonis function of the *vegan* software package with the option sqrt.dist = T when using abundance tables, but not when using UniFrac distances, and 10,000 permutations. Tests for differential abundance were performed using zero-truncated generalized linear models (GLMs) with negative binomial distribution on taxonomic groups present in at least 2 samples per group and 10 samples in total.

Data availability

Anonymized data will be shared by request from any qualified investigator.

Results

Study participants

Subject characteristics of the 23 patients with NMDAR encephalitis and 24 unaffected controls are given in table e-1 (links.lww.com/NXI/A156). Forty-three of 47 participants were female, and the mean age was 34 years in patients and 40 years in controls. There were no differences between groups regarding smoking, obesity, place of residence, defecation at the time of stool collection with general stool consistency and stool frequency per week, occurrence of hematochezia, occasional abdominal pain, nutritional intake, diet, travel history, and general medication, such as for hypertension, contraception, or hypothyroidism (table e-1). Five (22%) patients with NMDAR encephalitis with persisting symptoms still received immunosuppression or neuroleptic medication (rituximab [n = 3], cortisone [n = 1], bortezomib [n = 1], risperidone [n = 2], and lamotrigine [n = 1]), which could theoretically alter the gut microbiome. Two patients and 3 controls had probiotic intake.

Similar microbiome profiles in patients with NMDAR encephalitis and controls

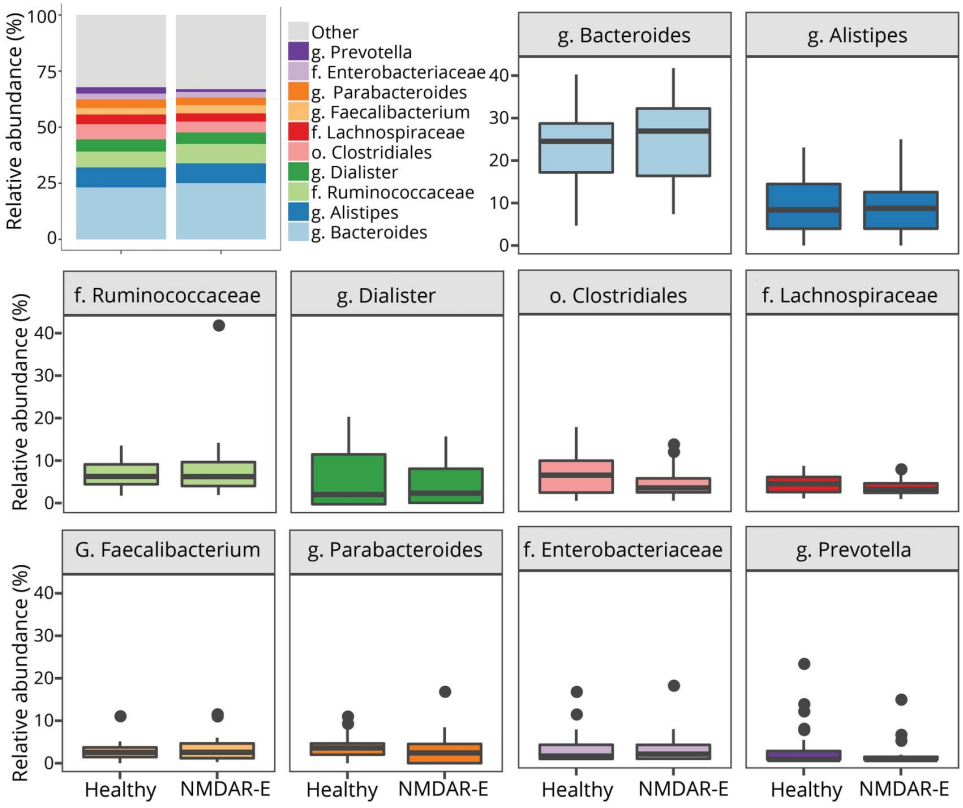
The average microbiome profile of patients with encephalitis and healthy controls did not reveal any differences between groups (figure 1). Regarding alpha-diversity (reflecting intraindividual bacterial diversity), no difference in species diversity within the gut microbiota was observed. The Shannon diversity index based on OTU distribution (groups of closely related individuals) did not reveal any significant difference between both groups. Similarly, the Chao1 index was not different (Mann-Whitney *U* test, figure 2).

Beta-diversity (interindividual dissimilarity) also did not differ significantly between patients and healthy controls. The lack of differences for genus and OTU is visualized using MDS plots (figure 3), i.e., showing the level of similarity of individual cases (adonis analysis: genera: $p > 0.3$; OTU: $p > 0.8$). All OTUs and genera with a mean abundance greater than 50 reads per sample (equivalent to 0.5% relative abundance) were tested in a GLM or hurdle model. None of the results were significantly different (all $q > 0.05$) after correction for multiple testing.

Alpha- and beta-diversity in patients with NMDAR encephalitis with a teratoma

Three of the 23 patients with NMDAR encephalitis had a teratoma, thus representing a subgroup with potentially different

Figure 1 Average gut flora profiles of patients with NMDAR encephalitis and healthy controls



Shown are all families and genera with a mean abundance of more than 2.5%. Remaining genera are summarized in the group "other."

immunologic mechanisms. Patients were compared with age- and sex-matched controls living in the same household. None of the 3 patients received immunosuppressive medication at the time of stool collection, and 2 had L-thyroxine for hypothyroidism. None of the controls required medication including antibiotics. All subjects were on omnivore diet, and the stool was inconspicuous.

No differences in microbiome variation were seen between patients with NMDAR encephalitis after teratoma removal and controls. Alpha-diversity was equal between both groups regarding Shannon diversity (genera/OTUs), the number of genera/OTUs, or the Chao1 estimator at the generic and OTU levels. Also, beta-diversity was not significantly different at the genus ($p = 0.62$) and OTU levels ($p = 0.32$, data not shown).

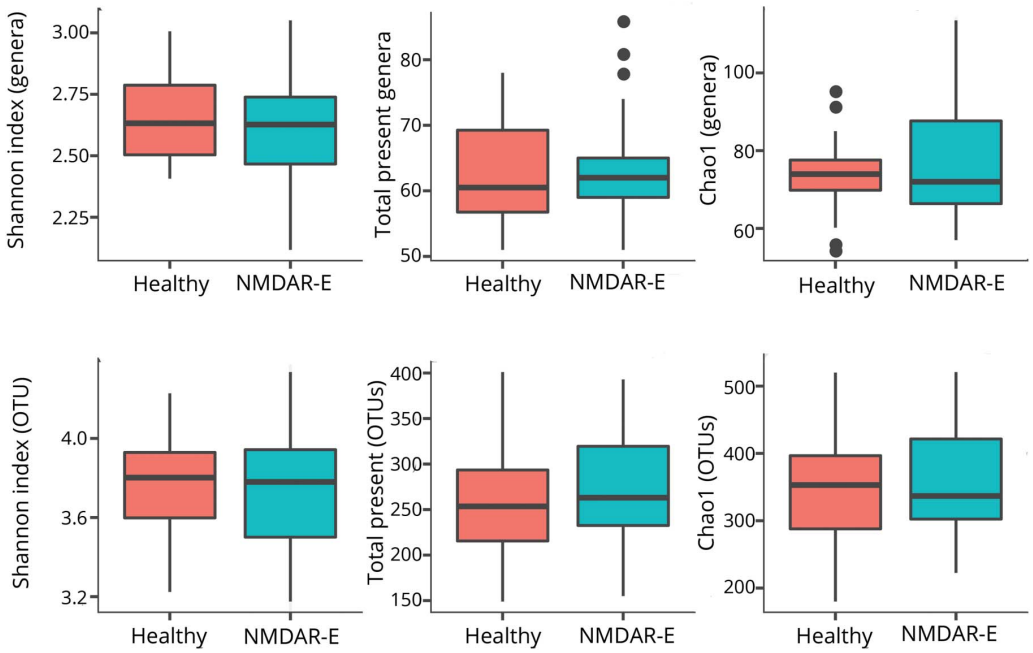
Alpha- and beta-diversity in patients with NMDAR encephalitis with acute illness

Eight of the 23 patients still had clinical symptoms of NMDAR encephalitis at the time of examination. Seven (88%) were female, and 5 (63%) received immunosuppressive therapy at the time of stool collection. These patients were compared with the 15 NMDAR encephalitis after disease recovery (14 [93%] females, 2 [13%]) on immunosuppressive therapy. All participants had normal stool habits and stool conditions at the time of examination. Comparing the intraindividual bacterial diversity between 2 patient groups showed no significant differences in the Shannon diversity index. However, significant differences in the number of genera were detected for *Clostridium XVIII*, *Clostridium IV*, *Oscillibacter*, *Prevotella*, and *Blautia* (figure 4), although significance was lost after Bonferroni correction (table e-2, links.lww.com/NXI/A156). Regarding beta-diversity, there were again no differences at the genus ($p = 0.082$) and OTU levels ($p = 0.15$).

Discussion

In this first study on the gut microbiome in patients with NMDAR encephalitis, data showed no overabundance of certain bacterial taxa compared with controls. Both patients and healthy participants had a "normal" microbiome consistent with previous reports of the healthy gut where gram-negative Bacteroides and gram-positive Firmicutes, including Clostridiales and Lactobacillaceae, dominate.[24,25] Similarly, no

Figure 2 Boxplots of the alpha-diversity indices

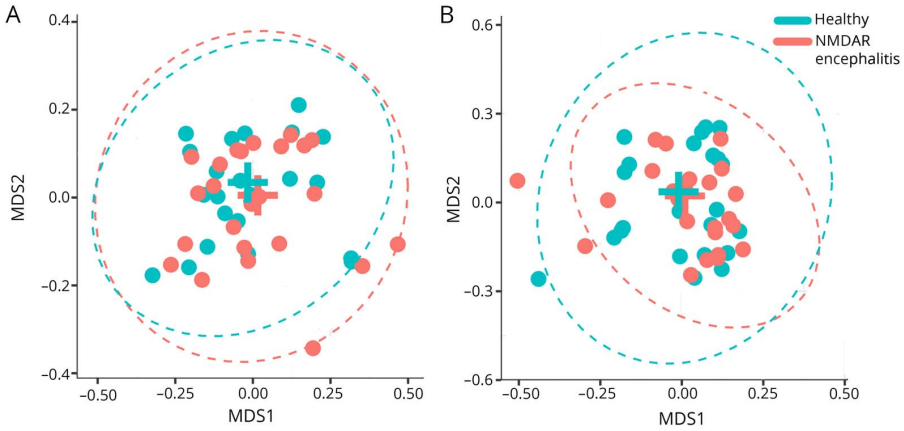


There were no significant differences in diversity (Shannon Index) and number or richness (Chao1) of gut microbiome genera and species within genera (OTUs) between patients with NMDAR encephalitis and healthy controls.

differences were seen in subgroups of patients with ovarian teratomas or in patients during the active disease phase.

