The process of somatic hypermutation increases polyreactivity for central nervous system antigens in primary central nervous system lymphoma

by Manuel Montesinos-Rongen, Monica Terrao, Caroline May, Katrin Marcus, Ingmar Blümcke, Martin Hellmich, Ralf Küppers, Anna Brunn, and Martina Deckert

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Running head: Impact of GC reaction on self-/polyreactivity in PCNSL.

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Abstract

The immunoglobulin (Ig) heavy and light chain variable gene mutational pattern of the B cell receptor (BCR) in primary central nervous system (CNS) lymphoma (PCNSL) cells suggests antigenic selection to drive pathogenesis and confinement to the CNS. This hypothesis is supported by the observation that the tumor B cell receptor (tBCR) of PCNSL is polyreactive and may be stimulated by CNS proteins.

To obtain further insight into the role of the germinal center (GC) reaction on BCR reactivity, we constructed recombinant antibodies (recAb) with Ig heavy and light chain sequences of the corresponding naive BCR (nBCR) by reverting tBCR somatic mutations in 10 PCNSL. Analysis of nBCR-derived recAb reactivity by a protein microarray and immunoprecipitation demonstrated auto- and polyreactivity in all cases.

Self-/polyreactivity was not lost during the GC reaction; surprisingly, tBCR significantly increased self-/polyreactivity. In addition to proteins recognized by both the nBCR and tBCR, tBCR gained self-/polyreactivity particularly for proteins expressed in the CNS including proteins of oligodendrocytes/myelin, the S100 protein family, and splicing factors.

Thus, in PCNSL pathogenesis, a faulty GC reaction may increase self-/polyreactivity, hereby facilitating BCR signaling via multiple CNS antigens, and may ultimately foster tumor cell survival in the CNS.

Keywords: primary CNS lymphoma; B cell receptor; antigen; CNS proteins; germinal center reaction
Introduction

Primary lymphoma of the central nervous system (CNS, PCNSL) is a distinct diffuse large B cell lymphoma (DLBCL) entity confined to the central nervous system (CNS)\(^1\).

PCNSL tumor cells carry somatically mutated rearranged immunoglobulin (Ig) heavy and light chain variable genes\(^2\)\(^3\), revealing a germinal center (GC) experience of the lymphoma cells\(^4\). A prerequisite for the GC reaction is a unique micromilieu, in which follicular dendritic cells present cognate antigen to B cells and helper T cells mediate B cell selection, aiming to increase B cell receptor (BCR) affinity for the antigen. Considering confinement to the CNS, the observation of ongoing somatic hypermutation (SHM)\(^2\), a GC B cell-specific process, raises the intriguing question as to the impact of the target organ, particularly of CNS antigens, on this organ-specific DLBCL entity. While T helper cells and antigen presenting cells are present in the PCNSL-infiltrated CNS, their \textit{in vivo} characteristics and function have not yet been elucidated. Thus, it is still unknown whether a GC reaction indeed occurs in the CNS. Despite the observation of follicle-like structures in the leptomeninges, but not the brain parenchyma, in some patients with late multiple sclerosis phases\(^5\), formal proof of GCs and a GC reaction in the brain is still lacking. In addition to a lack of classical, fully functional GCs in the brain, the CNS microenvironment may also, at least in part, be responsible for the absence of Ig class switch in PCNSL due to a lack of signals in the GC required for class switching. The recent demonstration that SHM and class switch recombination occur independently and topographically distinct is in line with this notion\(^6\).

So far, studies of PCNSL aiming at the identification of foreign antigens that might trigger the tumor B cells failed; particularly, viruses able to persist in the CNS, e.g. HHV6, HHV8, and SV40, were excluded\(^7\)\(^-\)\(^9\). Taking advantage of the fact that the
tumor cells of PCNSL express a functional BCR, we previously reconstructed the
tumor cell BCR (tBCR) as recombinant antibodies (recAb) in 23 PCNSL to identify
their antigen recognition pattern on a large-scale protein microarray. This approach
aiming at the identification of proteins that were expressed in the CNS and on the cell
surface, thus, enabling their recognition as antigen by the BCR, revealed that the
tumor cells are polyreactive including reactivity with proteins physiologically
expressed in the CNS. Neuronally expressed GRINL1A, centaurin, and BAIAP2 were
recognized by tBCR \(^{10}\). The majority of tBCR recognized galectin-3, upregulated on
microglia/macrophages, astrocytes, and endothelial cells upon CNS invasion by
PCNSL \(^{10}\). Thus, proteins differentially expressed by resident CNS cell populations
may trigger the tBCR and support PCNSL survival in the CNS.

These data prompted us to revert somatic mutations of the tBCR to their preimmune
sequence, yielding recAb with sequences similar to the BCR of the naive B cell
(nBCR) from which the tumor cells originated, to obtain further insight into the impact
of SHM on BCR characteristics of PCNSL. To this end, we focused on IGHV4-34\(^{+}\)
and IGHV3\(^{+}\) PCNSL, as the IGHV4-34 gene is preferentially rearranged in PCNSL,
while genes of the large IGHV3 subgroup also occur frequently, but without biased
usage \(^{2-4}\). Comparison of nBCR with tBCR reactivity demonstrated that autoreactivity
and polyreactivity increased upon SHM. Thus, in PCNSL, B cells exhibit features
that, principally, qualify for elimination; however, they are rescued by paradoxical
redemption in the GC reaction.
Methods

Patients

Stereotactic PCNSL biopsies (tumor load >80%) from 50 HIV-negative patients were included (Suppl. Table 1). PCNSL were diagnosed according to WHO guidelines. The study was approved by the Ethics Committee of the University Hospital of Cologne (06-187, 07-109) and performed according to the Declaration of Helsinki.

TBCR reversion

TBCR sequences (PCNSL #01-#10, Suppl. Tables 1, 2) were reverted to their unmuted form using IMGT database (www.imgt.org) similar to the approach reported by Hervé et al. reverting V-derived sequences of the VH and VK/VL domain. Mutated nucleotides were replaced by those in the variant region being most similar to the germline gene segment according to IMGT, which was considered as naive sequence. These sequences were synthetized by GeneArt (Thermo Fisher Scientific, Rockford, USA); correctness was controlled by Sanger sequencing.

Generation of recAb with binding specificity identical to tBCR and nBCR

Monoclonal recAb with binding specificity identical to the tBCR and their corresponding nBCR were generated as described (Suppl. Fig.1). Ig V gene sequences are detailed in Suppl. Table 3.

