Synaptic proximity enables NMDAR signalling to promote brain metastasis

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Metastasis—the disseminated growth of tumours in distant organs—underlies cancer mortality. Breast–to–brain metastasis (B2BM) is a common and disruptive form of cancer and is prevalent in the aggressive basal–like subtype, but is also found at varying frequencies in all cancer subtypes. Previous studies revealed parameters of breast cancer metastasis to the brain, but its preference for this site remains an enigma. Here we show that B2BM cells co-opt a neuronal signalling pathway that was recently implicated in invasive tumour growth, involving activation by glutamate ligands of N–methyl–D–aspartate receptors (NMDARs), which is key in model systems for metastatic colonization of the brain and is associated with poor prognosis. Whereas NMDAR activation is autocrine in some primary tumour types, human and mouse B2BM cells express receptors but secrete insufficient glutamate to induce signalling, which is instead achieved by the formation of pseudo–tripartite synapses between cancer cells and glutamatergic neurons, presenting a rationale for brain metastasis.

There has been substantial progress in the past decade in defining general mechanisms by which cancer cells in solid tumours acquire the hallmark capability to metastasize1,2. Brain metastases originating from breast tumours are the most prevalent in women3. Parameters of B2BM have been identified using cancer cells selected for proficiency at seeding lesions in the brain2. These parameters include activation of a sialyltransferase that promotes extravasation of cancer cells through the blood–brain barrier4, activation of inducers and modulators of reactive astrocytes5,6 and evasion of the activin B–activin receptor-like kinase-7 (ALK7) homeostatic barrier8. Although brain metastatic cancer cells can acquire neuronal cell characteristics8,9, the potential interplay between cancer cells and neurons is largely unexplored.

The neuronal glutamate receptor NMDAR promotes invasive tumour growth in neuroendocrine and ductal pancreatic cancers10–12. Autocrine secretion of glutamate stimulates multimeric NMDAR receptors comprising GluN1 and GluN2B subunits—the latter containing phosphorylation sites critical for NMDAR signalling14–20—in response to glutamate, which is transduced via the cytoplasmic adaptor protein GKAP to evoke an invasive growth program characterized by broad changes in cellular regulatory pathways12.

Implicating NMDAR in breast cancer metastasis

Seeking to more broadly assess glutamate-mediated signalling in human cancer, we evaluated the expression of a core set of parameters of NMDAR and other glutamate receptor component genes across multiple human cancer types, which led us to focus on breast cancer, encouraged by histological clues11. From the analysis of 1,100 primary breast cancers (TCGA)21, we derived transcriptional signatures and patient-specific signature scores for each of the four major glutamate receptors (the NMDA, AMPA, kainate and metabotropic receptors), which revealed distinct glutamate receptor-associated scores and expression in different subtypes (Extended Data Fig. 1a–d). In particular, basal-like tumours exhibited higher NMDAR scores but lower AMPA and kainate receptor scores than the other subtypes (Extended Data Fig. 1a–c). This association was driven by expression of GluN2 components (especially GluN2B; encoded by GRIN2B) and GKAP (encoded by DLGAP1) (Extended Data Fig. 1e). An NMDAR signature based on the four GluN2 genes and GKAP confirmed a strong association with basal-like breast cancer (Fig. 1a–c), which was validated using a recently described 148-gene signature diagnostic of NMDAR pathway activity12 (Extended Data Fig. 1f, g). Basal-like breast cancer is characterized by unfavourable prognosis, lack of expression of hormone receptors (estrogen receptor and progesterone receptor) and of the growth factor receptor HER2 (in triple-negative breast cancer or TNBC), and by a high risk of recurrence (ROR). We found that increased expression of GRIN2B was particularly strongly associated with TNBC status (Fig. 1d) and with a high ROR across all breast cancers (Fig. 1e). Elevated GRIN2B expression could be detected in more than 40% of all breast cancers tested, whether segregated by subtype or ROR status, but especially in ROR-high and basal-like cases (Fig. 1f). By contrast, the obligatory GluN1 subunit of NMDAR (encoded by GRIN1) was uniformly expressed in a large majority (more than 90%) of all breast cancer subtypes (Fig. 1g).

Notably, in an independent clinical cohort of breast cancers, expression of GRIN2B was associated with poor distant relapse-free survival, reflecting metastasis (Fig. 1h). The association with metastasis was highly significant within the subset of basal-like and TNBC tumours (Fig. 1i, j), along with a moderate association with worse prognosis (metastasis) for luminal A tumours and no association for the other breast cancer subtypes (Extended Data Fig. 1h).

Motivated by these results, we sought to explore the possibility that GluN2B-containing NMDAR is functionally involved in B2BM.

NMDAR activation in breast–to–brain metastases

To begin, we immunostained paired primary human breast tumours and brain metastases in a tissue microarray for phosphorylated GluN2B expression and brain metastases in a tissue microarray for phosphorylated GluN2B.
Expression of GluN2B–NMDAR signalling components is associated with poor prognosis across all human breast cancer types. a, mRNA expression of five NMDAR-associated genes (GRIN2A, GRIN2B, GRIN2C, GRIN2D and DLGAP1) in 1,100 patients with breast cancer. These results are based upon data generated by the TCGA Research Network (https://www.cancer.gov/tcga)\(^1\). Samples were sorted by NMDAR score (b), which combines, for each sample, Z-scores of normalized expression values of all five genes. Box plot comparison of NMDAR scores among PAM50 breast cancer subtypes, ranked by median score within each subtype. NMDAR scores in basal-like breast cancer were tested against scores obtained for all other subtypes. Wilcoxon two-tailed test. ANOVA, analysis of variance. c, NMDAR mRNA expression score for the TCGA breast cancer cohort (n = 1,100). Scores for all breast cancer samples are ranked in increasing order and colour coded on the basis of PAM50 subtype. d, Box plot comparison of GRIN2B mRNA expression (Z-scores of log$_2$-normalized RNA-seq data, normalized using RSEM) between samples classified as triple-negative breast cancer (TNBC; red dots) versus remaining samples (grey dots). Wilcoxon two-tailed test. e, Box plot comparison of GRIN2B mRNA expression (Z-scores of log$_2$-normalized RNA-seq RSEM-normalized values) between samples with high ROR (red dots) versus medium–low ROR (blue dots). Wilcoxon two-tailed test. f, g, Percentage of tumours with detectable GRIN2B (f) and GRIN1 (g) mRNA (RSEM-normalized counts > 0) classified by high ROR, medium-to-low (med–low) ROR and the most frequent PAM50 subtypes. h, Distant relapse-free survival analysis of an independent cohort of patients with breast cancer (GSE25065) with overexpressed GRIN2B (red line, top 25%) versus remaining patients (blue line). Two-sided log-rank test. i, j, Distant relapse-free survival analysis of basal-like (i) or TNBC (j) patients (GSE25065) with overexpressed GRIN2B (red line, top 25%) versus remaining patients (blue line). Two-sided log-rank test. The thick central line of each box plot represents the median number of significant mRNAs, the bounding box corresponds to the 25th–75th percentiles and the whiskers extend up to 1.5 times the interquartile range.

(pGluN2B) as well as total GluN2B protein. GluN2B phosphorylations at Tyr1472 and Tyr1252 (pGluN2B(Y1472/Y1252)) are instrumental for and diagnostic of cell-surface localization of NMDAR and induction of downstream signalling in response to glutamate\(^{14–20}\). GluN2B-mediated NMDAR signalling (revealed by the percentage of pGluN2B(Y1472/Y1252)-positive cancer cells) was high in 7 out of 9 and 10 out of 12 brain metastases, respectively, and was frequently increased compared with matched primary breast tumours (Fig. 2a, b, Extended Data Fig. 2a–e, Supplementary Table 1). Notably, total GluN2B and GluN1 were similarly expressed in primary and brain metastases (Extended Data Fig. 2f–k).