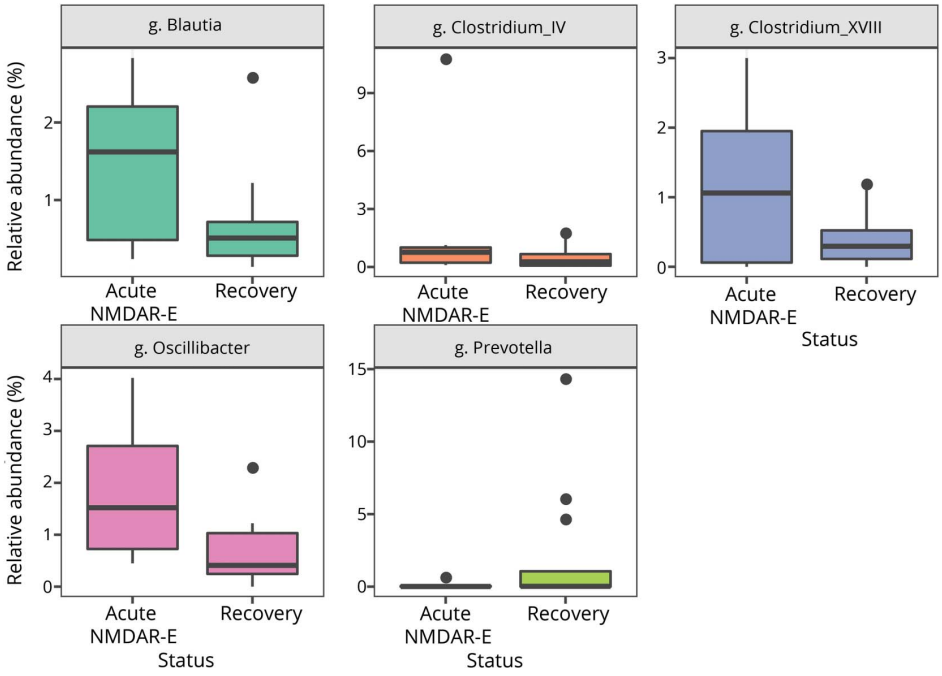
The study therefore provides no support for the hypothesis that microbiome changes are major contributors to pathogenesis, disease course, or prognosis in NMDAR encephalitis, although it is still possible that higher patient numbers could reveal differences. It is also possible that the microbiome is different only during the initial phase of the disease compared with remission. Indeed, our subgroup of

Figure 3 MDS plots of Bray-Curtis distances at the genera and OTU levels



Data show a high level of similarity regarding interindividual differences between patients with NMDAR encephalitis and healthy controls for genera (A) and OTU level (B). The ellipses represent the 99% CIs of the SDs within the groups around the mean values marked by the position of the group names.

Figure 4 Overabundance of bacterial species in active NMDAR encephalitis



Five genera showed significant difference in overall abundance in patients with acute NMDAR encephalitis compared with recovered patients. The differences were not present after correction for multiple testing.

patients with acute encephalitis had overabundance of 5 genera including *Clostridium XVIII* and *IV*, which, however, did not withstand statistical correction for multiple testing.

In the somewhat related neurologic disease NMOSD with autoantibodies targeting the water channel aquaporin-4 (AQP4), *C perfringens* was enriched in the gut microbiota of 16 patients vs 16 controls.[5] As T cells from NMOSD cross-react with a homologous sequence of a *C perfringens* adenosine triphosphate-binding cassette transporter,[26] the authors concluded that the microbiota might be involved in NMOSD pathogenesis.

It is therefore likely that different mechanisms initiate and drive disease in NMOSD and NMDAR encephalitis. For example, approximately one-third of female patients with NMDAR encephalitis have an ovarian teratoma, which contains neuronal elements expressing NMDAR and is thought to trigger the encephalitis.[16,27] In contrast, tumors are rare in patients with NMOSD. Another potential difference is the role of T cells for disease. Although T cells in patients with NMOSD proliferate in response to AQP4 protein,[26,28] no such role has yet been established for T cells from patients with NMDAR encephalitis. In fact, neuropathologic findings including the absence of relevant numbers of T cells in the brain suggested that NMDAR encephalitis is a predominantly humoral autoimmune disease.[27,29] Furthermore, molecular mimicry of NMOSD T cells between AQP4 and a *Clostridium* protein might contribute to NMOSD pathogenesis, whereas no cross-reaction of T cells and antibodies were so far observed in NMDAR encephalitis. The absence of detection of a consistent infectious agent makes an immune response by molecular mimicry unlikely.[14]

Differences in immunosuppression between groups had no effect on the composition of the gut microbiota, although conclusions about a clear effect would likely require higher patient numbers or stool samples from untreated patients with NMDAR encephalitis. These might be difficult to obtain as immunotherapy is usually started immediately after confirmation of the diagnosis with positive serum and CSF antibodies. In NMOSD, rituximab treatment did not account for changes in the microbiome.[5]

Despite the limitations of relatively small sample sizes, heterogeneity of the groups, and the majority of patients being in the recovery phase, the present work argues against a major influence of the gut microbiome in NMDAR encephalitis. It thereby reminds us of how little we still know about etiology and disease mechanisms in NMDAR encephalitis, in particular about the role of T cells and further immune cells. It is finally possible that microbiota-driven effects are still in place—not reflected by mere

overrepresentation of certain bacterial species, but rather by dysfunctional immune responses to numerically normal microbiota, potentially shaped by genetic, metabolic, or psychosocial factors.

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Corinna Bang, PhD	Christian Albrechts University of Kiel, Germany	Author	Major role in the acquisition of data and drafted the manuscript for intellectual content
Malte C. Rühlemann, PhD	Christian Albrechts University of Kiel, Germany	Author	Major role in the acquisition of data and drafted the manuscript for intellectual content
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Normal gut microbiome in NMDA receptor encephalitis

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RESEARCH ARTICLE

Human Gestational *N*-Methyl-D-Aspartate Receptor Autoantibodies Impair Neonatal Murine Brain Function

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Objective: Maternal autoantibodies are a risk factor for impaired brain development in offspring. Antibodies (ABs) against the NR1 (GluN1) subunit of the *N*-methyl-D-aspartate receptor (NMDAR) are among the most frequently diagnosed anti-neuronal surface ABs, yet little is known about effects on fetal development during pregnancy.
Methods: We established a murine model of in utero exposure to human recombinant NR1 and isotype-matched non-reactive control ABs. Pregnant C57BL/6J mice were intraperitoneally injected on embryonic days 13 and 17 each with 240μg of human monoclonal ABs. Offspring were investigated for acute and chronic effects on NMDAR function, brain development, and behavior.
Results: Transferred NR1 ABs enriched in the fetus and bound to synaptic structures in the fetal brain. Density of NMDAR was considerably reduced (up to −49.2%) and electrophysiological properties were altered, reflected by decreased amplitudes of spontaneous excitatory postsynaptic currents in young neonates (−34.4%). NR1 AB-treated animals displayed increased early postnatal mortality (+27.2%), impaired neurodevelopmental reflexes, altered blood pH, and reduced bodyweight. During adolescence and adulthood, animals showed hyperactivity (+27.8% median activity over 14 days), lower anxiety, and impaired sensorimotor gating. NR1 ABs caused long-lasting neuropathological effects also in aged mice (10 months), such as reduced volumes of cerebellum, midbrain, and brainstem.
Interpretation: The data collectively support a model in which asymptomatic mothers can harbor low-level pathogenic human NR1 ABs that are diaplacentally transferred, causing neurotoxic effects on neonatal development. Thus, AB-mediated network changes may represent a potentially treatable neurodevelopmental congenital brain disorder contributing to lifelong neuropsychiatric morbidity in affected children.

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Proper fetal development in mammals requires complex mechanisms regulated from both fetus and mother. One maternal immune mechanism to protect the fetus is passive immunity, where maternal immunoglobulin G (IgG) is transferred via neonatal Fc receptors (FcRn).[1] Transfer of maternal antibodies (ABs) in humans starts from gestational week 13 in the second trimester,[2] when the fetal blood–brain barrier (BBB) is still permeable,[3] creating a critical window for potentially harmful anti-neuronal ABs to compromise fetal brain development. Maternal immune activation including such ABs may therefore influence the etiology of neurodevelopmental disorders, including autism spectrum disorders (ASD), learning disabilities, and schizophrenia.[4,5]

Murine models of gestational transfer of maternal ABs have already revealed deleterious effects of some anti-neuronal ABs, for example, anti-NR2B (GluN2B),[6] anti–fetal brain ABs from mothers of children with ASD,[7,8] and anti-Caspr2 ABs.[9,10] So far, little is known about maternal ABs against the NR1 (GluN1) subunit of the *N*-methyl-D-aspartate receptor (NMDAR), although it is among the most frequent anti-neuronal ABs in clinically asymptomatic individuals, with an IgG seroprevalence of 1%.[11,12] This creates a considerable subgroup of pregnancies in which NR1 ABs can be diaplacentally transferred. A pathogenic role for impairment of fetal brain development by such ABs seems possible for several reasons. First, monoclonal human NR1 ABs cloned from anti-NMDAR encephalitis patients were directly pathogenic by disrupting synaptic NMDAR currents.[13] Second, in vitro and in vivo studies in adult animals demonstrated human NR1 AB-mediated internalization of NMDARs, resulting in NMDAR hypofunction.[14–16] Third, during fetal brain development, NMDARs are crucial for proper axonal[17] and dendritic growth,[18] neuronal survival,[19] and glutamatergic synaptic transmission at early stages.[20] Fourth, animal models of NMDAR hypofunction during neurodevelopment, induced by either transient NMDAR blockage[21] or NR1 mutations,[22] were characterized by major developmental deficits, for example, growth retardation and impaired cognitive functions.

We therefore hypothesized that maternally transferred NR1 ABs can cause fetal NMDAR hypofunction, leading to impaired brain development and sustained deficits persisting into childhood and possibly adulthood. If verified, this could have far-reaching clinical implications, including therapeutic options to prevent developmental brain abnormalities and lifelong psychiatric morbidity in affected children. Hence, we established a mouse model of gestational transfer of human monoclonal NR1 ABs and determined effects on NMDAR function, brain development, and behavior in the offspring.

Materials and Methods

Animal Experiments

Animal experiments were carried out in accordance with the ARRIVE guidelines, the EU directive (2010/63/EU) for animal experiments, and were approved by the local ethics committee for Animal Welfare (LaGeSO, Berlin, G0175/15).