As control, the recombinant anti-tetanus toxoid antibody recSA13 was generated from the SA13 cell line (HB8501, LCG Standard, Wesel, Germany) using the same strategy. For specificity of recSA13 see Suppl. Table 4.

Protein microarray processing
Protein microarray slides (9374 human proteins, ProtoArray, Thermo Fisher Scientific) including internal controls were analysed as described previously\textsuperscript{10,13,14}.

**Immunohistochemistry**

RecAb of PCNSL #01, #03, #09, labeled with green fluorescent dye CF488A (Antibody Labeling Kit, Sigma, Deisenhofen, Germany), were applied to PCNSL sections, followed by incubation with monoclonal rabbit anti-SNRPC (clone EPR16034, Abcam, Cambridge, UK) and species-specific Cy3-coupled immunoglobulin (Dianova, Hamburg, Germany).

Double immunofluorescence was performed with rabbit anti-SNRPC and mouse anti-GFAP (clone GA-5, Biogenex, Fremont, CA), mouse anti-Olig2 (clone OLIG2/2400, Abcam), mouse anti-NeuN (clone A60, Merck, Burlington, MA), mouse anti-CD68 (clone KP1, DCS, Hamburg, Germany), and mouse anti-CD34 (clone Qbend/10, Biogenex, Palo Alto), respectively, including appropriate positive and negative controls (Suppl. Figs. 2, 3) was performed.

**Analysis of gene expression profiling (GEP)**

GEP (10 normal CNS tissues, 21 PCNSL) obtained by the Affymetrix U95Av2 microarray (Santa Clara, CA, USA) published previously\textsuperscript{15} were analysed for SNRPC mRNA expression.

**Statistics**

To test for statistical significance between the number of proteins recognized by the recAb derived from nBCR and tBCR, the exact Wilcoxon signed rank test (calculated with R version 3.5.0, R Foundation for Statistical Computing, Vienna, Austria) was applied. Statistical significance between SNRPC mRNA expression in normal CNS
and PCNSL was assessed by Student’s *t*-test. *p*<0.05 was considered significant. All experiments were performed at least in duplicate.

### Results

**Ig heavy and light chain variable gene rearrangements in PCNSL used for generation of recAb**

All PCNSL harbored a monoclonally rearranged Ig heavy chain variable and a monoclonally rearranged Ig light chain variable gene. A gene of the IGHV3 and the IGHV4 subgroup was rearranged in five tumors each with five distinct members of the IGHV3 subgroup and five IGHV4-34+ PCNSL (Suppl. Table 2). All PCNSL had introduced somatic mutations into their rearranged Ig heavy and light chain variable genes. All Ig heavy and light chain sequences harbored mutations varying from 2%-15% with mean mutation frequencies of 9.9% and 8.3% for the heavy and the light chain V-genes, respectively. Mutations corresponded to point mutations in the heavy and light chain of all 10 PCNSL. In addition, case #01 harbored an insertion of six base pairs which extended the heavy chain CDR2 from eight to ten amino acids (Suppl. Table 3). As we attributed this insertion to SHM, it was eliminated in the revertant sequence (Suppl. Table 3). Deletions and stop codons were absent. Thus, all tumors harbored rearranged and somatically mutated Ig heavy and light chain variable genes encoding a potentially functional BCR, in accordance with previous data.

**RecAb of nBCR and tBCR show self-reactivity with common autoantigens**

Complete IgG recAb of nBCR and tBCR were compared for reactivity with common self-antigens using permeabilized human HEp-2 cells as target. In five cases, a positive reaction indicated autoreactivity (Table 1, Suppl. Fig. 4). In four of them, the
SHM-induced modification of the nBCR yielded tBCR HEp-2 reactivity not noticed for the nBCR (Table 1). In the remaining case, both nBCR and tBCR were HEp-2 reactive (Table 1). None of the nBCR exhibited reactivity that was lost upon SHM. Remarkably, HEp-2 reactivity was much below the standard (Suppl. Fig. 4), detectable at high, but not low recAb concentration (250 µg/ml vs. 10 µg/ml), indicating low affinity of the recAB.

ANA/ANCA ELISA detected reactivity in two IGHV4-34+ PCNSL (Table 1). In one case (PCNSL #10), both nBCR and tBCR reacted with autoantigens, while in the other case (PCNSL #08) only the tBCR, but not the nBCR was reactive (Table 1). Again, BCR binding was much below the standard of the ELISA requiring a high antibody concentration (200 µg/ml). At a concentration recommended for the detection of high-affinity antibodies in patients with clinically active autoimmune disease (10 µg/ml), in this regard differing from PCNSL-derived nBCR and tBCR, reactivity was abolished, further suggesting low affinity.

Together, these data show that the recAB of six of 10 (60%) PCNSL exhibit weak reactivity with self-antigens, mostly in the tBCR, indicating that SHM fosters self-reactivity.

**NBCR and tBCR differ in their protein recognition pattern on the ProtoArray**

All recAb specifically recognized proteins on the ProtoArray. A total of 821 proteins was specifically recognized by at least one recAb (mean: 165; median: 140; range: 106-300). Regarding all proteins recognized, 341 and 725 proteins were recognized by at least one of the nBCR and tBCR, respectively. The nBCR exclusively recognized 96 proteins including several ribosomal proteins, B cell linker, and ligase III (Suppl. Table 5), whereas the tBCR exclusively recognized 480 proteins including histones, myelin associated proteins (i.e. myelin basic protein, MBP; myelin-
associated oligodendrocyte basic protein, MOBP; myelin protein zero-like protein 1, MPZL1), ribosomal proteins, and splicing factors (SNRP70, SNRPA, SNRPB) (Suppl. Table 5). 245 proteins were recognized by both the nBCR and the tBCR including galectins, S100A1 protein, and SNRPC (Suppl. Table 4). With respect to the proteins recognized by the nBCR, 107 and 58 proteins were recognized exclusively by the nBCR derived from IGHV3+ and IGHV4-34+ PCNSL, respectively; 176 proteins were recognized in common. Regarding the proteins recognized by the tBCR, 248 and 179 proteins were recognized exclusively by the tBCR derived from IGHV3+ and IGHV4-34+ PCNSL, respectively; 298 proteins were recognized in common (Fig. 1A, Suppl. Table 5).