Next, we assessed levels of GluN2B, pGluN2B(Y1472/Y1252) and GluN1 in breast cancer cells (human MDA-MB-231 and MDA231) or mouse TS1 cells that had been selected for increased brain-metastatic proficiency in mice. We found that both subunits (GluN2B and GluN1), as well as pGluN2B, were significantly upregulated in each selected B2BM cell line (MDA231-BrM and TS1-BrM) in comparison to the poorly brain-metastatic parental MDA231 and TS1 cells (Fig. 2c, Supplementary Fig. 1), consistent with the possibility that upregulated NMDAR has a role in the acquired brain-metastatic proficiency of the B2BM cells. To assess the potential activation of GluN2B-mediated NMDAR signalling in the primary and metastatic sites, we inoculated mice with MDA231-BrM cells, either into the mammary fat pad to form orthotopic primary breast cancers, into the left cardiac ventricle (ICD) to elicit brain metastasis, or into the lateral tail vein to induce lung metastasis (Fig. 2d). Essentially 100% of brain metastases showed clear membrane staining for pGluN2B(Y1472/Y1252) in contrast to the much lower frequency of staining in orthotopic breast tumours and lung metastases (Fig. 2e, f, Extended Data Fig. 2l–n).

Paracrine source of glutamate to B2BM cells

To investigate why there was preferential activation of GluN2B–NMDAR signalling in brain metastases, we first established that l-glutamate, the major agonist for the NMDAR\(^{22}\), could increase GluN2B-mediated NMDAR signalling (as indicated by increased pGluN2B(Y1472/Y1252)) when supplied in a dose-dependent manner to MDA231-BrM cells in culture (Fig. 3a, Supplementary Fig. 1). Furthermore, using fluorescence imaging and patch-clamp recordings in 2D cultures of TS1-BrM cells, we verified that exogenously applied NMDA or glutamate both activated single-channel currents and elevated intracellular calcium, indicating that NMDAR-mediated signalling was functional in B2BM cells (Extended Data Fig. 3).

An ancillary question was where the NMDAR-activating l-glutamate might come from in vivo. One possibility was autocrine supply,
on the basis of several considerations: First, glutamate is secreted by TNBC cells in culture, and is detectable in cultured parental and B2M cells (Fig. 3b). Additionally, human glioma cells secrete extracellular glutamate, which triggers neuronal death and facilitates tumour expansion. Furthermore, pancreatic cancer cells increase their cellular glutamate, which triggers neuronal death and facilitates tumour expansion. Furthermore, pancreatic cancer cells increase their cellular glutamate, which triggers neuronal death and facilitates tumour expansion. Furthermore, pancreatic cancer cells increase their cellular glutamate, which triggers neuronal death and facilitates tumour expansion.

**Fig. 2** | GluN2B–NMDAR signalling is highly activated in human brain metastasis. a, b, Representative images (a) and quantification (b) of immunohistochemistry staining of GluN2B (Y1472) in paired human primary breast tumours (primary) and brain metastases (brain mets). Data are mean ± s.e.m. Two sided Wilcoxon two-tailed test. Scale bars, 100 μm. Breast cancer subtype: three HER2+, one luminal and five unknown. c, Western blot of pGluN2B (Y1252), pGluN2B (Y1472), GluN2B and GluN1 in pairs of human (left, MDA231) and mouse (right, TS1) parental breast cancer cell lines and their corresponding B2BM derivatives. Three independent experiments. Protein levels in B2BM cells were quantified relative to cognate parental cells, after normalization to β-actin or GAPDH controls, for which blotted membranes were cut into two parts. <50 kDa for β-actin or GAPDH staining and 50–250 kDa for pGluN2B (Y1252) (Fig. 3c). Additional, equally loaded gels were blotted and stained for GluN2B or GluN1, using the loading control from the pGluN2B (Y1252) gel. Relative protein levels are shown above the blot lanes. d, Schematic of assays performed with luciferase-expressing B2BM cells either inoculated orthotopically into the mammary fat pad (MFP) to develop primary tumours, ICD to seed brain metastases, or intravenously (IV) into the tail vein to seed lung metastases. e, f, Immunohistochemistry staining of pGluN2B(Y1472), total GluN2B and luciferase (e), and quantification of pGluN2B(Y1472) staining (f) in primary tumours, brain metastases and lung metastases formed by MDA231-BM cells (days 28–35 after injection). Scale bar, 10 μm. These images correspond to the insets shown in Extended Data Fig. 2. Two-tailed Student’s t-test, n = 3 mice per group; data are mean ± s.e.m.

**Fig. 3** | Autocrine secretion of l-glutamate is not sufficient to explain the brain metastasis-specific induction of GluN2B–NMDAR signalling. a, Western blot of pGluN2B (Y1252) and pGluN2B (Y1472) in MDA231-BM cells treated with increasing concentrations of l-glutamate and 10 μM glycine for 1 h in artificial cerebrospinal fluid. The numbers below the blot indicate levels of pGluN2B (Y1252), with pGluN2B (Y1472) expression normalized to GAPDH. Three independent experiments. GAPDH control was run in the same gel as pGluN2B (Y1252), and on separate gels from pGluN2B (Y1472). b, Secreted l-glutamate concentrations in conditioned medium from MDA231 and TS1 parental and derivative B2BM cells after 48 h of culture. Two-tailed Student’s t-test, n = 3 independent experiments. c, RT-qPCR analysis of glutamate transporter gene expression in MDA231 parental and B2BM cells. Two-tailed Student’s t-test; data are mean ± s.e.m., n = 3 independent experiments. d, Immunofluorescence detection of apoptotic cells (TUNEL, green) in mouse brains with metastases formed by MDA231-BM cells (days 28–35 after injection). Luciferase (magenta) marks the cancer cells, and NeuN (red) shows neurons. Bottom, DNAse1 treatment of brain tissue sections was used as a TUNEL-positive control. Images shown are representative of an analysis of >20 brain metastases from 4 mouse brains, 2 sections per mouse brain. Scale bars, 10 μm. Three independent experiments.

Given the reports that autocrine secretion of glutamate by glioma cancer cells elicits neuronal cell death, we immunostained mouse brain tissue sections bearing B2B metastases for apoptotic neurons (revealed as TUNEL+ NeuN– or cleaved-caspase-3–NeuN–), and found that apoptotic neurons were infrequent (Fig. 3d). By contrast, apoptotic neurons were frequently found at the margins of glioma brain tumours (Extended Data Fig. 4d). These results indicate that the concentration of extracellular glutamate in the B2B metastatic microenvironment was relatively low. Collectively, these results suggest that autocrine secretion of glutamate is not sufficient to explain the brain metastasis-specific induction of GluN2B–NMDAR signalling.