At gestational days E13 and E17, 8- to 10-week-old pregnant C57BL/6J mice were either not treated (n = 6) or injected intraperitoneally with 240μg human monoclonal IgG1 ABs each (nonreactive control clone: #mGo53 [n = 40; CTL], high-affinity NR1-reactive [amino-terminal domain] IgG1 clone: #003-102 [n = 47; NR1])[13,23,24] in sterile phosphate-buffered saline (PBS). Further controls included identical amounts of monoclonal IgG1 ABs against glial fibrillary acidic protein (GFAP; clone: #011-116[13] [n = 15]) and human immunoglobulins (intravenous immunoglobulin [IVIG]; Grifols, Barcelona, Spain [n = 15]). AB amounts were 10- to >100-fold lower than in comparable passive immunization studies[25,26] and resulted in maternal serum NR1 AB levels of 1:100 to 1:320 in routine cell-based assays. Animals were sacrificed as indicated in Figure 1. Offspring were housed in treatment-mixed groups of 2 to 5 mice of both sexes.

Production of Human Monoclonal ABs

Recombinant human monoclonal NR1-reactive IgG1 ABs (clones #003-102 and #007-168) were generated from 2 female patients with acute NMDAR encephalitis and produced together with a nonreactive human monoclonal isotype-matched ABs (#mGo53) and an anti-GFAP ABs (#011-116) as described previously.[13,27] All ABs were IgG1; these cross the human placenta and murine yolk sac with highest efficiency of all IgG subclasses.[28,29]

Fetal AB Distribution

Embryos were harvested at E19, and whole embryo sections were methanol-fixed and stained with rat anti-CD31 (1:150; BD Biosciences, Franklin Lakes, NJ; #55370), rabbit anti-VGLUT1 (1:500; Synaptic Systems, Göttingen, Germany; #135303), and rabbit-anti-NR1 (1:250; Merck, Darmstadt, Germany; #AB9864). Secondary ABs included goat anti-human IgG-488 (1:1,000; Dianova, Hamburg, Germany; #109-095-003), goat anti-rabbit-568, and goat anti-rat-568 (1:1,000; Life Technologies, Carlsbad, CA; #A11036, #A11077).

Serum AB Kinetics

Trunk blood from postnatal day (P) 0, P7, P10, and P14 pups and respective dams was taken by decapitation, and human AB levels were determined using a commercial antihuman IgG enzyme-linked immunosorbent assay (ELISA; Mabtech, Nacka Strand, Sweden).

IgG Extraction from Mouse Brain

Brain-bound IgG in immunized pups was extracted from whole unperfused brains at P0 and P7 by an acid-based method adapted from a previous protocol.[15] Brains from P0/P7 pups (~100/200mg) were homogenized in 6/10ml PBS with

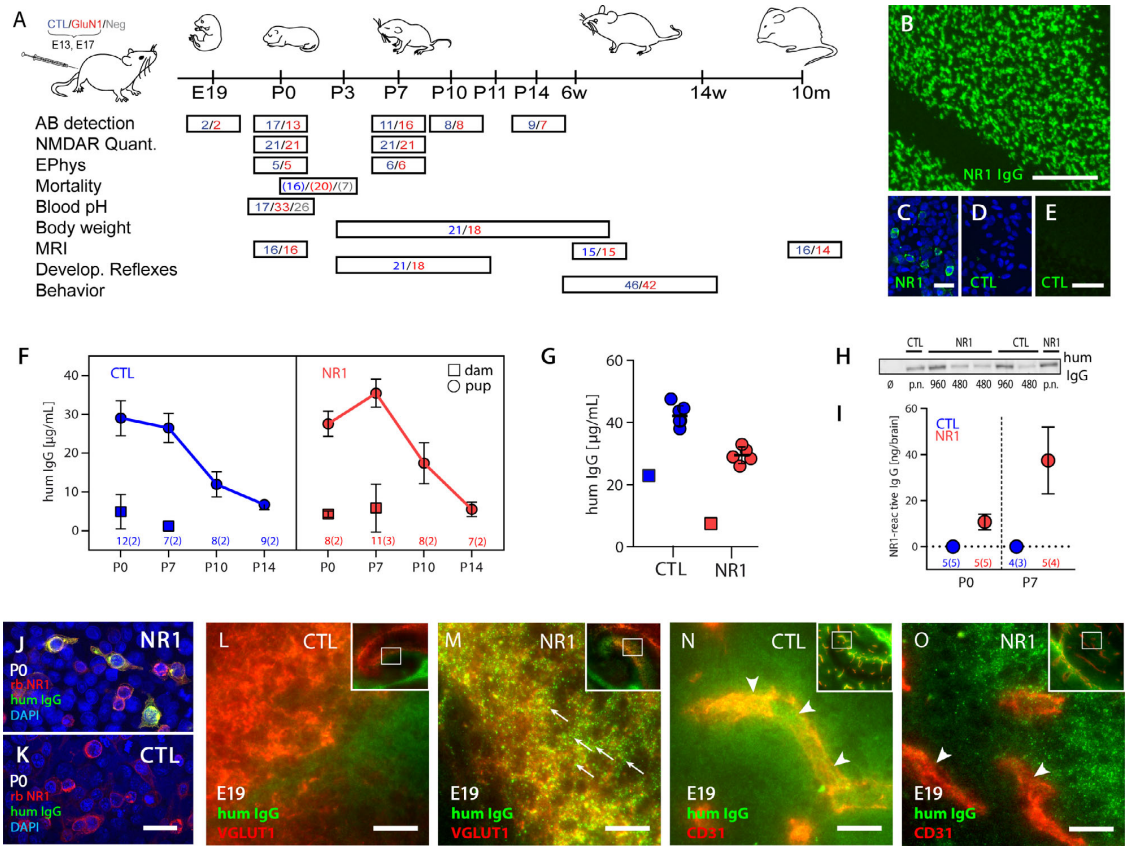

FIGURE 1: Experimental design and characterization of transfer of antibodies (ABs) in a gestational mouse model of maternal NR1 ABs. (A) Experimental design for characterization of AB transfer and neonatal development at different time points (n = pups; litters in parentheses) of control (CTL)/NR1/untreated dams. Blue = control, red = NR1, gray = untreated. (B, C) Human recombinant monoclonal NR1 ABs used for this study showed the typical staining on murine brain sections, for example, granular cells in cerebellum (B), and on NR1-transfected HEK cells (C). (D, E) The nonreactive human control ABs used throughout this study did not bind NR1-expressing HEK cells (D) or brain tissue (E). (F) Injected human ABs were transferred into the neonatal circulation with the highest concentrations at postnatal day (P) 0 for CTL and at P7 for NR1 AB-treated offspring, with declining level afterward. (G) AB injections into dams starting only postnatally (P0 and P3) resulted in high levels of neonatal ABs in both groups (P7), confirming that IgG ABs were also sufficiently transferred via breast milk. (H) Western blot (cropped) of neonatal brains detected human IgG in both CTL and NR1 AB-treated animals (960µg per dam; 480µg; Ø = untreated; p.n. = postnatal injection [480µg]). (I) Enzyme-linked immunosorbent assay quantification of NR1-specific ABs in brain IgG extracts revealed increasing levels of brain-bound IgG from P0 (mean = 10.7ng) to P7 (mean = 37.4ng) in the NR1 group. (J, K) Brain IgG extracts from NR1 AB-immunized neonates (P0) retained their binding capability to NR1 (J), whereas extracted IgG from the CTL group did not show any binding (K). (L, N) CTL ABs distributed diffusely in the fetal brain (L), mainly around blood vessels (N, *arrowheads*). (M, O) In contrast, NR1 ABs colocalized with punctate presynaptic VGLUT1 clusters (M, some clusters depicted with *arrows*), mainly outside vessels in the brain parenchyma (O). Inserts show the area of the micrograph in the hippocampus (L–O). Scale bars: B, E, 100µm, C, D, J, K, 20µm; L–O, 100µm. E = embryonic day; EPhys = electrophysiology; hum = human; MRI = magnetic resonance imaging; NMDAR = *N*-methyl-D-aspartate receptor; rb = rabbit.

proteinase inhibitors (PBS-PI; Roche, Basel, Switzerland; cOmplete ethylenediaminetetraacetic acid [EDTA]-free tablets). Six milliliters each were centrifuged, and the pellet was washed 3 times with 1ml PBS-PI to eliminate unbound IgG located in blood vessels. For extraction of bound IgG, the pellet was dissolved for 5 minutes in 258µl 0.1M Na-citrate buffer (pH 2.7) and centrifuged, and the supernatant was neutralized with 52µl 1.5M Tris (pH 8.8). Supernatant of the last wash (with 0.3ml) was analyzed as pre-extraction fraction to exclude residual free human IgG in the extract.

ELISA Quantification of Human NR1 ABs in Brain Extracts

Concentration of recombinant human NR1 AB #003-102 in brain extracts was determined in 96-well plates coated overnight at 4°C with donkey anti-rabbit IgG (20µg/ml, Dianova, #711-005-152). After blocking with 2% bovine serum albumin (BSA) in PBS/0.05% Tween-20 (PBS/T) at room temperature (RT), cell culture supernatants of HEK293 cells that expressed the amino-terminal domain (amino acids 1–400) of human NR1 fused to rabbit Fc were applied. Mouse brain extracts were

diluted 1:25/1:100 in 0.4% BSA-PBS/T and added in duplicates. Plates were washed with PBS/T and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-human IgG (1:5,000, Dianova, #709-035-149). After washing, HRP activity was measured using 1-Step Ultra TMB-ELISA substrate (Thermo Fisher Scientific, Waltham, MA). The concentrations of #003-102 in the extracts were deduced from a calibration curve generated with purified #003-102.

NR1-Specific Cell-Based Assay

NR1-transfected HEK cells were grown on coverslips, methanol-fixed, blocked for 30 minutes, incubated overnight with murine sera or IgG extracted from neonatal brains, and visualized as described.[13]

Quantification of NR1 Protein, GluR1, and Human IgG with Western Blots

Synaptosomal fractions were prepared from P0 pups with Syn-PER (Life Technologies, #87793). For membrane and total fractions, brains from P0 and P7 mice were homogenized in 10 volumes of homogenization buffer (0.32M sucrose, 10mM hydroxyethylpiperazine ethane sulfonic acid [HEPES] pH 7.4, 2mM EDTA) and centrifuged at $1,000 \times g$ for 10 minutes to remove nuclear fraction. An aliquot of supernatant was kept as total fraction. The remainder was centrifuged at $10,000 \times g$ for 15 minutes, and supernatant was centrifuged at $100,000 \times g$ for 60 minutes. Pellets from P0 and P7 pups were resuspended in 100 and 300µl sample buffer, respectively. CTL and NR1 AB-treated samples were loaded on 8% acrylamide gels, transferred via TurboBlot (Bio-Rad Laboratories, Hercules, CA) onto nitrocellulose, blocked, stained with rabbit-anti-NR1 (1:1,000, Merck, #AB9864) or rabbit anti-GluR1 (1:8,000; Millipore, Billerica, MA; #AB1504), and incubated with goat anti-rabbit IgG-HRP (1:6,000; Vector Laboratories, Burlingame, CA; #PI-1000). Membranes were developed with Western Lightning (PerkinElmer, Waltham, MA). Consecutive incubation was carried out with primary ABs for reference proteins (synaptosomal and total cell fraction: mouse anti-mortalin, 1:5,000, NeuroMab [Davis, CA], #75-217; membrane fraction: rabbit anti–β-actin, 1:3,000, Sigma-Aldrich [St Louis, MO], #A5060) and respective secondary ABs.