According to the Human Protein Atlas Project (www.proteinatlas.org), 482 proteins recognized by the recAb are expressed in the CNS, albeit mostly not exclusively (mean: 96; median: 82; range: 63-184). Of those CNS proteins recognized, 202 and 425 CNS proteins were recognized by at least one of the nBCR and the tBCR, respectively (Fig. 1B). NBCR exclusively recognized 57 proteins including BLNK and LIG23 (Suppl. Table 5), whereas tBCR exclusively recognized 280 proteins including MBP, MOBP, MPZL1, SNRPA, SNRPB2, and S100A13 (Suppl. Table 5); 145 proteins were recognized in common including endoglin, galectins, and SNRPC (Suppl. Table 5). Regarding CNS proteins recognized by nBCR, 64 and 33 CNS proteins were exclusively recognized by nBCR derived from IGHV3+ and IGHV4-34+ PCNSL, respectively; 105 proteins were recognized in common (Fig. 1B). Regarding CNS proteins recognized by tBCR, 143 and 112 CNS proteins were exclusively recognized by the tBCR derived from IGHV3+ and IGHV4-34+ PCNSL, respectively; 170 proteins were recognized in common (Suppl. Table 5).

Focusing on individual PCNSL, tBCR of both IGHV3+ and IGHV4-34+ PCNSL recognized significantly increased numbers of proteins compared to nBCR (Fig. 2A-
D). Proteins recognized exclusively by tBCR expressed in the CNS included histones, ribosomal proteins, CNS hormones (arginine vasopressin-induced 1, neuropeptide Y), proteins involved in glial cell metabolism (S100-A13, MPZL1, MBP, MOBP), and mitochondria (ATP/ATPases, cytochrome C) (Suppl. Table 5).

Thus, nBCR alter their protein recognition pattern with B cell maturation. In addition to proteins recognized by both nBCR and tBCR and those exclusively bound by tBCR, we also detected proteins that were recognized by the nBCR and lost reactivity with modification towards the tBCR. Important and of potentially functional relevance, the BCR increases its protein reactivity upon SHM enabling recognition of increased numbers of antigens expressed in the CNS.

**Reactivity of nBCR and tBCR with CNS proteins determined by immunoprecipitation**

Normal brain biopsies were used for immunoprecipitation with all recAb. 270 proteins (mean: 80.2, median: 82.5, range: 44–124) co-immunoprecipitated with at least one recAb (Fig. 3A). 214 and 241 proteins co-immunoprecipitated with at least one nBCR and tBCR, respectively. NBCR and tBCR exclusively immunoprecipitated 29 and 56 proteins, respectively. 185 proteins were recognized in common. Regarding nBCR corresponding to IGHV3⁺ and IGHV4-34⁺ PCNSL, 59 and 42 proteins were exclusively immunoprecipitated, respectively; 113 were recognized in common. With respect to the tBCR, 47 proteins each were immunoprecipitated exclusively by IGHV3⁺ and IGHV4-34⁺ PCNSL; 147 proteins were immunoprecipitated in common (Fig. 3A-C).

While a lack of information on protein characteristics on the ProtoArray and within human brain samples regarding isoform(s) and post-translational modifications hampers direct comparison of the proteins identified in these two assays, both
techniques allowed the conclusion that tBCR recognize significantly increased numbers of CNS proteins and identified proteins recognized exclusively by the tBCR. Among the proteins differentially recognized by the tBCR, but not the nBCR, were ribosomal proteins, histones, and proteins involved in mitochondrial and glial cell metabolism (laminin subunit beta-1, annexin A7, protein S100-A9) (Suppl. Table 6). Immunoprecipitation of the nBCR and tBCR with PCNSL lysates was limited due to tissue scarcity with low protein content; nevertheless, there was a tendency towards increased numbers of proteins recognized by tBCR compared to nBCR, which, however, did not reach statistical significance (Suppl. Fig. 5). RecAb binding in dot blots confirmed that Western blot conditions were too stringent (Suppl. Fig. 6,7).

**Immunofluorescence studies confirm binding of recAb derived from nBCR and tBCR of proteins recognized on the ProtoArray**

To confirm the ProtoArray data by an independent method, double immunofluorescence of SNRPC expression was performed. SNRPC was differentially recognized by recAb derived from nBCR and tBCR on the ProtoArray (nBCR: negative, tBCR: positive, PCNSL #01, #03, #09). RecAB derived from the nBCR of PCNSL #01, #03, and #09 did not co-localize with the commercial anti-SNRPC antibody (Fig. 4A-C). In contrast, the recAb derived from the tBCR co-localized with the commercial anti-SNRPC antibody demonstrating tumor cell SNRPC expression (Fig. 4D-F). RecAb staining was remarkably less strong compared to the commercial monoclonal anti-SNRPC antibody, further indicating their lower affinity. This observation was further supported by ELISA analysis of the recAb compared to the commercial anti-SNRPC antibody (Suppl. Fig. 8). Here, only a very high concentration of the recAb together with a much lower concentration of the commercial anti-SNRPC antibody revealed reactivity (Suppl. Fig. 8).
Thus, these *in situ* topographical proof-of-principle analyses confirmed the ProtoArray data further supporting low-affinity and polyreactivity of nBCR and tBCR.

**Proteins recognized by recAb are expressed in PCNSL**

Topographical analysis revealed that 90% (18/20) of PCNSL (PCNSL #11-#30) showed a nuclear SNRPC expression in the majority of the tumor cells (Suppl. Table 7, Fig. 5A). Neurons, astrocytes, oligodendrocytes, a few microglial cells and macrophages also expressed SNRPC (Fig. 5B-F). GEP \(^{15}\) confirmed a significantly increased SNRPC expression in PCNSL compared to normal brain (Fig. 5G).

**Discussion**

This study demonstrates that reverting somatic mutations of the V-derived sequences of the VH and VK/L domain of PCNSL tBCR can yield a low-affinity, self- and polyreactive BCR of the naïve precursor B cells. Comparison of the protein recognition pattern of nBCR and tBCR reveals increased polyreactivity of the GC-experienced B cell with a particularly increased reactivity with proteins expressed in the CNS.