**Pseudo-tripartite synapses supply glutamate**

We therefore studied the possibility that a paracrine supply of glutamate from the brain microenvironment was activating NMDAR signalling in metastatic lesions. High levels of glutamate are released locally as neurotransmitters from excitatory glutamatergic presynaptic neurons. The secreted glutamate is then rapidly absorbed by NMDAR-expressing postsynaptic neurons as well as by astrocytes surrounding the synaptic cleft, the latter serving to ensure the fidelity of neurotransmission and to avoid high extracellular glutamate-induced neurotoxicity. To investigate whether B2BM cells could be obtaining glutamate from glutamatergic synapses, we immunostained tissue sections of metastatic lesions and adjacent normal brain for B2BM cells (revealed by luciferase), for presynaptic neurons (revealed by vGluT2), and for an indicator of NMDAR signaling.
Luciferase vGlut2 pGluN2B

![Image](https://example.com/image1)

**Fig. 4 | B2BM cancer cells form pseudo-tripartite synapses with neurons.** a, b, Immunofluorescence staining of luciferase (B2BM; blue), pGluN2B (Y1252) (green) and vGlu2 (red) in a mouse brain metastasis formed by MDA231-BrM cells (day 25 after injection), imaged by STED super-resolution microscopy. a, Image with 3D volume. The white dotted border box indicates a normal synapse (shown in Extended Data Fig. 5c). b, Tilted 3D image of the area circumscribed by the solid white border in a shows a pseudo-tripartite synapse between B2BM cell(s) and neurons, revealed by vGlut2+ neuronal puncta (arrow) and pGluN2B+ puncta in B2BM cells and presumptive neuronal processes in close proximity (arrowhead). Images are representative of nine brain metastases from three mice and two sections per mouse brain. Side length for each square in the 3D view is 5 μm (a) and 400 nm (b). The images are projected in Supplementary Videos 1, 2, e. Electron micrograph of a brain metastasis formed by MDA231-BrM cells in the cerebral cortex. The cancer cell is shown in purple. Scale bar, 5 μm. Two independent experiments. d, Three serial electron microscopy images from the region indicated by the black box in c showing protrusions from a B2BM cell in close apposition to an excitatory synapse. Representative of six regions within two ultrathin sections from one mouse brain. B2BM cells, purple; D, dendrite (yellow); Ax, axon (green); R, postsynaptic density. Scale bar, 1 μm. e, Three-dimensional reconstruction of the region in d, illustrating protrusion of a metastatic cell proximal to pre- and postsynaptic elements and the synaptic density (red). f, Brain-metastatic cancer cells supplant astrocytes at neuronal synapses to enable NMDAR-dependent colonization. The tripartite synapse comprising pre- and postsynaptic neurons and astrocytes (left) is subverted by metastasizing breast cancer cells, which mimic astrocytes to form pseudo-tripartite synapses (right) that provide a source of glutamate ligand, activating NMDAR signalling to stimulate tumour growth in the brain.

NMDAR drives metastatic colonization

We next determined whether GluN2B–NMDAR signalling promotes brain metastasis. The conditionally knocked down (KD) B2BM cells were inoculated into mice by intracardiac injection concomitant with induction of the shRNAs. The GluN2B-KD B2BM cells produced lower brain tumour burden and significantly prolonged survival (Fig. 5a, b). Notably, EMRF, a candidate effector upregulated by NMDAR in pancreatic cancer cells12, was expressed in brain lesions of mice injected with control shRNA and markedly reduced in GluN2B-KD brain lesions (Extended Data Fig. 7a, b), consistent with ongoing NMDAR signalling in wild-type brain metastases. By contrast, the GluN2B-KD did not affect orthotopic primary tumour growth in the mammary fat pad or transplant-derived lung metastases (Extended Data Fig. 7c, d). To exclude possible off-target effects of the shRNA, GluN2B was re-expressed in GluN2B-KD MDA231-BrM cells, which rescued their brain metastatic capability (Extended Data Fig. 7e). Similar results were

![Image](https://example.com/image2)

![Image](https://example.com/image3)
observed with mouse TS1-BrM cells with the GluN2B KD (Extended Data Fig. 8, Supplementary Fig. 1).

To further define the stage at which GluN2B–NMDAR signalling contributes to brain metastasis, we performed a short-term metastasis assay with MDA231-BrM cells to assess initial seeding and survival. Circulating cancer cells complete their extravasation across the blood–brain barrier and evasion of tissue barriers 6–8. Given that subsets of all breast cancer subtypes show elevated NMDAR expression in association with comparatively poor distant relapse-free and/or overall survival, it is possible that breast tumours commonly produce cancer cells whose growth can be promoted by NMDAR signalling in the brain microenvironment. This realization builds on an earlier study documenting the ability of metastatic breast cancer cells to couple via gap junctions with astrocytes, reprogramming them to create a supportive tumour microenvironment 7. Thus, breast cancer cells functionally interact with both neurons and astrocytes to orchestrate their metastatic growth. Moreover, the capability for brain metastasis also depends on other components, including alterations that facilitate extravasation across the blood–brain barrier and evasion of tissue barriers 8–9. Given that subsets of all breast cancer subtypes show elevated NMDAR expression in association with comparatively poor distant relapse-free and/or overall survival, it is possible that breast tumours commonly produce cancer cells whose growth can be promoted by NMDAR signalling. Since lethality is not governed exclusively by brain metastasis, these observations suggest that NMDAR signalling may contribute more broadly to breast tumour progression. To assess this possibility, we envisage future studies probing large breast cancer biobanks via immunostaining of tissue microarrays with the phospho-specific antibodies that are diagnostic of active NMDAR signalling. Finally, it remains to be determined whether brain metastases from other primary tumour types similarly parasitize neuronal synapses to stimulate NMDAR-dependent colonization, and whether certain primary tumours and metastases to other sites characterized by ‘peri-neural invasion’ 34 might establish pseudo-tripartite synapses with peripheral nerves to activate and sustain NMDAR-dependent invasive growth.

Discussion

In sum, these results demonstrate an interaction between brain metastatic cells and neurons that serves to activate the glutamate-stimulated GluN2B–NMDAR signalling axis in cancer cells in the brain microenvironment. This realization builds on an earlier study documenting the ability of metastatic breast cancer cells to couple via gap junctions with astrocytes, reprogramming them to create a supportive tumour microenvironment. Thus, breast cancer cells functionally interact with both neurons and astrocytes to orchestrate their metastatic growth. Moreover, the capability for brain metastasis also depends on other components, including alterations that facilitate extravasation across the blood–brain barrier and evasion of tissue barriers. Given that subsets of all breast cancer subtypes show elevated NMDAR expression in association with comparatively poor distant relapse-free and/or overall survival, it is possible that breast tumours commonly produce cancer cells whose growth can be promoted by NMDAR signalling. Since lethality is not governed exclusively by brain metastasis, these observations suggest that NMDAR signalling may contribute more broadly to breast tumour progression. To assess this possibility, we envisage future studies probing large breast cancer biobanks via immunostaining of tissue microarrays with the phospho-specific antibodies that are diagnostic of active NMDAR signalling. Finally, it remains to be determined whether brain metastases from other primary tumour types similarly parasitize neuronal synapses to stimulate NMDAR-dependent colonization, and whether certain primary tumours and metastases to other sites characterized by ‘peri-neural invasion’ might establish pseudo-tripartite synapses with peripheral nerves to activate and sustain NMDAR-dependent invasive growth.
The demonstrated dependency of brain metastatic cells on GluN2B–NMDAR signalling for successful colonization of metastatic lesions suggests a therapeutic opportunity. In practice, however—recognizing the insidious interplay whereby brain metastatic cancer cells co-mingle with NMDA-dependent neurons—directly inhibiting NMDAR signalling in brain-metastatic cancer cells might elicit concomitant central nervous system neurotoxicity. Future studies are likely to identify downstream effectors of GluN2B–NMDAR signalling specific to, and responsible for, brain metastatic cell growth, representing potential vulnerabilities that could be amenable to therapeutic targeting while sparing neurons. In conclusion, this investigation has revealed a mechanism whereby GluN2B-mediated NMDAR signalling is activated in brain-metastatic cancer cells via the formation of pseudo-tripartite synapses to achieve paracrine supply of the glutamate ligand, a sobering manifestation of the ‘seed and soil’ theory of organ-selective metastasis.