Quantification of NR1 Protein Expression with Immunohistochemistry

NMDAR clusters were detected with human monoclonal AB clone #007-168. To exclude that this monoclonal "detection AB" competes with the "treatment AB" (#003-102) for the same epitope, 1mg of each AB was labeled with 8.4µl CruzFluor 488 succinimidylester (10mg/ml; Santa Cruz Biotechnology, Santa Cruz, CA; #sc-362617). Murine brain sections were incubated with a fixed concentration of labeled AB plus increasing concentrations of either the identical unlabeled AB, the other AB (competition assay), or a control AB (Homer-1, Synaptic Systems, #160004). For NR1 cluster analysis, brain sections were incubated for 48 hours at 4°C with Homer-1 (1:200) and #007-168-Biotin (biotinylation kit, Thermo Fisher Scientific, #QE217779, 15µg/ml), followed by goat anti–guinea pig-568 (Invitrogen, Carlsbad, CA; #A-11075) and streptavidin-488 (Invitrogen, #A-32360). Two pictures from the CA3 region were taken from 3 individuals of ≥3 litters. Homer-1–positive synaptic sites were marked manually, and fluorescence intensities for human NR1 AB #007-168 were calculated automatically for each marked site and corrected for background fluorescence. Overlapping puncta of Homer-1 and human NR1 were counted and displayed as ratio of colocalized to synaptic puncta.

Electrophysiological Recordings

Horizontal hippocampal vibratome slices (350µm) from P0 or P7 pups of CTL and NR1 AB-immunized dams were transferred to a submerged holding chamber and stored in artificial cerebrospinal fluid (in mM: 129 NaCl, 1.25 NaH_2PO_4, 1.6 $CaCl_2$, 3.0 KCl, 1.8 $MgSO_4$, 21 $NaHCO_3$, 10 glucose) at 32 to 35°C for 30 minutes, followed by RT until recording. Whole-cell patch clamp recordings on CA1 pyramidal neurons were performed with borosilicate pipettes (1.5mm outer diameter) filled with internal solution containing (in mM) 125 CsCl, 2 Mg_2Cl, 10 HEPES, 2 ethyleneglycoltetraacetic acid, 2 Na2ATP, 0.3 NaGTP. Access resistance did not exceed 20MΩ and varied less than 20%. Signals were low-pass filtered at 2kHz and sampled at 10kHz. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded at −70mV for at least 4 minutes. To avoid depolarizing γ-aminobutyric acid (GABA) currents, GABAA-R antagonist SR-95531 was applied (1µM; Tocris Bioscience, Bristol, UK).

Determination of Blood pH

P0 pups from CTL and NR1 AB-treated animals were decapitated, and trunk blood was mixed with 2 volumes of heparinized NaCl to reach the minimal volume required for analysis with an ABL-800 system (Radiometer, Brønshøj, Denmark).

Measurements of Activity

Transponders were implanted into offspring at 12 to 14 weeks. Activity was tracked for 14 days with a Social Activity Monitoring system (PhenoSys, Berlin, Germany) based on an ID-Grid sensor plate recording individual spatiotemporal information.

Magnetic Resonance Imaging–Based Determination of Brain Volume

Magnetic resonance imaging (MRI) was performed on P0 pups and adult mice using a 7T small animal scanner (7T BioSpec +1H-Cryoprobe; Bruker, Karlsruhe, Germany). Animals were euthanized using isoflurane. Volumes of P0 animals were assessed using a high-resolution morphological T2-weighed TurboRARE sequence (repetition time/echo time = 4,000/34.5 milliseconds, 10 averages, rapid acquisition with relaxation enhancement [RARE] factor = 8, 36 coronal slices per 0.25mm, field of view = $12.8 \times 12.8mm^2$, matrix = 170×170, in-plane resolution = $75 \times 75\mu m^2$, scan time = 14 minutes). Volumetry was performed using Analyze 10.0 software (AnalyzeDirect, Overland Park, KS). For adult mice (repetition time/echo time = 4,250/33 milliseconds, 2 averages, RARE factor = 8,

40 coronal slices per 0.40mm, field of view = 19.2 × 19.2 mm^2, matrix = 192 × 192, in-plane resolution = 100 × 100μm^2, scan time = 3 minutes 24 seconds), volumetry was performed automatically using the MATLAB (MathWorks, Natick, MA) toolbox ANTX to nonlinearly register magnetic resonance images to the Allen brain atlas.[30]

Neurodevelopmental Scoring

Neonates were scored from days P3 to P56 based on established protocols.[31] The cutoff for surface righting reflex, cliff avoidance, and negative geotaxis reflex was 30 seconds.

Behavioral Assessment

A modified SHIRPA test to identify general abnormalities[32] showed a normal phenotype in all offspring prior to behavioral assessment.

Three-Chamber Test (Sociability). After 10 minutes of habituation to a 3-chambered arena for 6-week-old mice, an empty cage and a cage containing an unfamiliar C57BL/6J mouse (same sex) were placed in opposite side chambers. The location of cages was systematically alternated between animals. The number of times mice entered the chambers and came close to the cages were recorded by the video tracking system Viewer-3 (Biobserve, Bonn, Germany).

Barnes Maze. Seven-week-old mice were tested for learning and memory in a Barnes maze. They were trained for 4 days on a white platform to find a hidden escape box under 1 of 20 holes. Orientation cues were placed around the platform on the wall. Behavior and distance traveled into the 4 quadrants were recorded with Viewer-3. Cutoff was 180 seconds during training (4 trials every day for 4 days) and 90 seconds during testing (days 5 + 12, memory recall during 90 seconds).

Elevated Plus Maze. Eight-week-old mice on a white polyvinyl chloride (50 × 50 × 53cm) elevated plus maze with 2 open arms and 2 closed arms explored for 5 minutes, then activity was tracked using Viewer-3.

Prepulse Inhibition. Each test session in 9-week-old mice consisted of 5 trial types according to established protocols[33]: white noise (N56dB), acoustic startle (P120dB, 40 milliseconds), and 3 prepulse acoustic startle trials (PP69dB, PP73dB, and PP81dB for 20 milliseconds, 100 milliseconds before acoustic startle of P120dB for 40 milliseconds). Each session started with 10 minutes of acclimation, then 5 acoustic startle trials (P120dB), followed by 10 blocks of the 5 prepulse trials (PP69dB–P120dB, PP73dB–P120dB, PP81dB–P120dB) in pseudorandom order and another 5 acoustic startle trials (P120dB). Intertrial intervals ranged between 12 and 30 seconds.

Home Cage Scan. Natural behaviors in the home cage of freely moving mice were determined over 24 hours using HomeCageScan software (CleverSys, Reston, VA), including durations of grooming, twitching (indicator for repetitive behavior), consumption (eating and drinking), and sleeping.

Nest Construction Test. Nestlets (5 × 5cm squares of pressed white cotton) were placed in the cage on 2 consecutive days, and the nest was scored the next day for complexity according to established protocols.[34] The remaining intact nestlet material was weighed.

Flow Cytometry–Based Quantification of NR1 Reactivity in Human Maternal Sera

Serum from 120 healthy mothers of children with psychiatric disorders (MCPD) and 105 serum samples from healthy control mothers of unaffected children (CTLM) were collected at the Vivantes Department for Child and Adolescent Psychiatry, Berlin-Friedrichshain, Germany. NR1 autoreactivity was quantified in a flow cytometry–based approach using live HEK cells overexpressing native human NR1 protein as described previously.[23]

Methods to Prevent Bias

Pregnant dams were randomly assigned for injection. After weaning, mice of both treatment groups were mixed-housed. Within all experiments, animals or samples were used in an alternating manner. The investigator was blinded to group allocation and analysis for MRI, electrophysiology, and NR1-positive cluster quantification. For behavioral paradigms, sample size was determined by publicly available datasets, extracted numeric data by the use of PlotDigitizer (http://plotdigitizer.sourceforge.net/), and subsequent a priori power analysis using G*Power (http://www.gpower.hhu.de/).

Statistical Analysis

Statistical analyses were performed with Prism 7 (GraphPad Software, San Diego, CA) and R (https://www.r-project.org/). Data are presented as scatterplots with mean ± standard deviation or median with 95% confidence interval. Statistical analyses included *t* test, analysis of variance (ANOVA), and repeated measurement ANOVA following Holm–Sidak or Tukey multiple comparison test. Mann–Whitney and Kruskal–Wallis tests were used for nonparametric data, followed by Dunn multiple comparison test. Wilcoxon test was used to compare total activity of animals, 1-sided Fisher exact test for frequency distribution, and Kaplan–Meier log-rank test for survival with Bonferroni correction; $p < 0.05$ was considered statistically significant.

Results

Human NR1 ABs Were Diaplacentally Transferred and Bound to Synaptic Structures within the Neonatal Brain

Human monoclonal recombinant NR1 IgG1 ABs and isotype-matched nonreactive CTL ABs were injected at gestational days E13 and E17 (240μg each), and AB-mediated effects were investigated in the offspring at

different developmental stages (see Fig 1). Monoclonal human NR1 ABs used in this study showed the characteristic binding on murine brain sections, for example, to granular cells in the cerebellum and to NR1 protein in routine cell-based assays, whereas CTL ABs were nonreactive on NR1-transfected cells and brain sections. ELISA quantification in murine serum confirmed that human IgG were transferred into and enriched in neonates (up to P7 in NR1 AB-treated mice) with declining levels afterward. Injections starting postpartum proved transfer of human IgG also via breast milk. Western blot analyses detected comparable amounts of human total IgG in the brains of NR1 and CTL AB-treated animals. NR1-specific human IgG was found in neonatal whole brain extracts via ELISA only following prenatal NR1 AB injections with increasing concentrations between P0 and P7.

We next examined whether brain-accumulated NR1 ABs retained the capability of binding the NMDAR. Extracts of brain-bound IgG from NR1 AB-treated but not from CTL AB-treated animals were reactive to NR1-transfected HEK cells at days P0 (see Fig 1J, K) and P7 (not shown). The anatomical distribution was strikingly different between groups, as confirmed with immunohistochemical colabeling of fetal brain tissue (E19). Whereas CTL ABs were diffusely distributed in proximity to CD31-positive blood vessels, NR1 ABs overlapped with the presynaptic marker VGLUT1 in a characteristic punctate parenchyma pattern not confined to vasculature (see Fig 1).