Gain of self- and polyreactivity during SHM was supported by several experiments, i.e. reactivity with self-antigens in immunofluorescence with HEp-2 cells, ANA/ANCA ELISA at high, but not low BCR concentration, ProtoArray analysis, immunoprecipitation and topographical immunohistochemistry. Comparability between the various methods is hampered by technical limitations due to specific characteristics of the respective analyses, e.g. different isoforms of proteins expressed may be present in the human brain and on the ProtoArray, a lack of knowledge of post-translational protein modifications and other metabolic modifications in the human brain. It is of note that our recAbs were expressed as IgG,
a well-established technique frequently used by many groups\textsuperscript{16, 17}. Recent studies demonstrated that the \textit{in vitro} binding of at least certain polyreactive Igs differed from the IgM-BCR counterpart\textsuperscript{18}, that the C region may change the fine antibody specificity for particular antigens\textsuperscript{19}, and that the isotype shapes epitope specificity, antibody affinity, and functional activities\textsuperscript{20}. Thus, one cannot exclude with certainty that some specificities may be confined to the soluble IgG antibody. However, it is unlikely that this holds true for all of the many specificities of all recAbs of our series. Moreover, the fact that the IgGs generated from the tBCR did not show the same autoreactivity as their corresponding IgGs generated from the nBCR, further supports that the expression of an IgM BCR as a soluble IgG antibody does not systematically lead to a gain of autoreactivity and hence serves as a kind of internal control. N- and P-nucleotides of the CDR3 sequence cannot be reverted due to lack of corresponding germline sequences. However, it is unlikely that mutations persisting in CDR3, which can also modify antibody affinity and specificity, may affect the overall results of the entire PCNSL series, in particular, as PCNSL are characterized by unrestricted CDR3 length and amino acid composition and by an absence of stereotyped BCR sequences\textsuperscript{4}. Nevertheless, despite these technical limitations, all analyses indicated increased poly-/autoreactivity of the tBCR and a faulty GC reaction, thus, extending previous data on polyreactive BCR of PCNSL and an involvement of numerous antigens in tumor cell selection\textsuperscript{4, 10, 21}.

These observations fit into the concept that antigen selection plays an important role in driving BCR activity in PCNSL. Increasing evidence suggests that BCR signaling in response to self and/or foreign antigens present in the target organ microenvironment sustains BCR signaling in various mature B cell lymphoma entities including PCNSL, follicular lymphoma (FL), splenic marginal zone lymphoma, MALT lymphoma of the eye, and CLL\textsuperscript{10, 12, 16, 22, 23}. In CLL, B cells with unmutated BCR
express highly polyreactive antibodies whereas most mutated CLL B cells do not. Mutated non-autoreactive CLL antibody sequences reverted into their germline counterparts encoded poly-/autoreactive antibodies, indicating that SHM plays an important role in disease development by altering original BCR autoreactivity and negatively selecting for auto-/polyreactivity. In this regard, SHM performs its physiological function in B cell differentiation to eliminate self-reactive B cells. SHM also strongly impacts on BCR reactivity in PCNSL, however, with opposite results, at least in some instances. In PCNSL, a failure of the selection process in the GC reaction may be a crucial event broadening autoantigenic reactivity and, thereby, fostering survival upon antigen encounter.

Physiologically, low-affinity, autoreactive B cells with IgD+ IgM+ immunophenotype, a hallmark of PCNSL, are anergic, short-lived and have long been considered to be excluded from the GC. However, it was recently shown that in particular conditions autoreactive B cells may enter a GC reaction, but lose their autoreactivity as a result of SHM. Such a mechanism has also been reported for B cells using the IGHV4-34 gene that often encodes autoreactive antibodies. PCNSL also preferentially rearrange the IGHV4-34 gene. On the other hand, B cells can acquire poly-/self-reactivity during the GC reaction, which may increase antibody affinity for a pathogen. Whether such a mechanism depicted for memory B cells may also underlie the increased poly-/self-reactivity of the tumor cells of PCNSL and whether they have been selected for a foreign antigen, is unknown. In this regard it is of note that the mutation pattern of the IG genes of PCNSL suggested triggering even by a (viral) superantigen.

Similar to autoreactive VH4-34+ B cells redeemed from elimination in the GC, 72% of IGHV4-34+ PCNSL showed a mutational loss of the CDR2 sequence motif that promotes N-linked glycosylation of CDR2, an event increasing accessibility of the...
binding site to eliciting foreign antigen 26. Thus, one may hypothesize that in PCNSL naive precursor B cells may enter the GC, where they undergo SHM as part of the rescue process.

However, if the B cells in the process of developing into lymphoma cells or already corresponding to tumor cells were rescued by redemption of autoreactive non-malignant B cells, this process, nevertheless, failed, because it did not yield B cells with reduced auto-/polyreactivity, but even with increased polyreactivity although some autospecificities were lost. Interestingly, with progression from the naive to the mutated BCR, overall, the tBCR gained autoreactivity for proteins expressed in the CNS including S100 protein family members and constituents of myelin/oligodendrocytes, including MPLZ1, MBP, and MOBP, which are widely and prominently expressed in the CNS, particularly in the white matter and also, at low level, by neurons 30. This expression pattern may explain, at least in part, the preferential growth of PCNSL in deep brain structures of the cerebral hemispheres, along fiber tracts, and in the basal ganglia 31.

Another antigen recognized by nBCR and/or tBCR was SNRPC, which is involved in pre-mRNA splicing 32. In conditions with chronic B cell activation, such as systemic lupus erythematoses and other rheumatic diseases, autoantibodies against various ribonucleoproteins are frequent 33-36. SNRPC, expressed by the majority of tumor cells in 90% of PCNSL, may become accessible to tBCR upon necrosis, which is frequent in PCNSL 37. These data extend our observation on galectin-3, also expressed on PCNSL tumor cells 10, as potential target for interactions with the BCR. Additionally, nBCR and tBCR may recognize intrinsic BCR structures, ultimately inducing cell-autonomous signaling similar to CLL 38.

Our data on polyreactive BCR in PCNSL are in line with reports from other mature B cell lymphomas 16, 39-41, suggesting a role for chronic antigenic stimulation in PCNSL
pathogenesis. This hypothesis is supported by a recent study on PCNSL which extended the list of autoantigenic targets by abnormally hyperglycosylated SAMD14 and neurabin-I. SAMD14 reactivity was shown for 8 of 12 (67%) PCNSL with hyperglycosylation of SAMD14/neurabin-I in 6 (50%). We noticed a comparable neurabin-I reactivity in 14 of 20 (70%) PCNSL in an independent series (Suppl. Table 1); however, Western blots bands were mostly weak (Suppl. Fig. 9). These data indicate neurabin-I, expressed by neurons and axons (Suppl. Fig. 10), as potential antigen in a fraction, but not all PCNSL. Our recAb, however, did not bind to proteins in PCNSL lysates (Suppl. Fig. 6), because experimental conditions of Western blot were too stringent. A lack of further concordant data between our and Thurner’s work may be attributed to technical differences. The ProtoArray and the Unipex differ in their proteins. Focussing on high-affinity antibodies, Thurner et al. pooled Fab fragments while we designed complete IgG antibodies which were studied individually, considering all recAb-reactive proteins in subsequent analyses.