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METHODS

Glutamate receptor expression analysis in human breast cancer patient cohorts. To examine transcriptional levels of glutamate receptors, we defined a gene signature for each receptor as composed by its core components. Glutamate receptor signatures used in this study were defined as follows. NMDAR: GRIN1, GRIN2A, GRIN2B, GRIN2C, DLGAP1; AMPAR: GR1A, GR1A2, GR1A3, GR1A4; kainate receptor: GRK1, GRK2, GRK3, GRK4, GRK5; metabotropic receptor: GRM1, GRM2, GRM3, GRM4, GRM5, GRM6, GRM7; and GRMR. Next, for each gene signature, we derived patient-specific gene-expression signature scores. RSEM gene-normalized RNA-seq data from the TCGA breast cancer cohort was collected from the FireBrowse data portal (http://firebrowse.org/) and RSEM counts were log2-transformed (log2(RSEM + 1)) and normalized as z-scores. For each patient, we took the sum of the z-scores obtained for the genes within each signature as the ‘signature score’ corresponding to that patient. As a control, we performed the same analysis by applying a rank-based transformation to the RSEM-normalized counts (rq-transformation) instead of the log2. The obtained scores were highly correlated (Pearson’s coefficient = 0.96). In the manuscript, we decided to use the score obtained from the log2-transformed data as it better reflects the magnitude of mRNA expression differences among the analysed genes, including the cases where mRNA expression of a given gene was not detectable (RSEM = 0). Given the association between NMDAR and basal-like breast cancer was primarily driven by GluN2 components, a second NMDAR signature was defined by excluding GRIN1 from the initial signature (see Fig. 1a–c).

Recently, a 148-gene expression signature was associated with NDMAR-activity in mouse tumours11,12. To compare our NMDAR score with this previously derived 148-gene signature, we first separated genes that were upregulated in NMDAR active tumours (100 genes) from genes that were downregulated (48 genes). Next, we mapped up- and downregulated genes to the corresponding orthologues in human using the biomaRt R package. Upon mapping, we found that the lists of up- and downregulated human genes had non-empty intersections, hence we removed genes present in both lists. Finally, we computed an expression signature score for each TCGA breast cancer patient applying the GSVA method13 (GSVA R package) to the list of unique upregulated genes (91 genes). Pearson’s correlation between signature scores and NMDAR scores was computed and tested for statistical significance using the R function cor.test (Extended Data Fig. 1a).

Within the TCGA breast cancer cohort, NMDAR scores have been compared among patients attributed to distinct PAM50 breast cancer subtypes (luminal A, luminal B, HER2-enriched, basal-like and normal-like) by the Wilcoxon two-tailed test. GRIN2B (encoding GluN2B) mRNA expression had been compared between patients with high or medium–low ROR by Wilcoxon two-tailed test. PAM50 subtypes and ROR had been previously determined for these samples21.

For distant relapse-free survival analysis, we analysed an independent dataset of 198 patients with breast cancer22 (Gene2S8065). Patients were stratified on the basis of GRIN2B expression into a ‘high’ expression group and a ‘low’ expression group. The obtained score sheet of license 3070. To monitor the growth of brain or lung metastases, mice were intraperitoneally injected with n–luciferin (150 mg kg−1), and then imaged and analysed using the IVIS Spectrum imaging system (Caliper Life Sciences) weekly after the injection. For the short-term brain metastasis assay, 5 × 105 MDA231-BrM or TS1-BrM cells suspended in 100 µl PBS were injected into the left cardiac ventricle of the mice. Any mice with abnormal respiratory signs and locomotion behaviour (ticked as score 3–4 according to the score sheet of license 3070) were euthanized.

For the lung metastasis assay, 2 × 105 MDA231-BrM cells suspended in 200 µl PBS were injected into the lateral tail vein of the mice. Any mice with abnormal respiratory signs and locomotion behaviour (ticked as score 3–4 according to the score sheet of license 3214) were euthanized.

Isolation of primary cortical neuron cells and co-culture with cancer cells. The isolation of primary cortical neurons was performed as described previously21. In brief, the brains of E17 embryos of FVB/N mice (Charles River) were dissected out in Hibernate E medium (Thermo Fisher Scientific). Cerebral cortices were collected under a dissecting microscope, and then digested by TrypLE Express (Gibco) in 37 °C for 10 min. The obtained primary cortical neurons were counted and suspended in neuron basal medium with 5% B27 and 0.5 mM l-glutamine (33) and then plated onto poly-l-lysine (Sigma, P6407). Total 2,000 cancer cells (mKate2) were seeded in 1 ml complete neuron basal medium and were added to the primary neuronal culture. After maintaining the co-cultures for 14 days, mKate2 positive cells were detected by taking five random pictures (10× objective lens, both bright and fluorescent fields) in each plate using a Fluorescence microscope (Leica DMi4000B). The quantification of mKate2 tumour cells was performed using ImageJ-64bit.

TUNEL staining. Mouse brains with metastatic lesions formed by MDA231-BrM cells (28–35 days after the ICD injection) were used for TUNEL staining. In brief, sections were de-paraffinized, treated with 1% proteinase K for 8 min at room temperature, and then incubated with equilibration buffer for 10 min, followed by TdT buffer in 37 °C for 30 min. Sections were then incubated with anti-digoxigenin antibody conjugated with POD (for DAB staining) or fluorescein (for FITC staining). The slides were incubated with the secondary antibody using the Bond Polymer Refine Kit (Leica Biosystems) for 15 min. Subsequently, samples were incubated with the chromogen 3-3′-diaminobenzidine (DAB) for 10 min, counterstained with haematoxylin for 5 min and finally mounted with Aquatex (Merck Millipore). The stainings were scored semiquantitatively as 0–100 percentage of positive cells.

Tissue microarray staining of human samples. Immunohistochemical staining of human samples was carried out in the automated system BOND RX (Leica Biosystms). All sections were deparaffinized and rehydrated using Dewsolution (Leica Biosystems) at 72 °C for 30 s. Endogenous peroxidase activity was blocked with 3% H2O2 solution (Leica Biosystems) for 5 min. Samples were incubated with the following primary antibodies at room temperature for 30 min: NMDA receptor type 1 (GluN1) rabbit polyclonal antibody (ThermoFisher; PA3-102) at 1:200 diluted in polyclonal antigen-free blocking solution (ThermoFisher; PA3-105) at 1:100 dilution or Glna2R(B1252) or Glna2B (Y1472) rabbit polyclonal antibody (ThermoFisher Scientific; 485200 or 387000) at 1:200 dilution. Antigen retrieval was performed using tris-EDTA buffer (pH 9) at 95 °C for 30 min.
10 HEPES/Na, pH adjusted to 7.4 with NaOH. Patch pipettes were pulled from capillary glass (Harvard Apparatus, borosilicate capillary glasses, GC100F-7.5). For current-clamp (membrane-potential) recordings and some voltage-clamp recordings, pipettes were filled with a solution containing 105 K glumonate, 30 KCl, 10 HEPES, 4 Mg-ATP, 0.3 GTP Na+, 10 creatine phosphate-Na, pH adjusted to 7.3 with NaOH. For low noise voltage-clamp recordings, pipettes were filled with a caesium-based solution, containing (mM): Cs methane sulphonate 125, CsCl 5, KCl 10, EGTA 1, 10 HEPES, 4 Mg-ATP, 0.3 GTP Na+, 10 creatine phosphate-Na, pH adjusted to 7.3 with CsOH. Open pipette resistance was between 5 and 10 MΩ, and the membrane potential signal was corrected for nulling of the liquid junction potential before seal formation. Whole-cell recordings were established using an Axon Multiclamp 700B patch-clamp amplifier (Molecular Devices) using a feedback resistor of 50 Ω, low-pass filtered at 4 kHz (Bessel, 8-pol) and sampled at 20 kHz with 16-bit resolution (X-series PCIe board, National Instruments). Further Gaussian digital filtering (low-pass at 500–1000 Hz, high-pass at 0.25 Hz) was applied offline. Glutamate and NMDA responses were measured by pressure ejection of agonist dissolved in the Ringer solution, through pipettes with tip diameters of 5–10 μm, and pressures of 5–10 mBar, using a solenoid valve-controlled pressure ejection system. Recording and perfusion pipettes were positioned with LM-Mini stepper-motor-controlled micromanipulators (Luigs and Neumann). All recordings were carried out at room temperature (23°C).