Maternal NR1 ABs Reduced NMDAR Density and Changed Electrophysiological Properties in Early Postnatal Life

Observed binding of human ABs to NR1 led to the question of whether transferred ABs reduce density of NMDAR in the neonatal brain. Western blots of purified synaptosomes showed a significantly reduced density in NR1 AB-treated neonates, indicating profound loss of synaptic NMDAR, which was absent at P7 (Fig 2). Similarly, brain membrane fractions and total-cell fractions contained significantly less NR1 protein than in CTL AB-treated animals at P0, but returned to normal levels at P7. As a control, NR1 AB-treated animals showed no reduction of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs; GluR1) in total-cell fractions at P0. Corresponding histological quantification of NMDAR clusters colocalizing with synaptic Homer-1 revealed strong reduction (up to 49.2%) in synaptic NMDAR cluster densities in the early postnatal brain, which was less marked at P7. The human monoclonal NR1 AB used for detection (#007-168) did not compete with the human treatment AB (#003-102) or the Homer-1 AB. Reduction of synaptic NMDAR was paralleled by electrophysiological changes in brain slices. NR1 AB-treated animals at day P0 showed significantly reduced amplitudes of sEPSCs, which normalized until P7. The frequency of sEPSCs was not altered.

Increased Mortality and Altered Physiological Parameters in NR1 AB-Exposed Offspring

NR1 AB-treated neonates displayed significantly reduced survival rates within the first 3 postnatal days (66.7%) compared to CTL AB-treated (#mGo53, 93.9%; GFAP, 100%; IVIG, 100%) and untreated (95.2%) offspring (Fig 3). Survival rates markedly varied in the NR1 group, ranging from 0 to 100%, whereas they never fell below 60% in controls. As animals did not die later than P3, we investigated vegetative functions related to acid–base metabolism early after birth. Blood gas analysis showed a highly significantly elevated blood pH in NR1 AB-treated neonates. Surviving animals in the NR1 group gained less body weight during breastfeeding. The difference persisted after weaning during adolescence in both female and male mice and diminished in adulthood (8 weeks). Changes were not attributable to dysfunctional maternal behavior, as dams were asymptomatic, and showed normal maternal care (pup retrieval test[35]), breastfeeding, and nesting (data not shown), and the litter size was not different excluding prepartum death of embryos. Whole brain volumes and hippocampal volumes were not affected between both groups at birth using MRI. However, brain volumes were significantly reduced in the NR1 AB-treated offspring after 8 weeks and 10 months. The strongest reduction was detectable in cerebellum, midbrain, and brainstem (Table 1). Activity recordings revealed significantly higher baseline activity (+27.8% comparing median over 14 days) in the home cages of NR1 AB-treated animals during the dark (active) phase.

Maternal NR1 ABs Delayed Neurodevelopment in Neonates, Reduced Anxiety Behavior, and Impaired Prepulse Inhibition in Adult Offspring

We next examined whether the development of early postnatal reflexes was impaired. Compared to controls, mice of the NR1 group had significantly reduced abilities for righting between P5 to P8 (Fig 4). Similarly, development of the cliff avoidance reflex was significantly impaired, and the negative geotaxis reflex was significantly delayed between P4 and P9 in NR1 AB-treated animals. During adolescence (6–7 weeks), animals of both groups had similar social preference in the 3-chamber test and equal abilities in spatial learning and memory in the Barnes maze test. NR1 animals at 9 weeks showed decreased prepulse

inhibition (PPI; −8.9%) at 69dB only, indicating subtle changes in sensorimotor gating function that persisted into adulthood. Likewise, NR1 AB-treated animals spent more time in and entered more often the open arms of the elevated plus maze, which was not related to faster locomotion, thus indicating lower anxiety. In the home cage, nests built by either group were similarly complex, and the naturally expressed behaviors (ie, climbing, drinking, sleeping) did not quantitatively differ between groups.

Serum Anti-NR1 IgG Reactivity in Mothers of Children with Psychiatric Disorders

To estimate whether NR1 autoreactivity can also be found in the serum of human mothers, we compared samples of 120 MCPD versus 105 CTLM. Children from MCPD had various psychiatric disorders ranging from hyperkinetic or emotional disorders to depressive episodes and pervasive developmental disorders; the pregnancies were 4 to 20 years before serum sampling (Table 2). Using our

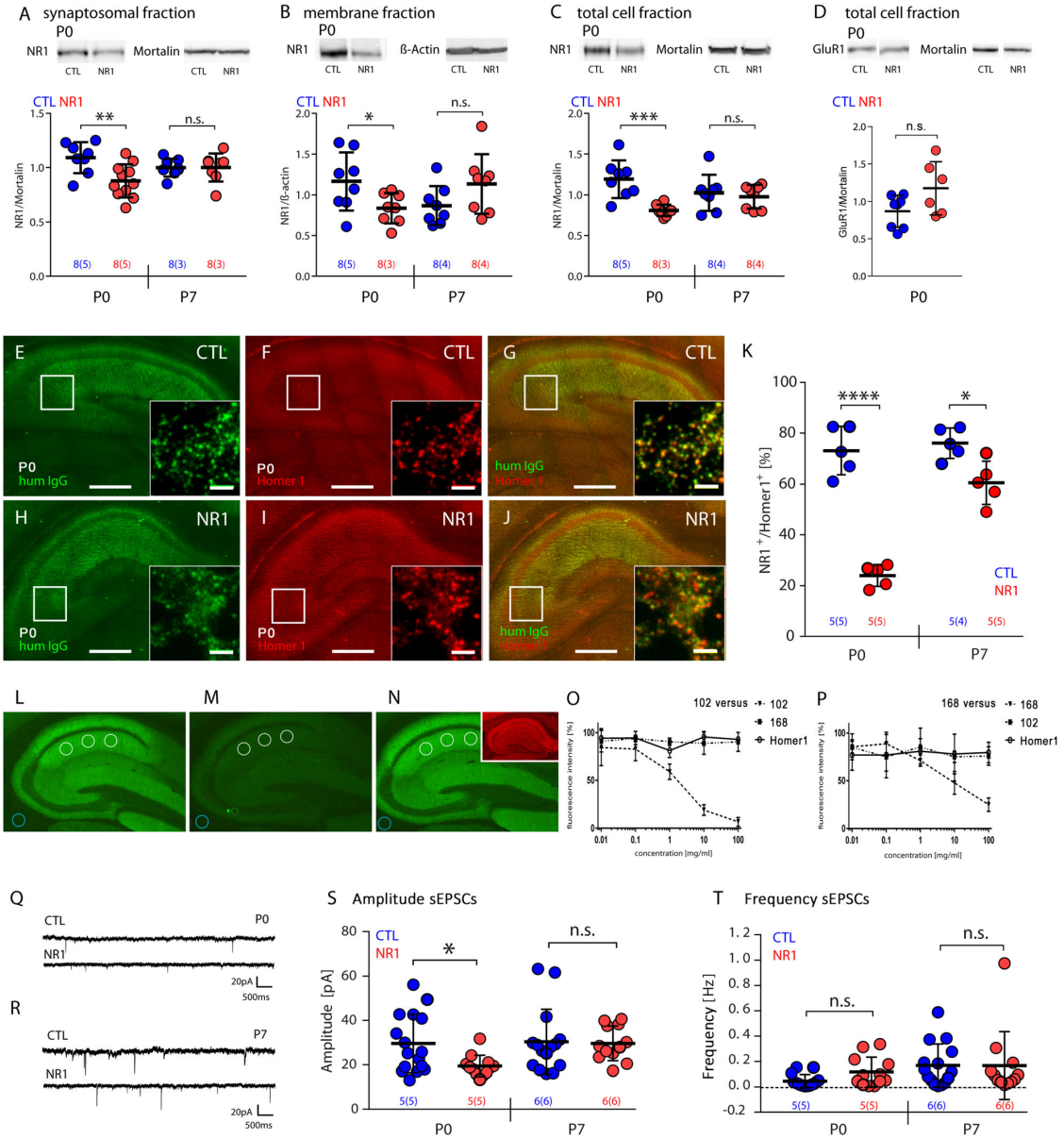

FIGURE 2: Legend on next page.

flow cytometry–based approach to objectively measure the continuous spectrum of NR1 IgG autoreactivity, titers were slightly higher in MCPD samples compared to CTLM (p = 0.038; Fig 5). Dose-titration curves with human monoclonal NR1 ABs and isotype-matched nonreactive CTL ABs revealed high sensitivity with flow cytometry even at a concentration of 1ng/ml. No specific psychiatric disorder was favored in children of MCPD with the highest titers. We next determined whether the enrichment of ABs in the fetal circulation can lead to clearly detectable IgG titers in the pups despite negative AB results in dams using a routine ELISA. Stepwise titration of injected human IgG resulted in this "seropositivity/seronegativity" discrepancy in the 6.6μg group, suggesting that pregnant women testing "negative" for NR1 ABs in clinical routine assays may not exclude fetal enrichment.

Discussion

Human IgG NR1 ABs were detected with cell-based assays in 1% of asymptomatic controls in previous studies,[11,12] creating a considerable subgroup of pregnant women at risk of transferring this anti-neuronal AB to the fetus. The prevalence of low-titer NR1 ABs might even be higher, as routine cell-based assays work with clear cutoff values for detection of patients with suspected NMDAR encephalitis. To model the underlying risk in mice, we administered human recombinant NR1 ABs into pregnant animals. The offspring had reduced densities of synaptic NMDAR, increased mortality, altered physiological functions, and impaired neurodevelopment. Most importantly, mild behavioral changes persisted into adulthood and were accompanied by significantly reduced brain volumes, indicating the potential of lifelong neuropsychiatric consequences from transient exposure to maternal NR1 ABs during pregnancy. Given the increased mortality in NR1 AB-treated offspring, surviving animals might have been more mildly affected, resulting in underestimation of the AB effect.