**Conclusion**

In conclusion, B cells with auto-/polyreactive nBCR may enter the GC where SHM may further increases BCR auto-/polyreactivity (Suppl. Fig. 11). Together with mutation-induced sustained active BCR signaling and the inability to terminate SHM, the cells may become eligible for reaction with a plethora of antigens, particularly of CNS antigens. Thus, in the target organ, a microenvironment fertile for PCNSL proliferation may result from stimulating CNS antigens, autoantigens liberated by dying tumor cells or expressed on the surface of tumor cells, resulting in a vicious cycle of uncontrolled proliferation sustained by the interaction of an active tBCR with (auto)antigens, as a consequence of a faulty GC reaction. While this concept does not fit into the “classical” view that, physiologically, the GC reaction exclusively
selects for high-affinity antibodies, it extends novel findings on low-affinity BCR that play a role in immunological memory by adding PCNSL as lymphoma resulting from a dysregulated GC reaction.

**Author contribution**

Contribution: M.M.R., M.T., A.B., M.D., C.M. performed experiments; M.M.R., M.T., A.B., M.D. analyzed results and made the figures; M.M.R., A.B., M.D. designed the research; I.B. provided crucial material; I.B., K.M. discussed the manuscript; M.M.R., R.K., M.D. discussed the results and provided important intellectual contribution; M.D. wrote the paper. M.H., M.M.R., M.T. has done statistical analysis.

**Conflict of interest statement**

The authors disclose potential conflicts of interest. Informed consent was obtained from all individual participants included in the study.

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Table 1: Autoreactivity of the recombinant antibodies tested by HEp-2 immunofluorescence, ANAcombi and ANCAcombi ELISA.

<table>
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<tr>
<th>PCNSL</th>
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a: RNP-70 (small nuclear ribonucleoprotein U1 subunit 70),
b: lysozyme,
c: lysozyme, elastase, myeloperoxidase, BPI (bactericidal permeability-increasing protein),
d: lysozyme, elastase, proteinase 3.

Figure Legends

Figure 1 Recognition pattern of recAb derived from nBCR and tBCR of PCNSL on the ProtoArray

A: Quantitative Venn diagrams show numbers of proteins recognized by at least one recAb (upper panel). The numbers of proteins recognized exclusively by nBCR or tBCR are shown in the middle panel. The bottom panel depicts numbers of proteins recognized exclusively by nBCR or tBCR derived from IGHV3<sup>+</sup> or IGHV4-34<sup>+</sup> PCNSL.
Figure 2  Recognition pattern of recAb derived from nBCR and tBCR of PCNSL
A: BoxPlot diagram of ProtoArray analysis showing numbers of proteins recognized by at least one nBCR derived recAb or tBCR derived recAb. Numbers of proteins recognized exclusively by either nBCR or tBCR in individual PCNSL were used for calculation. Statistical significance was determined by exact Wilcoxon signed rank test.
B: Number of proteins on the ProtoArray that are recognized by recombinant antibodies derived from nBCR and tBCR are shown for individual PCNSL cases. In addition to proteins recognized by either nBCR or tBCR derived recAbs, the number of shared target proteins is depicted.
C: BoxPlot diagram shows the numbers of proteins known to be expressed in the CNS that were recognized by at least one nBCR and tBCR recAb. Proteins known to be expressed in the CNS recognized exclusively by either nBCR or tBCR in individual PCNSL were used for calculation. Statistical significance was determined by exact Wilcoxon signed rank test.
D: The number of proteins on the ProtoArray known to be expressed in the CNS that are recognized by recombinant antibodies derived from nBCR and tBCR is shown for individual PCNSL cases. In addition to proteins recognized by either nBCR or tBCR derived recombinant antibodies, the number of shared target proteins is depicted.

Figure 3  TBCR recognize increased numbers of proteins in the CNS as compared to nBCR in immunoprecipitation studies
A: Quantitative Venn diagrams of immunoprecipitation analysis show the number of proteins that co-immunoprecipitate with at least one recAb (upper panel). The middle
panel shows numbers of proteins co-immunoprecipitating exclusively with nBCR or tBCR. The bottom panel depicts numbers of proteins co-immunoprecipitating nBCR or tBCR derived from either IGHV3+ or IGHV4-34+ PCNSL. Proteins co-immunoprecipitating with both recAb are shown in the intersection.

B: BoxPlot diagram of immunoprecipitation shows the numbers of proteins co-immunoprecipitating with at least one recAb. Only proteins co-immunoprecipitating with either nBCR or tBCR were analysed. Statistical significance was determined by exact Wilcoxon signed rank test.

C: Numbers of proteins co-immunoprecipitating with nBCR and tBCR shown for individual PCNSL cases. In addition, numbers of shared target proteins are depicted.

**Figure 4** Recognition of SNRP by recAb corresponding to nBCR and tBCR

A-C: In PCNSL #17, the nBCR (FITC, A) derived from PCNSL #09, which did not react with SNRPC on the ProtoArray (Suppl. Table 4), shows only a weak staining of single tumor cells (arrows, A). Prominent expression of SNRPC in the nuclei of the majority of tumor cells in PCNSL #17 is evidenced by application of the commercial anti-SNRPC antibody (Cy3, B). The overlay (C) shows that there is no co-localization of both antibodies (arrows, C). Insert: High-power magnification of C, x 1000. *: erythrocyte autofluorescence.

D-F: The tBCR (FITC, D) derived from PCNSL #09, which reacted with SNRPC on the ProtoArray (Suppl. Table 4), co-localizes with the commercial anti-SNRPC antibody (Cy3, E, F) that depicts prominent SNRPC expression by the tumor cells of PCNSL #17 (E). Arrows and arrowheads in D indicate prominent and moderate immunoreactivity of the tBCR, respectively, yielding a yellow (arrows, F) or orange (arrowheads, F) signal in the tumor cell nuclei in the overlay (F). Insert: High-power magnification of F, x 1000. *: erythrocyte autofluorescence.
Double immunofluorescence using rabbit anti-SNRPC (clone EPR16034, Abcam, Cy3) with nBCR (A) and tBCR (D) labeled with Mix’n Stain 488 Labeling Kit (Sigma). Microphotographs were taken with an Axiophot (Zeiss) and Zen 2 software (Zeiss). Original magnification x400 (objective: x40). Overlay of the microphotographs (C, F) and adjustment for contrast and brightness were performed with Adobe Photoshop software version CC. Similar results were obtained for staining with recAb derived from PCNSL #03 and #09 as well as for staining of sections derived from PCNSL #14, #21, and #24.

**Figure 5** Expression of SNRPC in PCNSL

A: Nearly all CD20⁺ (FITC) tumor cells of PCNSL show a nuclear expression of SNRPC (Cy3).