Intracellular calcium measurement. To record intracellular calcium signals, cells were loaded with the fluorescent indicator Oregon Green 488 BAPTA-1 AM (OGB, Life Technologies). OGB was dissolved in DMSO with 20% v/v Phoruncin F-127, at 200 mg ml⁻¹, then diluted to a final concentration of 5 μM in PBS. Cells were loaded in this solution at room temperature for 1 h, and imaged using epifluorescence (Olympus BX51W microscope, UPlanFI 60× objective, X-Cite 120 light source, EXFO Photonic Solutions), and an eCMOS camera (Orca-Flash4.0, Hamamatsu). Using custom programs with the Matlab Image Processing Toolbox, regions of interest were defined for individual cells were selected, and an automatic bleaching correction was applied by subtraction of a single exponential decay fitted by least-squares to the first 5–10 s of each response, preceding agonist application. Average signals across pixels in each region of interest were analysed as the change in fluorescence (∆F) relative to the median resting baseline level (F0), that is, ∆F/F0. Cells were bathed in the same Ringer solution used for electrophysiology (see above). Glutamate or NMDA was either puffed-applied through a pipette (see above), or added to the bath in concentrated aliquots.

Block face scanning electron microscopy. SCID/beige mice bearing brain metastases formed by MDA231-BrM cells (28–35 days after the ICD injection) were terminally anaesthetized with an overdose of inhalation anaesthetic (isoflurane) and then immediately perfused with a buffered solution of 2.5% glutaraldehyde and 2% paraformaldehyde (0.1M phosphate buffer, pH 7.4). A vibratome was used to slice the brain in the coronal plane at 80 μm thickness. Sections that showed mRNAs or proteins of interest were then further processed to be imaged in a block face scanning electron microscope. They were postfixed in potassium ferrocyanide (+) relative to the median resting baseline level (F0), that is, ∆F/F0. Cells were bathed in the same Ringer solution used for electrophysiology (see above). Glutamate or NMDA was either puffed-applied through a pipette (see above), or added to the bath in concentrated aliquots.

Measurement of glutamate concentration. MDA231 or TS1 parental and B2BM cells were seeded into one well of a six-well plate at a density of 3 × 10⁴ cells per well. Forty-eight hours later, the conditioned medium was collected and filtered using 10-kDa Amicon Ultra centrifugal units. l-Glutamate quantification was performed with the colorimetric Glutamate Assay Kit (Sigma, MAK004-1KT), following the manufacturer’s protocol.

Cell proliferation assay. Cell proliferation rate was measured using an MTT cell proliferation kit (Roche). In brief, cells were plated in triplicate in 96-well plates (5 × 10³ or 1 × 10⁴). Seventy-two hours later, 10 μl of MTT labelling reagent was added to each well and then incubated for 4 h at 37°C, followed by the addition of 100 μl MTT solubilization reagent overnight. Absorbance was measured at 595 nm on a plate reader (Tecan Safire).

Statistical analysis. Statistical analyses were performed with Prism 7 (GraphPad Software). Otherwise, the Student’s t-test was used for non-paired experiments (two-tailed). Wilcoxon matched pairs test (two-tailed) was performed for paired experiments that did not follow a Gaussian distribution. Values are given as mean ± s.e.m.
Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All materials are readily available from the corresponding author upon request or from standard commercial sources, including original IVIS data, Confocal, STED and electron microscopy images, immunohistochemical staining of human or mouse samples and full western blots. There are no restrictions on availability of the materials used in the study.

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Author contributions Q.Z., G.C. and D.H. conceptualized the project and designed the experiments; I.P.M. contributed to the experimental design. G.C., S.S. and P.Z. performed the bioinformatic analysis; I.P.M. and Q.Z. generated the knockdown and rescue approach. J.A.G. and I.Z. did the immunohistochemical staining on human breast cancer and brain metastasis samples; H.P.C.R. carried out electrophysiology and calcium imaging. J.W., Q.Z. and B.D.M. performed the STED analysis of mouse brain with brain metastases; G.K. did the electron microscopy analysis; Q.Z. performed in vivo, in vitro and primary culture experiments, data analyses and quantification; Q.Z., G.C., I.P.M. and D.H. prepared the manuscript.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Association of the NMDAR signalling pathway with human breast cancer. a, mRNA expression of six NMDAR-associated genes (GRIN2A, GRIN2B, GRIN2C, GRIN2D, GRIN1, and DLGAP1) in 1,100 patients with breast cancer from The Cancer Genome Atlas (TCGA). Samples were sorted by NMDAR score (bottom), which combines, for each sample, the Z-scores of normalized expression values of all six genes. b, mRNA expression of four AMPAR-associated genes (GRIA1, GRIA2, GRIA4, and GRIA3) in 1,100 patients with breast cancer from TCGA. Samples were sorted by AMPAR score (bottom), which combines, for each sample, the Z-scores of normalized expression values of all four genes. c, mRNA expression of 5 kainate-associated genes (GRIK2, GRIK4, GRIK3, GRIK1, and GRIK5) in 1,100 patients with breast cancer from TCGA. Samples were sorted by kainate score (bottom), which combines, for each sample, the Z-scores of normalized expression values of all five genes. d, mRNA expression of eight metabotropic glutamate-associated genes (GRM8, GRM1, GRM3, GRM4, GRM7, GRM2, GRM6, and GRM5) in 1,100 patients with breast cancer from TCGA. Samples were sorted by metabotropic score (bottom), which combines, for each sample, the Z-scores of normalized expression values of all eight genes. e, Box plot comparison of mRNA expression of GRIN2A, GRIN2B, GRIN2C, GRIN2D, DLGAP1, and GRIN1 (from left to right) across the five breast cancer PAM50 subtypes. P values were computed using a two-sided ANOVA test. f, Comparison between NMDAR score (x axis) and a previously derived NMDAR expression signature (y axis) across all breast cancer samples. Points are colour coded based on points concentration (warm colour = high concentration, cold colours = low concentration), n = 1,100. g, Box plot comparison of scores from the previously derived NMDAR expression signature across the five PAM50 breast cancer subtypes. h, Kaplan–Meier plots comparing distant-relapse free survival between samples with high GRIN2B expression (top 25%, red line) versus remaining cases (blue line) in luminal B, HER2-enriched, luminal B, normal-like and basal-like breast cancer subtypes (from left to right). The log-rank tests are two sided. In all comparisons, the number of samples for each breast cancer subtype (a–e, g) are: luminal A (n = 467), luminal B (n = 198), HER2-enriched (n = 72), normal-like (n = 28) and basal-like (n = 155). The thick central line of each box plot represents the median number of significant motifs, the bounding box corresponds to the 25th to 75th percentiles, and the whiskers extend up to 1.5 times the interquartile range. These results are based upon data generated by the TCGA Research Network (https://www.cancer.gov/tcga)21.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | GluN2B–NMDAR signalling is activated in brain metastasis. 