In a translational approach, data from the present study indicate that healthy MCPD may have higher serum NR1 IgG reactivity compared to CTLM. The effect was only subtle, potentially weakened due to spontaneous fading of NR1 AB titers over time (as known from autoimmune encephalitis) given the long interval (4–20 years) between pregnancy and AB testing. We compared the continuous spectrum of NR1 autoreactivity rather than categorizing only extremely high titers ("seropositive," which may indicate NMDAR encephalitis) as MCPD were asymptomatic (see Fig 5A). Interestingly, even below-threshold maternal serum titers of NR1 ABs may result in fetal IgG enrichment (see Fig 5C). Similar to human placental FcRn, IgG is actively transported into the fetal murine circulation via the FcRn in the yolk sac.[36] In contrast to humans, maternal IgG reaches the neonatal circulation in mice also via secretion into breast milk.[37] In both species, the fetal BBB is not fully developed,[38] allowing interference of ABs with fetal development while not crossing the intact maternal BBB. Binding to NMDAR might further retain and increase the ABs in the fetal brain, as in an injection model using animals with disrupted BBB.[39] Thus, high levels of ABs might not be required to distinguish MCPD from CTLM. In asymptomatic human mothers with low or even subthreshold serum NR1 AB titers, fetal NR1 IgG might still accumulate to a degree sufficient for permanent damage to the

FIGURE 2: Quantification of synaptic *N*-methyl-D-aspartate receptor density and characterization of electrophysiological properties in antibody (AB)-treated neonates. (A–C) Representative Western blots (cropped) of synaptosomal (A), membrane (B), and total-cell fractions (C) prepared from neonatal brains at postnatal day (P) 0 showed NR1 protein reduction, as compared with the respective reference protein. Quantification showed significant NR1 protein reduction in all 3 fractions (n = 8 from 3–5 different litters; mean ± standard deviation [SD], unpaired *t* test, p = 0.0063 [A], p = 0.0365 [B], p = 0.0005 [C]). (D) Control (CTL) blots showed no reduction of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (GluR1) protein at P0 in the total-cell fraction. (E, F, H, I) NR1 protein reduction was also observed when comparing immunohistochemistry of NR1-positive clusters (E, H) with expression of the synaptic protein Homer-1 (F, I). (G, J) The overlays demonstrated much less colocalization in NR1 AB-treated animals (J) compared to controls (G). (K) Quantification of the percentage of $NR1^+$ clusters of all Homer-1^+ clusters showed strong reduction at P0 (n = 5 from 5 different litters, mean ± SD, unpaired *t* test, $p < 0.0001$), which was less pronounced at P7 (n = 5 from 4–5 different litters, mean ± SD, unpaired *t* test, p = 0.0102). For cluster quantification in E–K, AB treatment was done with a monoclonal human NR1 AB (#003-102) different from the human detection AB (#007-168). This was possible as both ABs did not compete for the identical epitope (L–P), exemplarily shown for #003-102 alone (L, signal intensity measured in the hippocampal stratum radiatum [mean of the *white circles*] minus background fluorescence *[turquoise circle]*), #003-102 competing with 100μg/ml #003-102 (M) or 100μg/ml #007-168 (N, insert shows hippocampal binding of the Homer-1 AB). (P) Quantification of the respective dose curves showed that each monoclonal NR1 AB was only displaced by itself but not by the other NR1 or a Homer-1 AB (n = 4 independent experiments). (Q, R) Representative recordings of spontaneous excitatory postsynaptic currents (sEPSCs) in neonatal brain slices at P0 (Q) and P7 (R). (S) Quantification revealed marked reduction of sEPSC amplitudes at P0 (n = 16 [CTL] and n = 12 [NR1] cells from 5 neonates from 5 different litters, mean ± SD, unpaired *t* test, p = 0.0176), which normalized until P7 (n = 15 [CTL] and n = 12 [NR1] cells from 6 neonates from 6 different litters, p = 0.088). (T) sEPSC frequencies were not affected (mean ± SD, unpaired *t* test, p = 0.0688 [P0], p = 0.994 [P7]). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. hum = human; n.s. = not significant.

developing brain. Also, other factors could facilitate maternofetal AB transfer and induce neuropsychiatric disease, such as inflammation, genetic risk loci, placental microstructure, or psychosocial stress ("second hits").

Support for our model also comes from previous murine models of maternal anti-neuronal ABs affecting fetal development with long-term defects in the offspring.[6–9] Gestational transfer of IgG from mothers of children with ASD into mice resulted in reduced sociability and increased anxiety.[7,8] Active immunization against the NMDAR-NR2A/-NR2B (GluN2A/2B) subunits in pregnant dams led to death of female fetuses, altered histological properties, and

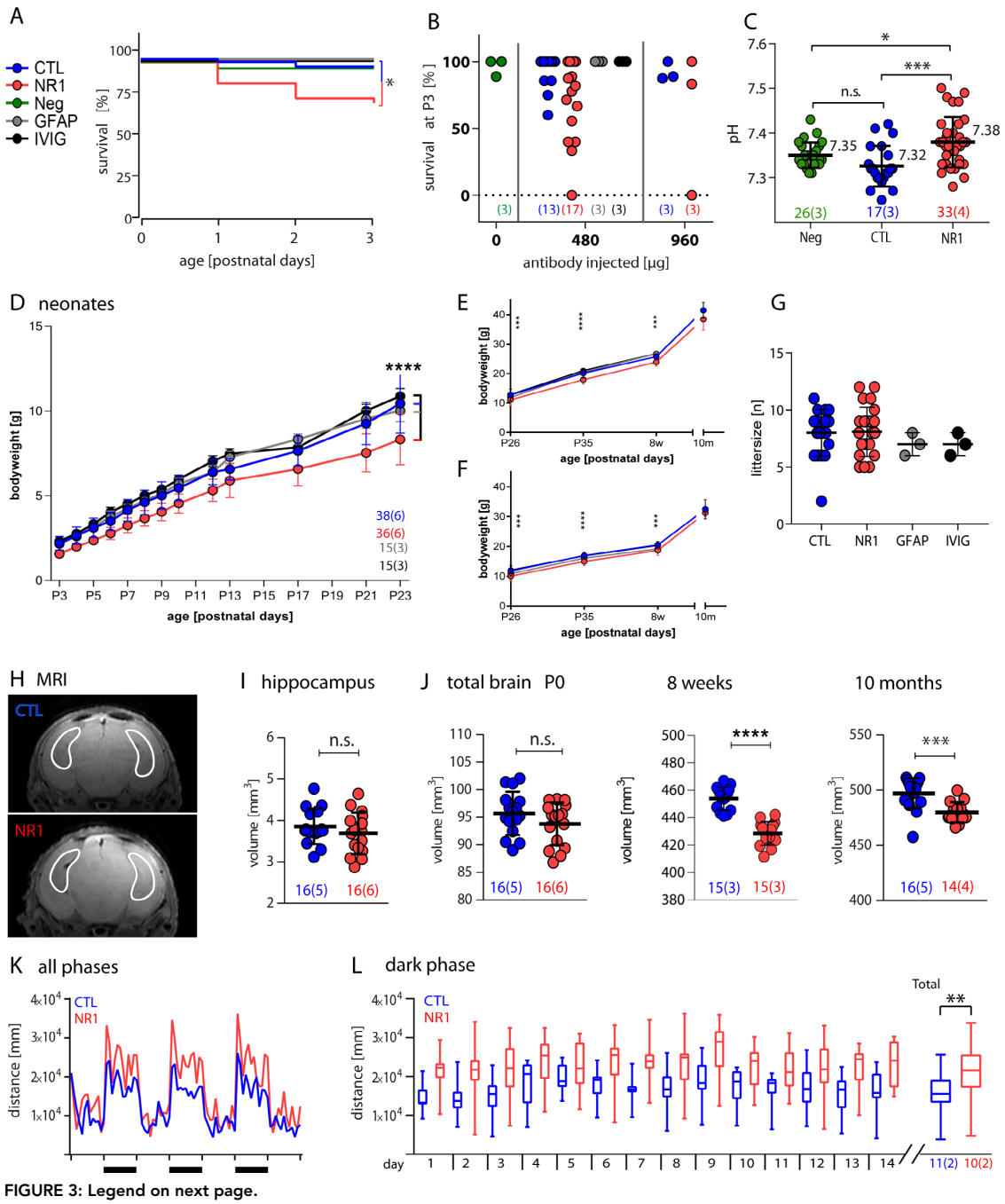

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TABLE 1. Comparison of Absolute Volumes of Different Brain Regions after 8 Weeks and 10 Months

	8 Weeks				10 Months			
Region	CTL, n = 15, Mean ± SD	NR1, n = 15, Mean ± SD	*p*	Sign.	CTL, n = 16, Mean ± SD	NR1, n = 14, Mean ± SD	*p*	Sign.
Cerebellum	45.32 ± 0.87	40.97 ± 1.72	1.79E-9	[a]	48.61 ± 1.49	44.61 ± 1.23	1.23E-8	[a]
Midbrain	22.74 ± 0.58	21.48 ± 0.65	5.90E-6	[a]	24.25 ± 0.96	22.92 ± 0.50	7.00E-5	[a]
Brainstem	86.39 ± 1.83	80.73 ± 2.73	3.00E-7	[a]	93.05 ± 3.11	88.47 ± 2.32	0.0001	[a]
Pallidum	8.02 ± 0.31	7.50 ± 0.36	0.0002	[b]	9.27 ± 0.42	8.94 ± 0.22	0.0133	
Hypothalamus	12.01 ± 0.71	11.20 ± 0.47	0.0004	[b]	13.53 ± 0.85	12.90 ± 0.60	0.0299	
Cerebral cortex	173.83 ± 3.61	166.45 ± 2.75	8.31E-7	[a]	190.90 ± 5.36	187.26 ± 3.68	0.0414	
Striatum	39.99 ± 1.29	34.54 ± 1.21	1.03E-5	[a]	41.52 ± 1.42	40.69 ± 0.96	0.0738	
Hippocampus	35.59 ± 0.98	33.64 ± 1.06	1.52E-5	[a]	39.26 ± 1.56	38.58 ± 1.83	0.2780	
Thalamus	19.44 ± 0.51	18.40 ± 0.53	5.49E-6	[a]	21.71 ± 0.71	21.38 ± 1.02	0.3027	
Amygdala	0.96 ± 0.05	0.92 ± 0.03	0.0041	[c]	1.07 ± 0.05	1.06 ± 0.03	0.6579	

Probability values were not adjusted. Values are expressed in mm^3. Changes in total brain volumes were treatment-specific (8 weeks: $p < 0.0001$; 10 months: $p = 0.0015$), but not sex-specific (8 weeks: $p = 0.284$; 10 months: $p = 0.677$; 2-way analysis of variance of treatment versus sex interaction).

[a] $p < 0.0001$; [b] $p < 0.001$; [c] $p < 0.01$.