B: Intermingled with tumor cells of a PCNSL, single CD68⁺ (FITC) macrophages also express SNRPC (arrows, Cy3); additionally, there are also SNRPC-negative CD68⁺ macrophages (arrowheads).

C: Cortical NeuN⁺ (FITC) neurons express SNRPC (arrows, Cy3).

D: Strongly activated GFAP⁺ (FITC) reactive astrocytes express SNRPC (arrows, Cy3).

E: In the white matter, some Olig2⁺ (FITC) oligodendrocytes express SNRPC (arrows, Cy3).

F: CD34⁺ (FITC) endothelial cells of neocortical cerebral blood vessels do not express SNRPC (Cy3).

G: mRNA expression of SNRPC recognized on the ProtoArray in the normal CNS and PCNSL. SNRPC is downregulated in normal CNS and upregulated in PCNSL samples. The line represents the SNRPC tag on the U95Av2 microarray (Affymetrix) in individual samples of 10 normal CNS tissues and 21 PCNSL published previously.
SNRPC was significantly differentially expressed between CNS and PCNSL (p < 0.01, Student's t test).

A-F: Double immunofluorescence with rabbit anti-SNRPC (clone EPR16034, Abcam, Cy3) and mouse anti-CD20 (clone L26, DCS, FITC; A), mouse anti-CD68 (clone KP1, DCS, FITC; B), mouse anti-NeuN (clone A60, Millipore, FITC; C), mouse anti-GFAP (clone GA-5, Biogenex, FITC; D), mouse anti-Olig2 (clone Olig2/2400, Abcam, FITC; E), and mouse anti-CD34 (clone QBend/10, Biogenex, FITC; F). Microphotographs were taken with an Axiophot (Zeiss, Jena, Germany) and Zen 2 software (Zeiss). Original magnification: x400 (objective: x40). Inserts: x750 (A-C), x1000 (D-F). Overlay of the microphotographs and adjustment for contrast and brightness were performed with Adobe Photoshop software version CC.
Figure 3

A

270

29

185

56

nBCR

IGHV3

IGHV4-34

113

42

47

147

47

IGHV3

IGHV4-34

B

p < 0.0274

C

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Title: The process of somatic hypermutation increases polyreactivity for central nervous system antigens in primary central nervous system lymphoma.

Authors: Manuel Montesinos-Rongen¹, Monica Terrao¹, Caroline May², Katrin Marcus², Ingmar Blümcke³, Martin Hellmich⁴, Ralf Küppers⁵, Anna Brunn¹, and Martina Deckert¹

Affiliations: ¹Institute of Neuropathology, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany; ²Medizinisches Proteom-Center, Ruhr-University Bochum, Bochum, Germany; ³Department of Neuropathology, University Hospital Erlangen, Erlangen, Germany; ⁴Institute of Medical Statistics and Computational Biology (IMSB), Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany; ⁵Institute of Cell Biology (Cancer Research), University of Duisburg-Essen, Medical School, Essen, Germany, and German Cancer Consortium (DKTK)

Supplemental Material
Supplemental Methods

Assessment of autoreactivity by indirect immunofluorescence with HEp-2 cells, anti-nuclear antibody (ANAcombi) ELISA and anti-nuclear and cellular antibody (ANCAcombi) ELISA

RecAb (ELISA: 200 ng/µl, HEp-2: 250 ng/µl as well as 10 µg/ml), were tested by ANAcombi and ANCAcombi ELISA and with the HEp-2 kit (Orgentec, Mainz, Germany) according to the manufacturer's instructions.

Determination of recAb reactivity against spliceosomal nuclear ribonucleoprotein C (SNRPC) by ELISA

Recombinant human SNRPC (OriGene, Herford, Germany) was coated (5 µg/ml) to a 96 well plate (Eppendorf, Hamburg, Germany) at 4°C overnight. After blocking (1% BSA in PBS-T), recAbs (200 µg/ml as well as 10 µg/ml) were applied, followed by donkey anti-rabbit IgG alkaline phosphatase (1:5,000, Jackson ImmunoResearch, Cambridgeshire, UK). Monoclonal rabbit anti-human SNRPC (clone EPR16034, Abcam, Cambridge) monoclonal mouse anti-MBP antibody (clone MBP101, Abcam) was used as positive control and negative control, respectively (400 ng/ml). Analysis was performed in triplicate. Experiments were repeated twice.

Immunoprecipitation of nBCR and tBCR with non-tumor CNS tissue

Twenty 10 µm frozen sections from brain biopsies of five epilepsy patients were lysed with RIPA buffer (Sigma, Deisenhofen, Germany) and pooled. Immunoprecipitation with purified recAb (5 µg) was performed according to the manufacturer (Miltenyi Biotech, Bergisch Gladbach, Germany). Proteins were eluted with Laemmli buffer, separated by SDS-PAGE (with proteins migrating up to 1 cm), and analysed by mass spectrometry. Immunoprecipitation in the absence of antibody served as control (10
replicates). RecSA13 was used as control (5 µg/ml). Cut-off for immunoprecipitated proteins was > 2-fold enrichment. Only proteins that yielded consistently positive results in triplicate analyses were considered.

**Immunoprecipitation of nBCR and tBCR with PCNSL**

Twenty 10 µm frozen sections from cryopreserved PCNSL (cases #01, #02, #03, #04, #05, #08, #09, #10) were lysed with 250 µl of RIPA buffer (Sigma). Immunoprecipitation was performed with recAbs (5 µg) and 25 µl of µMACS ProteinG MicroBeads (Milteny) according to the manufacturer’s instructions. Proteins bound were eluted with Laemmli buffer and separated by SDS-PAGE with proteins migrating up to 1 cm. Gel segments containing the proteins were dissected and analysed by mass spectrometry. Immunoprecipitation in the absence of recAb served as control. Cut-off for immunoprecipitated proteins was > 2-fold enrichment. Only proteins that yielded consistent positive results in triplicate analyses were considered.

**Semiquantitative evaluation of SNRPC expression**

Immunohistochemical expression of SNRPC (clone EPR16034, Abcam) was evaluated (PCNSL #11–#30) according to a semiquantitative grading system (0: no cells, +:0–30%, ++:30–50%, +++:50–80%, ++++:80–100%) of cells independently by two authors (M.D., A.B.), yielding similar results.