a, Representative haematoxylin and eosin-stained images of two paired human primary breast cancers (primary) and brain metastases (brain metastases) are shown. Scale bar, 100 µm. Two independent experiments. 
b, Quantification of pGluN2B(Y1472) staining in 16 primary breast tumours and 18 brain metastases, including 9 matched primary cancers and brain metastases, along with 7 unmatched primary tumours and 9 unmatched brain metastases. Mean ± s.e.m. Mann–Whitney test (two-tailed) was used. Subtype categorization for those patients: 4 TNBC, 6 HER2+, 2 luminal and 13 unknown. 
c–e, Representative images (c) and quantification (d) of immunohistochemical staining of pGluN2B(Y1252) in 12 paired human primary breast cancers (primary) and brain metastases (brain metastases). Mean ± s.e.m. Wilcoxon test analysis (two-tailed). Subtype categorization for these twelve patients: 1 TNBC, 4 HER2+, 2 luminal and 5 unknown. 
e, Quantification of pGluN2B(Y1252) staining in 16 primary breast tumours and 22 brain metastases, including 12 matched primary cancers and brain metastases, along with 4 unmatched primary tumours and 8 unmatched brain metastases. Mean ± s.e.m. The Mann–Whitney (two-tailed) statistical test was used. Subtype categorization for those patients: 4 TNBC, 6 HER2+, 2 luminal and 13 unknown. Scale bar, 100 µm. 
f–h, Immunohistochemical staining of GluN2B in 16 human primary breast cancers and 23 brain metastases: representative images (f), Wilcoxon test analysis (two-tailed) of the 15 matched primary cancers and brain metastases (g), and Mann–Whitney (two-tailed) test analysis (h) of all samples. Mean ± s.e.m. Note that it remains to be ascertained whether the few brain metastases lacking GluN2B expression have activated NMDAR signalling via another GluN2 subunit. Scale bar, 100 µm. Subtype categorization for those patients: 4 TNBC, 6 HER2+, 2 luminal and 13 unknown. 
i–k, Immunohistochemical staining of GluN1 in 16 human primary breast cancers and 16 brain metastases: representative images (i), Wilcoxon test (two-tailed) analysis of the 11 matched primary cancers and brain metastases (j) and Mann–Whitney test (two-tailed) analysis (k) of all samples. Mean ± s.e.m. Scale bar, 100 µm. Note that owing to variations in the presence of cancer in the sections taken from the tissue microarray block for analysis, not all 25 samples were represented in each of the immunostainings performed in Fig. 2 and in this figure. Subtype categorization for those patients: 4 TNBC, 6 HER2+, 2 luminal and 13 unknown. Scale bar, 100 µm. Note that owing to variations in the presence of cancer in the sections taken from the tissue microarray block for analysis, not all 25 samples were represented in each of the immunostainings performed in Fig. 2 and in this figure. 
l, Representative immunohistochemical staining of pGluN2B(Y1472), total GluN2B and luciferase in primary tumours, brain metastases, and lung metastases formed by MDA231-BrM cells (day 28–35 after injection). Scale bar, 100 µm. The areas indicated by black boxes are shown in Fig. 2e. Scale bar, 100 µm. The areas indicated by black boxes are shown in Fig. 2e. 
m, Immunohistochemical staining of pGluN2B(Y1252), total GluN2B, and luciferase (m) and quantification of pGluN2B(Y1252) staining (n) in primary tumours, brain metastases, and lung metastases formed by MDA231-BrM cells (day 28–35 after injection). Scale bar, 100 µm. Insets are magnified 3.2 × relative to the main field. Two-tailed Student’s t-test, mean ± s.e.m., n = 3 mice per group.
Extended Data Fig. 3 | NMDAR-mediated calcium signalling and single channel currents in B2BM cells. To test for functional NMDARs, fluorescence imaging and patch-clamp recordings were carried out in 2D cultures of TS1-BrM cells, 14 fields of view (301 × 301 µm) from 10 different culture dishes. Total of 151 responding cells out of 1,070 imaged cells (14.11%). Data from bath addition of 50–100 µl of 100 mM potassium glutamate, 50–200 µl of 10 mM NMDA, local perfusion-pipette puffing of 100 µM potassium glutamate or 500 µM NMDA, all of which produced calcium responses. 

a–c, Representative calcium imaging experiments. a, TS1-BrM cells were loaded with Oregon Green BAPTA-1 AM fluorescent calcium indicator, and imaged with a sCMOS camera at 5 frames per second. A subset of cells in the field of view (13/75) showed calcium elevation in response to the addition of glutamate to the bath (50 µl of 10 mM glutamate, final concentration 125 µM). b, Calcium signals from four cells as indicated in a; the arrow indicates glutamate application. 

c, Average signal over the 13 agonist-responsive cells during application of glutamate (blue trace) or preceding control application of the same volume of Ringer solution (red trace). 

d, Response to application of 50 µl of 10 mM NMDA (final concentration 125 µM), averaged over 6 responding cells in a field of view containing 30 cells (blue trace), compared to an example response averaged over 52 cells to the same amount of NMDA, but in the presence of 100 µM APV (2-amino-5-phosphono-pentanoic acid; a selective NMDA receptor antagonist) and 2 mM Mg²⁺ (red trace). Overall, calcium transients stimulated either by NMDA or glutamate were detectable in about 14% of cells (151 of 1,070 cells from 10 culture dishes). 

