ALG-2 interacting protein-X (Alix) is required for activity-dependent bulk endocytosis at brain synapses

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Competing interests

On behalf of all authors R.Sadoul declares no competing interests.

Abstract

In chemical synapses undergoing high frequency stimulation, vesicle components can be retrieved from the plasma membrane via a clathrin-independent process called activity dependent bulk endocytosis (ADBE). Alix (ALG-2 interacting protein X)/ PDCD6IP) is an adaptor protein binding to ESCRT and endophilin-A proteins and thereby driving deformation and fission of endosomal and cell surface membranes. In fibroblasts, Alix is required for clathrin-independent endocytosis. Here, using electron microscopy, we show that synapses from mice lacking Alix have subtle defects in presynaptic compartments, translating into flawed synaptic plasticity. Using cultured neurons, we demonstrate that Alix is required for ADBE. We further demonstrate that in order to perform ADBE, Alix must be recruited to synapses by the calcium-binding protein ALG-2 and interact with endophilin-A. Finally, we show that mutant mice lacking Alix in the forebrain undergo less seizures during kainate-induced status epilepticus. Furthermore, propagation of the epileptiform activity to the contralateral side of kainate injection is reduced. These results thus highlight Alix ko mice as an invaluable model to study the exact role of ADBE at synapses undergoing physiological or pathological stimulations.

Keywords: PDCD6IP**,** Clathrin-independent-endocytosis, Alix knock-out, endophilin-A, ALG-2, bulk endocytosis, synaptic vesicle recycling, synaptic transmission, calcium, epileptic seizures.

List of abbreviations:

4AP: 4-Aminopyridine
ADBE: Activity-Dependent Bulk Endocytosis
ALG-2: Apoptosis Linked Gene 2
Alix: ALG-2 Interacting protein X
BAPTA: 1,2-Bis(o-AminoPhenoxy)ethane-N,N,N',N'-Tetraacetic Acid
BE: Bulk Endosome
CA1: Cornu Ammonis 1
CA3: Cornu Ammonis 3
CHMP4B: Charged Multivesicular body Protein 4B
CGN: Cerebellar Granule Neuron
CIE: Clathrin-Independent Endocytosis
CME: Clathrin-Mediated Endocytosis
CTxB: Cholera Toxin chain B
DIV: Day *In Vitro*
EGTA: Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N',N'-Tetraacetic Acid
EM: Electron Microscope (Microscopic/ Microscopy)
ESCRT: Endosomal Sorting Complexes Required for Transport
$GABA_A$: γ-AminoButyric Acid type-A
GFP: Green Fluorescent Protein
HRP: Horse Radish Peroxidase
kDa: kiloDalton
KO: KnockOut
LTP: Long-Term Potentiation
N-BAR: N-terminal amphipathic helix- Bin-Amphiphysin-Rvs
PRD: Proline-Rich Domain
PSD95: Post-Synaptic Density Protein 95
ROI: Region of interest
SH3: Src Homology 3
SV: Synaptic Vesicle
Syp-pH: Synaptophysin-pHluorin
TSG-101: Tumour Susceptibility Gene-101
WT: Wild Type
YFP: Yellow Fluorescent Protein

Introduction

Neuronal communication in mammalian brain relies heavily on the activity-dependent release of chemical neurotransmitters from presynaptic boutons. Following fusion of synaptic vesicles (SV) with the presynaptic membrane, SV lipids and proteins are retrieved by endocytosis. Endocytosis avoids detrimental increase in the plasma membrane surface and allows recycling of the SV components to replenish the SV pool (Gan and Watanabe, 2018). At moderate levels of stimulation, retrieval of membrane involves clathrin-mediated (CME) and clathrin-independent endocytosis (CIE), in proportions which are still highly debated (Chanaday and Kavalali, 2018). Moreover, long-lasting high-frequency stimulations also lead to the clathrin-independent internalization of large stretches of pre-synaptic membranes. This calcium-dependent process, first discovered at the amphibian neuromuscular junction (Miller and Heuser, 1984) is referred to as activity-dependent bulk endocytosis (ADBE). It is meant to avoid abnormal increase of the synaptic bouton surface and to allow replenishment of SVs during sustained synaptic stimulations (Cheung and Cousin, 2012; Marxen et al., 1999). Thus, ADBE has been suggested to play key regulatory roles in physiological or pathological events like epilepsy, which are triggered and sustained by high frequency neuronal activity. However, decrypting the physiological role of ADBE has been hindered by the lack of identified molecules that are both specific and essential to this endocytosis mode.

We have recently demonstrated that ALG-2 interacting protein-X (Alix) is essential for clathrin-independent bulk endocytosis in fibroblasts (Mercier et al., 2016). In the adult brain, Alix is ubiquitously expressed but concentrates at hippocampal pre-synaptic terminals during epileptic seizures (Hemming et al., 2004). Alix is a cytosolic protein first identified through its calcium-dependent binding to the penta-EF-hand protein ALG-2 (apoptosis-linked gene 2)(Chatellard-Causse et al., 2002; Missotten et al., 1999; Sadoul, 2006). Since then, it was reported that following plasma membrane wounds, ALG-2 binds to inflowing calcium and helps recruiting Alix to the membrane where the latter organizes repair (Scheffer et al., 2014). Alix interacts with several membrane modifying proteins among which endophilin-A proteins (A1, A2 and A3) (Chatellard-Causse et al., 2002). These cytoplasmic proteins that contain an N-BAR (Bin/Amphiphysin/Rvs) domain capable of sensing and generating membrane curvature (Kjaerulff et al., 2011), are major actors of CME at synapses (Gad et al., 2000; Ringstad et al., 1999). They also drive CIE in fibroblasts (Boucrot et al., 2014; Renard et al.,

2014) and were shown to control the fast mode of CIE at ribbon synapses (Llobet et al., 2011) as well as in hippocampal neurons (Kononenko et al., 2014; Watanabe et al., 2018).