**Double immunofluorescence for neurabin-I and Neu-N**

Paraffin sections of normal brain were stained with mouse anti-human NeuN (clone A60, Merck Millipore, Darmstadt, Germany), horse anti-mouse BSP (Dianova, Hamburg, Germany), and extravidin-FITC (Sigma, Deisenhofen, Germany), followed
by rabbit anti-neurabin-I antibody (ab3483, Abcam, Cambridge, UK) and goat anti-
rabbit-Cy3 (Dianova). As negative control, primary antibodies were omitted.
**Supplemental Table 1: Patients’ data.**

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All patients were HIV-negative. Systemic lymphoma manifestation was excluded.
**Supplemental Table 2:** Immunoglobulin heavy and light gene characteristics of the PCNSL used for generation of naive BCR.

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**Supplemental Table 3:** Immunoglobulin heavy and light chain sequences.

As separate Excel file.

**Supplemental Table 4:** The recombinant anti-tetanus toxoid antibody recSA13 recognizes tetanus toxoid protein as dominant target in immunoprecipitation with epilepsy tissue and tetanus toxoid.

As separate Excel file.

**Supplemental Table 5:** Results of ProtoArray analysis.

As separate Excel file.

**Supplemental Table 6:** Results of immunoprecipitation analysis.

As separate Excel file.
Supplemental Table 7: Expression of SNRPC in PCNSL.

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0, no expression;
+, > 0 – 30 % of tumor cells immunoreactive;
+++, > 30 – 50 % of tumor cells immunoreactive;
++++, > 50 – 80 % of tumor cells immunoreactive;
+++++, > 80 – 100 % of tumor cells immunoreactive.
**Supplemental Figure Legends**

**Supplemental Figure 1** Generation of recAbs

A: For each recAb (3 µg), expression of the heavy (~56 KDa) and light chain (25-30 KDa) is demonstrated by SDS-PAGE.

B, C: To test for potentially functional folding, reactivity of the recAbs with anti-human IgG was studied by ELISA. The initial concentration was 10 ng/µl for each recAb. Human IgG (initial concentration: 10 ng/µl) was used as positive control (B,C), and anti-SNRPC (Abcam) was used as negative control (C). Data are shown for nBCR and tBCR of PCNSL #09 and #10 (B) and for PCNSL #02 (C). Similar results were obtained for all recAbs. Each experiment was performed in triplicate; standard deviation is given for each point of measurement.

**Supplemental Figure 2** Regular staining staining pattern of the antibodies used for protein expression analysed in appropriate positive control tissues

A: Nuclear expression of SNRPC (Cy3) in nuclei of seminal epithelium of a normal testis. Staining with monoclonal rabbit anti-SNRPC (clone EPR16034, Abcam).

B: Selective, prominent expression of the CD20 antigen (FITC) on the surface of malignant B cells of a PCNSL. Staining with monoclonal mouse anti-CD20 (clone L26, DCS).

C: In an inflammatory, demyelinating brain lesion macrophages (arrowheads) and activated microglial cells (arrows) prominently express the CD68 antigen (FITC). Staining with monoclonal mouse anti-CD68 (clone CS, PG-M1, DCS).

D: In the normal temporal cortex, neurons express NeuN in the nuclei (FITC). Staining with monoclonal mouse anti-NeuN (clone A60, Millipore).

F: In an anaplastic oligodendroglioma (WHO grade III), the tumor cells show a nuclear expression of Olig2 (FITC). Staining with monoclonal mouse anti-Olig2 (clone Olig2/2400, Abcam).

G: Capillary endothelial cells express the CD34 antigen (arrows, FITC). * indicates the vascular lumen. Staining with monoclonal mouse anti-CD34 (clone QBend/10, BioGenex).

Microphotographs were taken with an Axiophot (Zeiss, Jena, Germany) and Zen 2 software (Zeiss). Original magnification: x400 (objective: x40). Adjustment for contrast and brightness were performed with Adobe Photoshop software version CC.

Supplemental Figure 3 Absence of staining with omission of the primary antibodies used for protein expression analysed in appropriate positive control tissues

A: Omission of the CD20 (FITC) and the SNRPC (Cy3) antibodies abolishes immunostaining of the CD20⁺ SNRPC⁺ tumor cells of a PCNSL.

B: Macrophages/activated microglia intermingled between the tumor cells of a PCNSL are not stained when the CD68 (FITC) and SNRCP (Cy3) antibodies are omitted.

C: In the normal temporal cortex, NeuN⁺ neurons are not stained upon omission of the NeuN (FITC) and SNRPC (Cy3) antibodies. *: autofluorescence of intraneuronal lipofuscin.

D: GFAP⁺ activated astrocytes in the temporal grey matter are not stained upon omission of the GFAP (FITC) and SNRPC (Cy3) antibodies.

E: Olig2⁺ oligodendrocytes in the white matter are not stained upon omission of the Olig2 (FITC) and SNRPC (Cy3) antibodies.

F: CD34⁺ endothelial cells of cortical blood vessels are not stained upon omission of the CD34 (FITC) and SNRPC (Cy3) antibody.
Microphotographs were taken with an Axiophot (Zeiss, Jena, Germany) and Zen 2 software (Zeiss). Original magnification: x400 (objective: x40). Adjustment for contrast and brightness were performed with Adobe Photoshop software version CC.

**Supplemental Figure 4** Reactivity of recAb with HEp-2 cells

Representative staining pattern of nBCR and tBCR of PCNSL #01, #02, and #10 with HEp-2 cells. NBCR of PCNSL #01 and #10 did not react, while PCNSL #02 showed a weak immunostaining compared to the positive control (human serum containing anti-nuclear antibodies). Regarding tBCR, PCNSL #01 and #02 weakly reacted with HEp-2 cells, while PCNSL #10 was negative.

Fluorescence with FITC-conjugated anti-human IgG, original magnification x400. Microphotographs were taken with an Axiophot (Zeiss, Jena, Germany) and Zen 2 software (Zeiss). Original magnification: x400 (objective: x40).

**Supplemental Figure 5** Numbers of proteins recognized in PCNSL by recAb derived from nBCR and tBCR of PCNSL

BoxPlot diagram shows the numbers of proteins that were recognized by at least one nBCR and tBCR recAb in immunoprecipitation using total protein extracts from PCNSL. Only proteins co-immunoprecipitating with either nBCR or tBCR were analysed. Statistical significance was determined by the exact Wilcoxon signed rank test (calculated with R version 3.5.0, R Foundation for Statistical Computing, Vienna, Austria).

**Supplemental Figure 6** RecAb do not bind to SAMD14 / neurabin-I in Western blot analysis
Total protein extracts from the respective PCNSL (lane 1), epilepsy tissue (lane 2), glioblastoma multiforme (lane 3), and peripheral blood mononuclear cells (lane 4) were used. RecAb were used at 50 µg/ml as first antibody, followed by FITC-conjugated anti-human IgG (5 µg/ml, Thermo Fisher). Similar results were obtained with recAb at 10 µg/ml. Arrows indicate 45 kDa (SAMD14) and 151 kDa (neurabin-I), respectively.