e–g, Puff application (pipette at left) of NMDA (200 µm, grey bar) activates inward single-channel currents recorded through the pipette at right (e, f), with amplitudes of around 4.5 pA at a holding potential of −90 mV (g), equivalent to a chord conductance of 50 pS, characteristic of GluN2A- or GluN2B-containing NMDARs. Single-channel currents of 4.5 pA were detectable in low-noise whole-cell recordings on the application of glutamate (100 µM) or NMDA (200 µM) in 45% (9/20) of recorded cells. h, Resting membrane potentials were measured by whole-cell current-clamp using pipettes filled with cytoplasm-like high-potassium solutions (box plot, −52 ± 10.6 mV (mean ± s.e.m., n = 9 cells). For comparison, the voltage dependence of GluN2A and GluN2B inward current in physiological (1 mM) magnesium is superimposed, indicating that membrane potentials are sufficiently depolarized to overcome a substantial fraction of voltage-dependent magnesium block of NMDARs.
Extended Data Fig. 4 | Brain metastases are not inducing the neuronal apoptosis anticipated for autocrine secretion of glutamate by cancer cells. a, Western blot analysis of the xCT and vGlut2 glutamate transporters in pairs of human and mouse parental breast cell lines and their corresponding brain metastatic derivatives. Protein levels in B2BM cells are quantified relative to cognate parental cells, after normalization to GAPDH. Three independent experiments. GAPDH was run on separate gels from xCT and vGlut2 as sample processing controls. b, Immunohistochemical-(DAB)-based staining to detect apoptotic cells in mouse brains with metastases formed by MDA231-BrM cells (day 28–35 after injection). DNAase I treatment on brain tissue sections was used as a TUNEL+ control. Images shown are representative of an analysis of more than 20 brain metastases from 4 mouse brains, 2 sections per mouse brain. Scale bar, 100 µm. c, Immunofluorescent staining for cleaved-caspase 3 (green), luciferase (cancer cells; magenta) and NeuN (neurons; red) in mouse brain with metastases formed by MDA231-BrM cells (day 28–35 after injection). Images shown are representative of an analysis of >20 brain metastases from 4 mouse brains, 2 sections/mouse brain. Scale bar, 100 µm. d, Immunofluorescence staining for TUNEL (green), NeuN (red), GFAP (glioma cells; magenta) in a brain harbouring a glioma that arose in a genetically engineered mouse model. Images shown are representative of an analysis of more than six gliomas from six mouse brains, one section per mouse brain. As previously reported, glioma cells secrete glutamate—which causes neuronal apoptosis—in contrast to B2BM cells, which do not. Scale bar, 50 µm.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Interactions between B2BM cells and neurons. a–c, Immunofluorescence staining of luciferase (B2BM; blue), vGlut2 (red) and pGluN2B(Y1252) (green) in a mouse brain metastasis formed by MDA231-BrM cells (day 28–35 after injection), imaged by STED super-resolution microscopy. a, b, Tilted 3D images of the area circumscribed by the solid white-bordered box in Fig. 4a. Arrow, vGlut2+ in presynaptic neuron; arrowhead, pGluN2B(Y1252)+ in a blue-labelled B2BM cell, potentially in close apposition to a postsynaptic neuron. c, Tilted 3D image of the area circumscribed by the dotted white-bordered box in Fig. 4a, depicting a normal synapse revealed by close association between vGlut2+ puncta in a presynaptic neuron (arrow) with pGluN2B+ puncta in a postsynaptic neuron (arrowhead). Side length of each square in 3D view, 400 nm (a, b) and 300 nm (c). Images are representative of an analysis of nine brain metastases from three mouse brains, two sections per mouse brain. See Supplementary Videos further describing the images shown in a–c. d, e, Immunofluorescence staining of luciferase (green) and synaptobrevin 1 (red) in a mouse brain metastasis formed by MDA231-BrM cells (day 28–35 after injection), imaged by STED super-resolution microscopy. d, A merged image (left) and individual fluorescent images (middle and right). Scale bar, 10 µm. e, A tilted 3D image at two magnifications, highlighting a synaptobrevin 1+ bouton chain (red) localized proximal to luciferase+ tumour cells (green). Images are representative of an analysis of nine brain metastases from three mouse brains, two sections per mouse brain. Side length for each square in 3D view, 10 µm. f, g, Immunofluorescence staining for postsynaptic proteins PSD-95 (red, f), and neurexin 2 (red, g) along with luciferase (green) in mouse brains with metastases formed by MDA231-BrM cells (days 28–35 after injection; scale bar, 10 µm). The area circumscribed by the white box is shown at higher magnification (left; scale bar, 1 µm). Images are representative of an analysis of more than 20 brain metastases from 4 mouse brains, 2 sections per mouse brain.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | NMDAR signalling in B2BM cells is activated in coculture with neurons. a, Schematic of cancer cell–primary neuron co-culture system and experimental design. b, Immunofluorescence staining for MAP2 (neuronal marker, green) and GFAP (astrocyte marker, red) in primary cultures of cortical neurons, revealing the preponderance of neurons. The area circumscribed by the white box is shown at higher magnification (left). Scale bars, 1 μm (left), 10 μm (right). Three independent experiments. c, Representative bright-field (BF) and fluorescent (mKate+) images (left) and quantification (right) of MDA231 parental and MDA231-BrM cells co-cultured with primary cortical neurons for 14 days. Two-tailed Student’s t-test, mean ± s.e.m., n = 3 biological replicates over three independent experiments. Scale bar, 100 μm. d, Representative bright-field and fluorescent images (left) and quantification (right) of TS1 parental and TS1-BrM cells co-cultured with primary cortical neurons for 14 days. Data are mean ± s.e.m. Student’s t-test (two-tailed) was used. n = 3 biological replicates over three independent experiments. e–g, Quantification of MDA231 parental and MDA231-BrM cell numbers after 14 days of culture in poly-d-lysine-coated plates in three different conditions: in complete neuronal culture medium (e); in conditioned medium from primary cortical neuron cultures (f); and in a Boyden chamber with neurons in the top chamber and cancer cells in the bottom chamber (g). Mean ± s.e.m. Student’s t-test (two-tailed) was used. n = 3 biological replicates over three independent experiments. The panels below each bar graph show representative images of the cancer cells at the end of the assay, revealed by mKate fluorescence. h, Schematic inducible miR-E-based shRNA knock-down system carrying three distinct miR-E-based shRNA sequences that bind to different regions of a targeted mRNA (top vector), and tet-on inducible vector for GRIN2B open reading frame (ORF) used for rescue experiments (bottom vector). TRE, tet-on inducible promoter; rtTA, reverse tetracycline transactivator. i, Knockdown of GRIN2B in cultured MDA231-BrM cells with tet-on inducible shRNAs, as assessed by western blotting after DOX treatment (1 μg ml⁻¹) for two days. Three independent experiments. The numbers above the blots indicate levels of pGluN2B(Y1252) and total GluN2B protein normalized to GAPDH. GAPDH was run in the same gel as pGluN2B(Y1252), and run on separate gels from GluN2B as sample processing controls. j, Representative bright-field and fluorescent images (bottom) and quantification (top) of MDA231-BrM cells transfected with inducible shCtrl and shGRIN2B (1 μg ml⁻¹), co-cultured with primary cortical neurons for 14 days. Two-tailed Student’s t-test; mean ± s.e.m., n = 3 biological replicates over three independent experiments. Scale bar, 100 μm. k, Rescue expression of GluN2B in cultured MDA231-BrM cells with tet-on inducible shRNAs along with a GRIN2B cDNA, as assessed by western blotting after DOX treatment (1 μg ml⁻¹) for two days. Three independent experiments. GAPDH was run in the same gel as GluN2B. l, Representative fluorescent (mKate+) images (left), and quantification (right) of MDA231-BrM shCtrl, shGRIN2B and GluN2B-rescued cells co-cultured with primary cortical neurons for 14 days. Two-tailed Student’s t-test; mean ± s.e.m., n = 3 biological replicates over three independent experiments. Scale bar, 100 μm. m, Cell proliferation in shCTRL and shGRIN2B MDA231-BrM cells as determined by MTT assays, starting with 5,000 or 10,000 cells per well, after 72 h in culture. Two-way ANOVA; mean ± s.e.m., n = 3 independent experiments.
Extended Data Fig. 7 | In vivo assessment of the functional importance of GluN2B–NMDAR signalling in B2BM cells. a, b, Representative immunofluorescent images of FMRP (green), luciferase (red) and DAPI (blue) in brain metastases formed by shCtrl (control shRNA) and shGRIN2B MDA231 cells (a), and quantification of mean FMRP fluorescence in Luciferase+ tumour-cell clusters (b). Two-tailed Student’s t-test, mean ± s.e.m.; n = 25 for shCtrl and n = 32 shGRIN2B group, all from 3 mice per group. c, Weight of orthotopic breast tumours formed by MDA231-BrM cells transfected with inducible shCtrl or shGRIN2B inoculated into the fourth mammary fat pads of female mice. The mice were fed food containing DOX to induce the shRNAs concomitantly with mammary fat pad injection. Two-tailed Student’s t-test, mean ± s.e.m., n = 9 mice for the shCtrl group, and 10 mice for shGRIN2B group. d, Bioluminescence imaging (BLI) and quantification of lung metastatic lesions formed by MDA231-BrM cells transfected with inducible shCtrl or shGRIN2B inoculated intravenously. DOX food was added to induce the shRNAs concomitantly with intravenous injection. Two-tailed Student’s t-test; mean ± s.e.m., n = 5 mice per group, two independent experiments. e, BLI and quantification of brain metastatic lesions formed by MDA231-BrM cells transfected with inducible shCtrl or shGRIN2B, or additionally with rescue expression of a GRIN2B cDNA. DOX food was supplied concomitantly with ICD injection to induce shRNA expression. Tumour burden was assessed four weeks later by BLI. Two independent experiments. Two-tailed Student’s t-test, mean ± s.e.m., n = 10 mice for shCtrl group, n = 9 mice for shGRIN2B group and n = 9 mice for the rescue group.
Extended Data Fig. 8 | Functional analysis of GluN2B expression in B2BM cells. a, Knockdown of GluN2B expression in cultured mouse TS1-BrM cells with tet-on inducible shRNAs, as assessed by western blotting two days after DOX treatment in vitro (1 µg ml⁻¹). Three independent experiments. The numbers above the blot indicate levels of GluN2B normalized to GAPDH. GAPDH was run in the same gel as GluN2B. b, Weight of primary orthotopic breast tumours formed by TS1-BrM cells transfected with inducible shCtrl or shGrin2b, and inoculated into the fourth mammary fat pad of female mice. Mice were fed with food containing DOX to induce the shRNAs concomitantly with mammary fat pad injection. Two-tailed Student’s t-test; mean ± s.e.m., n = 6 mice for shCtrl group, and n = 8 mice for shGrin2b group. c–e, In vivo BLI (c) and quantification of brain (d) and thoracic cavity (e) photon flux in mice bearing TS-BrM cells transfected with inducible shCtrl or shGrin2b four weeks after intracardiac injection. DOX food was supplied concomitantly with ICD injection to induce shRNA expression. Two-tailed Student’s t-test; mean ± s.e.m., n = 9 mice per group, two independent experiments. f, Ex vivo BLI and quantification of excised brains with metastatic lesions formed by TS1-BrM cells transfected with inducible shCtrl or shGrin2b. Mice were fed food containing DOX concomitantly with ICD injection to induce shRNA expression. Two-tailed Student’s t-test; mean ± s.e.m., n = 9 mice per group, two independent experiments.
Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