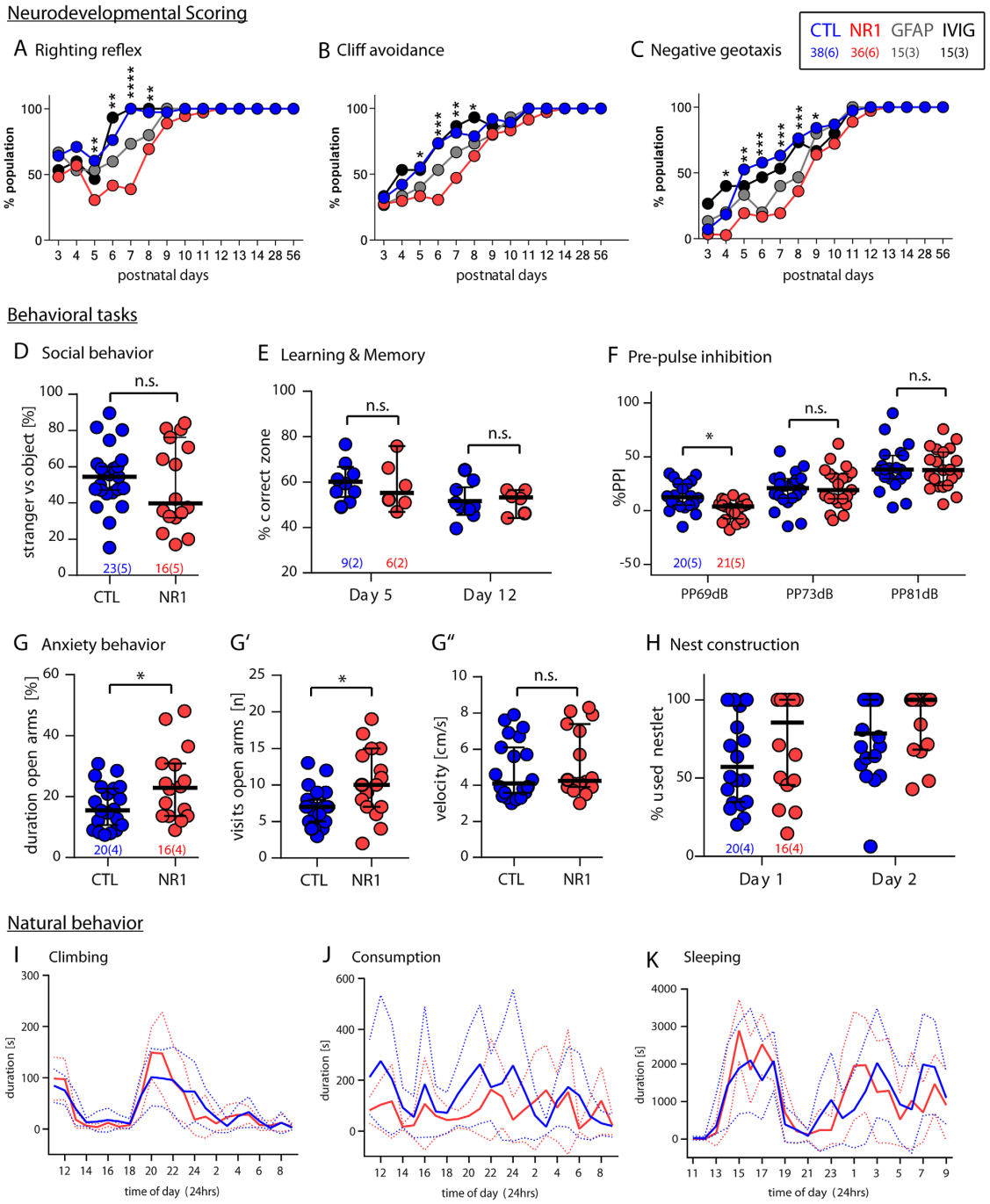
CTL = control; SD = standard deviation; Sign. = significance level.

long-lasting cognitive defects in male offspring.[6] In another study, human recombinant Caspr2 ABs were passively transferred into mice, resulting in abnormal cortical development in male offspring with decreased dendritic complexity and abnormal behavior.[9] Similarly, Caspr2 AB-containing maternal IgG fractions led to marked social interaction deficits in the offspring, disturbances in layer formation in somatosensory cortex, and increased microglial activation.[10]

Synaptic NMDAR activity is involved in different stages of fetal and neonatal brain development, crucial for circuit development and map formation, and deletion of the NR1 subunit is lethal.[40] In accordance with other

FIGURE 3: Mortality and physiological parameters of control (CTL) and NR1 antibody (AB)-treated offspring. (A) Kaplan–Meier analysis of neonate survival showed significantly increased mortality in NR1 AB-treated animals (log-rank test, corrected with Bonferroni correction for K = 5, adjusted *p* = 0.025 [NR1 vs CTL]) compared to all 4 control groups. (B) Survival rates of untreated, CTL, glial fibrillary acidic protein (GFAP), intravenous immunoglobulin (IVIG), and NR1 AB-treated neonates at postnatal day (P) 3 with different doses (untreated: n = 3 litters; 480µg: n = 13 [CTL], n = 17 [NR1], n = 3 [GFAP], n = 3 [IVIG]; 960µg: n = 3 [CTL], n = 3 [NR1]). (C) Blood pH of untreated, CTL, and NR1 AB-treated neonates at P0 showed significantly elevated pH in the NR1 group (untreated: n = 26 from 3 litters; CTL ABs: n = 17 from 3 litters; NR1 ABs: n = 33 from 4 litters; mean ± standard deviation [SD], 1-way analysis of variance [ANOVA], post hoc Tukey multiple comparison test, untreated vs CTL ABs: *p* = 0.1685; untreated vs NR1 ABs: *p* = 0.0488; CTL vs NR1 ABs: *p* = 0.0002). (D–F) Development of body weight of breastfed neonates (D), females (E), and males (F) after weaning (mean ± SD, repeated measures ANOVA, Sidak multiple comparison test, *p* < 0.0001 for NR1 vs all 3 control groups). (G) Litter size did not differ between all treatment groups (n = 17 [CTL], n = 20 [NR1], n = 3 [GFAP], n = 3 [IVIG]). (H) Representative brain magnetic resonance imaging (MRI) of CTL and NR1 AB-treated neonates (hippocampal regions highlighted). (I, J) MRI-based quantification showed similar volumes at P0 in both groups for hippocampus (I; n = 16, mean ± SD, unpaired *t* test, *p* = 0.3158) and total brain (J [left]; n = 16, mean ± SD, unpaired *t* test, *p* = 0.176). With long follow-up, total brain volumes of 8-week-old (J [middle]; CTL, n = 15; NR1, n = 15; mean ± SD, unpaired *t* test, *p* = 0.0001) and 10-month-old mice (J [right]; CTL, n = 16; NR1, n = 14; mean ± SD, unpaired *t* test, *p* = 0.0005) showed highly significant differences in total brain volumes. (K) Typical activity (mean of distance traveled) in home cages during 3 dark *(black bars)* and 4 light phases of adult offspring. (L) In dark phases, home cage activity (boxplots of distance traveled) of NR1 (n = 10) compared to CTL AB-treated animals (n = 11) was consistently higher during 14 days and in total (boxplots: median, 25th–75th quartile, minimum–maximum; Wilcoxon test of dark phase over all days, test score = 179, *p* = 0.0049). **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. n.s. = not significant.

in vitro and in vivo studies,[14,15] we here showed removal of NMDAR from the surface. This reduction was accompanied by decreased amplitudes of AMPAR-mediated sEPSCs, only present in young neonates (P0). Despite the lack of data for direct NMDAR function, affected AMPAR-mediated signaling was treatment-specific, and



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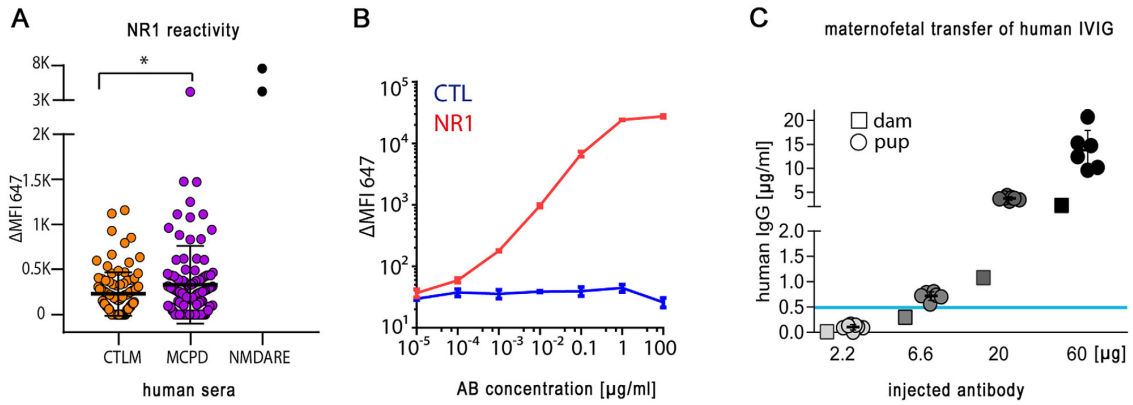

FIGURE 5: Serum NR1 autoreactivity in mothers of children with psychiatric disorders (MCPD) compared to healthy control mothers of unaffected children (CTLM). (A) Antibody (AB) reactivity (median fluorescence intensity [ΔMFI]) of sera against NR1-expressing HEK cells was increased in MCPD (n = 120) versus CTLM (n = 105, mean ± SD; unpaired *t* test, **p* = 0.038). Serum of *N*-methyl-D-aspartate receptor encephalitis (NMDARE) patients served as positive controls (right; n = 2). (B) The flow cytometry assay showed high sensitivity even in the low ng/ml range as validated with monoclonal human NR1 versus control (CTL) ABs. (C) Increasing doses of maternally injected human immunoglobulins consistently resulted in murine fetal AB enrichment. A given AB dose (here single maternal injection of 6.6µg human IgG) can lead to clearly detectable titers in pups *(circles)*, whereas it remains under the detection threshold (*blue line*, <0.5µg/ml in this routine enzyme-linked immunosorbent assay) in the mother (rectangles). IVIG = intravenous immunoglobulin.

likely resulted from disrupted NMDAR-mediated insertion of AMPAR,[20] normalizing at P7. The seeming discrepancy between increasing postpartum AB levels in serum and brain versus decreasing effects on NMDAR density and function indicate an early developmental window of high susceptibility. At this time, NMDAR are primarily responsible for glutamatergic transmission but are expressed at low levels. Thus, they cannot compensate the NR1 AB-mediated detrimental effect, whereas this is likely possible when NMDAR expression increases considerably later on.