**Supplemental Figure 7** TBCR recognize multiple proteins as evidenced by dot blot analysis

As target, protein lysates from PCNSL cases #01 - #05, #09, and #10 (row I, A-G), from epilepsy (row II, A-G), glioblastoma multiforme (GBM, row III, A-I), and peripheral blood mononuclear cells (PBMC, row IV, A-I) were used. As controls, the respective tBCR (row V, A-G), recSA13 (loading control, row VI, A-G), and HEK293T cells (row VII, A-G) were spotted. In H and I, tetanus toxoid protein (initial concentration: 0.5 µg, Sigma) was used as target, I serves as negative control with omission of the primary antibody. TBCR and recSA13 were used at a starting concentration of 10 ng. Peroxidase-conjugated polyclonal rabbit anti-human IgG (1:25,000 Sigma) was used as secondary antibody.

**Supplemental Figure 8** Affinity of recAb derived from nBCR and tBCR for SNRPC

A: RecAb derived from PCNSL #01 to #10 were tested for binding of SNRPC by ELISA. Compared to the positive control, there is only very low binding of the recAB derived from both the nBCR as well as the tBCR. Monoclonal rabbit anti-SNRPC (clone EPR16034, Abcam) and mouse anti-MBP (clone MBP101, Abcam) served as positive and negative control, respectively. RecAb as well the positive and negative controls were used at a concentration of 0.4 ng/µl.
B: RecAb derived from PCNSL #09 was tested in serial dilutions for binding to SNRPC by ELISA. Initial concentration of all antibodies was 0.4 ng/µl. Note only a slight binding of recAb at highest concentration of the recAb. Analysis was performed in triplicate. Data represent one of three tests yielding similar results.

C: SNRPC was recognized by the recAb derived from the nBCR as well as the tBCR of PCNSL #09 only when tested at a concentration 500-fold increased to the positive control, anti-SNRPC (Abcam). Despite low-affinity, the tBCR showed increased binding as compared to the corresponding nBCR. Analysis was performed in triplicate. Data represent one of three tests yielding similar results.

**Supplemental Figure 9** Neurabin-I expression in PCNSL tissue as determined by Western Blot

Total protein extracts from 20 PCNSL (cases #31 - #50, for patients characteristics see Suppl. Table 1) were used. A tumor cell content of >80% was assured by analysis of hematoxylin & eosin-stained cryostat sections above and below the tissue sections used for protein extraction. Jurkat cells (J) served as positive control recommended by the manufacturer showing a strong band at 151 KDa (arrows), corresponding to neurabin-I. M: marker. As primary antibodies, two different polyclonal antibodies against the N-terminus of neurabin-I were used, i.e. rabbit anti-neurabin-I (1:500, antibody online, ABIN2790677, A) and rabbit anti-neurabin-1 (1:500, antibody online, ABIN1108406, B). HRP-conjugated goat anti-rabbit (1:4000, ThermoFisher, Waltham, MA) was used as secondary antibody.

Rabbit anti-human transferring receptor (1:500, clone HPA028598, Sigma) and Ponceau staining were used as loading control.

**Supplemental Figure 10** Neurabin-I is expressed in CNS neurons and their axons
A,B: In the normal temporal cortex, neurabin-I (Cy3) is expressed in the cytoplasm of NeuN+ (FITC) neurons (arrowheads). An elongated swollen axon in the temporal grey matter also expresses neurabin-I (Cy3) (B, arrows).

C: In the frontal cortex, large neurons characterized by a prominent nucleolus (arrowheads) as well as their axons (arrows) express neurabin-I (Cy3).

D: Omission of the anti-neurabin-I and anti-NeuN antibodies abolishes Cy3 and FITC staining from neurons. *: autofluorescence of intraneuronal lipofuscin.

Microphotographs were taken with an Axiophot (Zeiss, Jena, Germany) and Zen 2 software (Zeiss). Original magnification: x400 (C, D), x1000 (A, B). Adjustment for contrast and brightness were performed with Adobe Photoshop software version CC.

**Supplemental Figure 11** Model for the impact of a faulty GC reaction on PCNSL pathogenesis

Instead of normal B cell maturation with SHM of a naïve B cell resulting in a terminally differentiated plasma/memory B cell (upper panel), a mutation prevents apoptosis of an auto-/polyreactive B cell, instead conferring a survival advantage. Upon GC entry, SHM starts, modifying IG and BCL6 genes. Through extension of SHM to proto-oncogenes (aberrant SHM, ASHM) and the occurrence of translocations, the B cell acquires further oncogenic hits while simultaneously becoming unable to stop SHM. This leads to increased auto-/polyreactivity without being (further) selected for high-affinity BCR antigen binding, thus, resulting in a PCNSL (precursor) B cell characterized by a GC exit geno-/phenotype with auto-/polyreactivity with a shift towards increased numbers of CNS proteins recognized.
Supplementary Figure 1

**A**

![Image of gel electrophoresis](image-url)

**B**

![Graph of antibody dilution vs. OD405](image-url)

**C**

![Graph of antibody dilution vs. OD405](image-url)
Supplementary Figure 5

![Graph showing number of CNS proteins bound exclusively to nBCR and tBCR with a p-value of < 0.5482]
Supplementary Figure 6

![Supplementary Figure 6](image-url)
Supplementary Figure 7

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<th>B (tBCR#02)</th>
<th>C (tBCR#03)</th>
<th>D (tBCR#04)</th>
<th>E (tBCR#05)</th>
<th>F (tBCR#10)</th>
<th>G (recSA13)</th>
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Dilution:
- 1
- 1:5
- 1:25
- 1:125
**Supplementary Figure 11**

- Bone marrow → Peripheral B cell → Naive B cell
- Bone marrow → Peripheral B cell → Polyreactive and/or autoreactive B cell
- Peripheral B cell → Mutated lymphoma precursor B cell (MYD88/CD79B)
- Peripheral B cell → Anergic/tolerant B cell → Apoptosis
- Germinal Center (GC) with BCL6 expression and class switch recombination (CSR)
  - SHM/ASHM, no CSR
- Terminally differentiated B cell
  - Plasma cell
  - Memory B cell
  - PCNSL precursor cell

CD20+ BCL6+ MUM1+ CD138-
- Highly mutated IG genes
- Ongoing SHM/ASHM
- IgM+/IgD+ → No CSR
- ‘GC exit phenotype’
- Polyreactivity with shift to CNS proteins