☐ Confirmed
☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐ The statistical test(s) used AND whether they are one- or two-sided
☐ Only common tests should be described solely by name; describe more complex techniques in the Methods section.
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☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
☐ Give P values as exact values whenever suitable.
☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
☐ Clearly defined error bars
☐ State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection
The TCGA breast cancer cohort was collected from the FireBrowse data portal (more information is provided in Methods section). All raw data are also provided in Source Data for Fig. 1 and ED Fig. 1.

Data analysis
GraphPad Prism 7.0.
R Version 3.3.2
Image; 64bit
Fiji (www.fiji.sc)
Arvins 3.0

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All materials are readily available from the corresponding author upon request or from standard commercial sources. There are no restrictions on availability of the materials used in the study.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/reportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample size for in vivo experiments was based on previous publications using the same cell lines and mouse experimental procedures (e.g. Chen Q. et al. Nature 2016). No statistical methods were used to predetermine sample sizes. |
| Data exclusions | No data were excluded from the analyses. |
| Replication | Replicates were used in all experiments as noted in text, figures legends and methods. Key in vivo experiments were repeated at least twice with consonant results. Key in vitro experiments were repeated at least at least three times with consonant results. |
| Randomization | Female SCID/beige mice (6-8 weeks old) were randomly allocated to shCTRL or shGRIN2B groups. |
| Blinding | Blinding was not performed during experiments to facilitate veterinary and staff monitoring. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|------|-----------------------|
| ☑ ☑ | Unique biological materials |
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |

Methods

| n/a | Involved in the study |
|------|-----------------------|
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

The tissue microarray (TMA) with human primary breast cancer tissues and matched brain metastasis tissue was generated at the Institute of Pathology, University of Bern, Switzerland, in accordance to the Swiss Federal Human Research Act (HFG 2014).
We state that no unique materials were used. TMAs are available upon request.

Antibodies

| Antibodies used | GAPDH, Cell Signaling, 2118S, 1:1000; |
|----------------| b-actin, Cell Signaling, 49705, 1:1000; |
pGluN2B Y1252, Life technologies, 485200; 1:1000 for WB and 1:200 for IHC and IF staining; pGluN2B Y1472, Biorbyt, orb65339 for WB (1:1000); pGluN2B Y1472, Life technologies, 387000, for both WB (1:1000) and IHC staining (1:200); GluN2B, Proteintech, 21920-1-AP for WB (1:1000), and for ThermoFisher Scientific, PA3-105 for IHC staining (1:100 for human tissues, 1:500 for mouse tissues); NMDAR1 (GluN1), Polyclonal Antibody Thermo scientific, PA3-102, 1:1000 for WB, 1:200 for IHC staining of human tissues and 1:500 for IHC staining of mouse tissues; Cleaved-caspas 3, Cell Signaling, 9664, 1:500; Luciferase, NOVUS, NB100-1677, 1:1000 for IHC and IF staining; MAP2, Synaptic system, 188 002, 1:500; vGlut2, Synaptic system, 135 404, 1:400; NeuN, Sigma, mab377, 1:200; Synaptobrevin 1, Synaptic system, 104002, 1:500; PSD-95, Abcam, ab18258, 1:500; Neuroligin 2 Prof. Peter Scheiffele, 1:200;

**Validation**

Information for the Neuroligin 2 antibody from Prof. Peter Scheiffele is described in: Scheiffele, P., Fan, J., Choih, J., Fetter, R. & Serafini, T. Neuroligin Expressed in Nonneuronal Cells Triggers Presynaptic Development in Contacting Axons. Cell 101, 657 - 669 (2000); Commercial per-validated antibodies were purchased form reputable sources (Cell signaling, Abcam, Life technologies, and Synaptic system), with validation data present on the manufacturer’s website as noted in the Methods section. Neuron related antibodies (pGluN2B Y1472 and pGluN2B Y1252 et al.) were further validated using primary neurons as positive controls (Supplementary Figure 1).

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**Eukaryotic cell lines**

**Policy information about:** cell lines

**Cell line source(s):** MDA231 parental and BRM2a cells have been described (Bos, P. D. et al. Nature 2009), as have TS1 parental and BRM cells (Sevenich, L. et al. Nat. Cell Biol. 2014). Isolation of the primary cortical neurons is described in the Methods.

**Authentication:** We received these two pairs of cell lines from Prof. Joan Massague’s and Prof. Johanna Joyce’s labs at MSKCC, New York. None of the cell lines were further authenticated.

**Mycoplasma contamination:** All cell lines were tested negative for mycoplasma contamination.

**Commonly misidentified lines**

(See [CCAC register](#))

The two pairs of cell lines used are not listed in the misidentified cell line database.

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**Animals and other organisms**

**Policy information about:** studies involving animals; ARRIVE guidelines recommended for reporting animal research

**Laboratory animals:** SCID/beige mice, female, 6-8 weeks old

**Wild animals:** We state that no wild animals were used in this study.

**Field-collected samples:** We state that no field-collected samples were used in this study.