Defects in our NR1 AB-treated animals likely were related to NMDAR dysfunction, as they resembled genetic and autoimmune models. For example, affected neonates had >27% increased mortality in the first postnatal days and markedly increased blood pH, a clinical parameter of acid-base metabolism and respiratory function. Loss-of-function mutations in the NR1 gene were lethal,[22] and NR1 knockout mice died within 8 to 15 hours after birth due to respiratory failure.[41] Small neonatal blood volumes prevented additional measurement of pCO_2, pO_2, or HCO_3 levels from further determining respiratory, neuromuscular, and metabolic contributions. Genetic models usually showed a more severe phenotype, whereas NR1 AB-treated animals performed well in paradigms of social behavior, spatial

FIGURE 4: Neurodevelopmental and behavioral impairment from postnatal to adult mice in NR1 antibody (AB)-treated offspring. (A–C) Delayed development of the righting reflex (A), cliff avoidance (B), and negative geotaxis (C) in NR1 AB-treated neonates compared to controls (3–6 litters, n = 17 [control (CTL)], n = 14 [NR1], n = 15 [glial fibrillary acidic protein (GFAP)], n = 15 [intravenous immunoglobulin (IVIG)], points symbolize % of population showing the respective reflex, 1-sided Fisher exact test for each day; **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001, NR1 vs CTL). (D) Normal social preference of adolescent mice from both treatment groups (n = 23 [CTL], n = 16 [NR1] from 5 litters, median ± 95% confidence interval [CI], Mann–Whitney *U* test, *p* = 0.4115). (E) Memory recall in the Barnes maze was not impaired 1 and 7 days after learning (n = 9 [CTL], n = 6 [NR1] from 2 litters, median ± 95% CI, Kruskal-Wallis-test, *p* = 0.1322). (F) Prepulse inhibition (PPI) determined by startle reaction showed impaired sensorimotor gating in NR1 AB-treated mice (n = 20 [CTL], n = 21 [NR1] from 5 litters, median ± 95% CI, Kruskal–Wallis test with Dunn post hoc test: PP69dB, *p* = 0.0263; PP73dB, *p* = 0.7964; PP81dB, *p* = 0.9650). (G, G′) Anxiety was lower in the NR1 group as displayed by longer duration in open arms (G, n = 20 [CTL], n = 16 [NR1] from 4 litters, median ± 95% CI, Mann–Whitney *U* test, *p* = 0.0494) and increased number of entries into open arms of the elevated plus maze (G′, *p* = 0.0139). (G″) Velocity was not affected by AB treatment (*p* = 0.3475). (H) Nest construction was normal as measured by the weight of unused nest material (n = 20 [CTL], n = 16 [NR1] from 4 litters, median ± 95% CI, Kruskal–Wallis test, *p* = 0.0718). (I–K) Individual behavior was recorded with a HomeCageScan system for each mouse and did not show significant differences in any recorded behavior, including climbing behavior (I; CTL, n = 9; NR1, n = 6, mean ± SD *[dotted lines]*, repeated measures analysis of variance; between-subject factor *p* = 0.9957), consumption (eating + drinking) behavior (J; between-subject factor *p* = 0.1857), and sleeping behavior (K; *p* = 0.9628). n.s. = not significant; N = white noise; P = pulse; PP = prepulse.

TABLE 2. Cohort Characteristics of Matched Mothers of Children with Psychiatric Disorders (n = 120, Mean Age = 42.3 ± 7.8 Years) and Healthy Control Mothers of Unaffected Children (n = 105, Mean Age = 43.6 ± 9.7 Years)

ICD-10	Disorder Classification	Cases	M	F
F1	Mental and behavioral disorders due to psychoactive substance use	2	1	1
F2	Schizophrenia, schizotypal, and delusional disorders	2	1	1
F3	Mood (affective) disorders	22	6	16
F4	Neurotic, stress-related, and somatoform disorders	19	11	8
F5	Behavioral syndromes associated with physiological disturbances and physical factors	1	0	1
F6	Disorders of adult personality and behavior	2	0	2
F7	Mental retardation	1	1	0
F84	Pervasive developmental disorders	8	8	0
F90	Attention-deficit hyperactivity disorders	32	24	8
F91; F92	Conduct disorders; mixed disorders of conduct and emotions	20	11	9
F93; F94; F98	Emotional disorders with onset specific to childhood; disorders of social functioning with onset specific to childhood and adolescence; other behavioral and emotional disorders with onset usually occurring in childhood and adolescence	12	9	3

Diagnoses of psychiatric disorders were coded according to the German modification of the ICD-10 as provided within health insurance data.
F = female; ICD-10 = International Classification of Diseases and Related Health Problems, 10th Revision; M = male.

learning, and nest construction, which is likely related to normalization of NMDAR function with fading AB titers. Clinical experience with pregnant NMDAR encephalitis patients further supports the pathogenic effects of in utero exposure to human NR1 ABs[42–46] and even demonstrated 4-fold higher titers in a newborn.[45] Obvious neonatal developmental impairment was seen in approximately half of the babies.[47] The lack of follow-ups prevented any conclusion on potential further deficits with later onset. Midgestational sera from a Danish biobank revealed significantly higher frequencies of NMDAR ABs (29%) in mothers with schizophrenia spectrum disorders (SSD) whose children were diagnosed with mental retardation and disorders of psychological development, compared to mothers with SSD and unaffected children.[48] Such difference was not seen in mothers without SSD.[48]

Offspring from our murine study displayed behavioral signs resembling several models of neuropsychiatric developmental disorders. For example, surviving animals showed impaired neurodevelopment, lower bodyweight, and delayed neonatal sensory–motor reflexes, similar to gestational transfer models of ASD[7] and learning disabilities related to anti-NR2A/B ABs.[6] In humans, motor delays are observed in 51% of individuals with ASD.[49] Moreover, behavioral changes manifesting later in life in NR1 AB-treated animals included elevated activity, decreased anxiety behavior in the elevated plus maze, and partially reduced PPI. Hyperlocomotion is a hallmark of several neuropsychiatric disorders, such as attention-deficit/hyperactivity disorder (ADHD), bipolar disorders, and schizophrenia, in both humans and rodent models[50] and in mice with reduced NMDAR expression.[51,52] Reduction of anxiety was equally well documented in ADHD models.[53] Finally, NR1 AB-treated animals were similar to animals with reduced NMDAR expression by decreased PPI, reflecting impaired sensorimotor gating and preconscious attention.[50–52] PPI is reduced not only in schizophrenia, but also in psychotic bipolar disorder and ADHD.[54]

The findings collectively suggest that diaplacentally transferred NR1 ABs are not linked to a single (NMDAR encephalitis-like) disorder, but could potentially contribute to a broader spectrum of behavioral abnormalities found in ADHD, ASD, bipolar disorders, schizophrenia, or learning disabilities. In line with this hypothesis, maternal NR1 ABs induced long-lasting neuropathological effects in the offspring, with reduced volumes of several brain areas in old mice. The mechanisms behind the delayed development of pathological changes require further research and may include dysfunctional formation of cortical layers or NR1-mediated effects on progenitor cells.[55]

Thus, gestational NR1 AB exposure may primarily be seen as a neurodevelopmental congenital brain disorder that is potentially amenable to immunotherapy, thus clearly deserving detailed analyses in future animal and prospective human studies with long follow-up. These should determine the kinetics of AB titers over time in mothers and neonates, presence of clinical neuropsychiatric symptoms and developmental delays, and contribution of additional risk factors.

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This work is part of B.J.'s PhD thesis. Datasets are publicly available and can be accessed at https://figshare.com/s/bfb41d12865ca5efc4e5.

Author Contributions

B.J., M.C., J.K., K.L., U.D., D.S., M.K., and H.P. contributed to the conception and design of the study; B.J., M.C., J.K., K.L., L.K., P.F., H.-C.K., L.-M. D., N.K.W., M.L., M.R., Y.W., J.L., J.H., S.Ma., S.Mu., P.B.-S., M.K., and H.P. contributed to the acquisition and analysis of data; B.J., M.C., J.K., L.K., H.-C.K., L.-M.D., D.S., M.K., and H.P. contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

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11.LEBENSLAUF

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

12. KOMPLETTE PUBLIKATIONSLISTE

1) Herken J and Prüss H (2017) Red Flags: Clinical Signs for Identifying Autoimmune Encephalitis in Psychiatric Patients. *Front. Psychiatry* 8:25. DOI: 10.3389/fpsyt.2017.00025 **Impact Factor: 2.849**
2) Herken, J., Bang, C., Rühlemann, M., Finke, C., Klag, J., Franke, A., Prüss, H. (2019). Normal gut microbiome in NMDA receptor encephalitis**.** *Neurol Neuroimmunol Neuroinflamm Nov 2019,* 6 (6) e632; DOI:10.1212/NXI.0000000000000632 **Impact Factor: 7.724**
3) Jurek, B., Chayka, M., Kreye, J., Lang, K., Kraus, L., Fidzinski, P., Kornau, H. C., Dao, L. M., Wenke, N. K., & Long, M., Rivalan M., Winter Y., Leubner J, Herken, J., Mayer S., Mueller S., Boehm-Sturm, P., Dirnagl, U., Schmitz, D., Kölch, M., Prüss, H. (2019). Human gestational N-methyl-d-aspartate receptor autoantibodies impair neonatal murine brain function. *Annals of Neurology, 86*, 656–670. DOI: (1) **Impact Factor: 9.037**

13. DANKSAGUNG

Kaum zu glauben, dass ich jetzt gerade hier sitze und meine Danksagung schreibe. Ich erinnere mich noch gut daran, wie ich mich vor 5 Jahren auf die Suche nach dem perfekten Thema für meine Doktorarbeit begab. Es sollte ein Thema sein, das mich wirklich interessiert, fasziniert und vor allem sollte es eine Relevanz für die Allgemeinheit haben. Damals wusste ich noch nicht, auf welchen Fachbereich ich mich später einmal spezialisieren würde. Vorstellen konnte ich mir in der inneren Medizin, in der Neurologie oder in der Gynäkologie zu arbeiten. 2015 absolvierte ich daher eine Famulatur in der Neurologie im Campus Mitte der Charité. Harald hielt damals einen internen Vortrag über autoimmune Enzephalitiden, speziell die Anti-NMDA-Rezeptor Enzephalitis, und sofort war ich fasziniert. Durch die Fortschritte in der modernen Neurologie war eine Erkrankung entdeckt worden, die noch vor weniger als einem Jahrzehnt als psychisch bedingt und unheilbar galt. Etliche Patientinnen, vor allem junge Frauen, konnten durch die Diagnosestellung geheilt und ihr langer Aufenthalt in der Psychiatrie beendet werden. Und schon damals war die Anti- NMDA-Rezeptor Enzephalitis längst nicht mehr die einzige Form der autoimmunen Enzephalitiden, die eine Psychose initiieren kann. Viel mehr war eine heterogene Gruppe neuro-immunologischer Erkrankungen entdeckt worden. Nach dem Vortrag schrieb ich Harald sofort eine E-Mail und erkundigte mich, ob die Kapazität und das Interesse für eine Zusammenarbeit im Rahmen einer klinischen Doktorarbeit bestünde. Und so begann unsere Zusammenarbeit.

Die letzten 4 Jahre waren voller persönlichen Herausforderungen: es galt neben dem Studium, und später der Arbeit, den langen Atem zu haben. Ich musste Ängste überwinden und immer weitermachen ohne sicher zu sein, ob ich jemals ankommen würde. Geholfen hat dabei eine intrinsische Motivation, die mich trotz berüchtigter Kämpfe, die jeder Doktorand kennt, auf Trab hielt.

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