The role of Alix in bulk endocytosis in fibroblasts, its capacity to interact with endophilin-A and to be recruited by calcium at membranes, together with its increased concentration at hippocampal synapses during kainate-induced seizures, brought us to test its possible function in ADBE. For this we made use of Alix ko mice which have normally organized but smaller brains (Campos et al., 2016; Laporte et al., 2017), a phenotype that we previously linked with an alteration of CIE in developing neurons (Laporte et al., 2017).

We now report that synapse morphology and function are both altered in Alix ko brains. One obvious feature is their reduced number of synaptic vesicles and increase in size correlating with impairments in synaptic facilitation and recovery during high frequency stimulation. Using cultured wt neurons, we bring evidence that sustained synaptic activity leads to calcium-dependent recruitment of ALG-2. ALG-2 in turn interacts with Alix, which binds and concentrates endophilin-A. This protein complex is indispensable for ADBE, which is selectively impaired in Alix ko neurons. Finally, we show that the number of seizures during status epilepticus induced by intracortical kainate injections is reduced in conditional ko mice deleted from *alix* in cortical and hippocampal neurons. In addition, the propagation of epileptiform activity to the contralateral side of injection is reduced. Thus, our results show that some molecular mechanisms involved in ADBE may also be involved in certain aspects of synaptic plasticity such as facilitation and accommodation to synaptic fatigue and recurrence of epileptic seizures.

Material and methods

Plasmids

Endophilin A2-mCherry was obtained by subcloning (In-Fusion Cloning kit, Clontech) endophilin A2 cDNA into a pmCherry-N1 vector (Clontech). GFP-ALG-2 was obtained by performing a reverse mutagenesis (Quick change II site directed mutagenesis kit, Stratagene) on a GFP-hALG2 Y180A (a generous gift from Masatoshi Maki) to acquire GFP-hALG2wt. hALG2 E47A-E114A cDNA was kindly provided by Masatoshi Maki (Shibata et al., 2004) and was subcloned into a pEGFP-C1 vector (Clontech) to obtain GFP-hALG2 E47A-E114A (GFP-ALG2ΔCa).

All constructs containing Alix cDNA (wt or mutants) were obtained by subcloning the relevant cDNAs from pCI vectors harbouring Alix cDNA or its mutants. Alix I212D (AlixΔCHMP4B) and AlixΔPGY (AlixΔALG2) cDNAs in pCI were generated by mutagenesis (Quick change II site directed mutagenesis kit, Stratagene) and Alix R757E (AlixΔendo) by in-fusion cloning, using the oligos given below.

mCherry-2Xflag-mAlix wt (mCherry-Alix) was obtained by subcloning 2xflag-mAlix wt cDNA into a pmCherry-C1 vector (Clontech). Alix-YFP was obtained by subcloning wild type Alix cDNA into a pEYFP-N1 vector (Clontech). GFP-flag-Alix (GFP-Alix) was described in (Mercier et al., 2016). GFP-Alix and its mutant forms (GFP-Alix R757E, GFP-AlixΔPGY) were obtained by subcloning the various cDNAs into a pEGFP-C1 vector (Clontech). DNA constructs used for the rescue experiments were prepared in two steps. First, IRES2-GFP cDNA was subcloned into pSIN lentiviral vector (kindly provided by F. Saudou) by using pIRES2-GFP (Clontech) as a template. Then the various cDNAs were subcloned into pSIN-IRES2-GFP.

Oligos used to generate mutants:

Alix I212D

sense: 5'-AAGATGAAAG ATGCCGACAT AGCTAAGCTG-3'

antisense: 5'-CAGCTTAGCT ATGTCGGCAT CTTTCATCTT -3'

Alix R757E

sense: 5'-CAGCCGAGCC TCCACCTCCT GTGCTTCCTG -3'

antisense: 5'-GAGGCTCGGC TGGAGGCTGG GGCTTAGCAG-3'

AlixΔPGY

sense: 5'-GCCACAGGCT CAGGGATGCC AAATGCCCAT GC-3' antisense: 5'-GCATGGGCAT TTGGCATCCC TGAGCCTGTG GC -3'.

Antibodies

Antibody	Supplier (reference)	Species (type)	Dilution
Anti-Actin	Millipore (MAB1501R)	Mouse (monoclonal)	1/10000
Anti-Alix	Covalab (ab0204)	Rabbit (polyclonal)	1/10000
Anti-phospho-p44/42 MAPK (T202/Y204)	Cell signaling (9106)	Mouse (monoclonal)	1/1000
Anti-PSD95	NeuroMab (73-028)	Mouse (monoclonal)	1/2000
Anti-PSD95	Millipore (MAB1598)	Mouse (monoclonal)	1/500
Anti-Synapsin-1	Millipore (AB1543P)	Rabbit (polyclonal)	1/1000
Anti-Synaptophysin	Merck Millipore (MAB5258)	Mouse (monoclonal)	1/5000
Anti-Flag	Sigma-Aldrich (F3165)	Mouse (monoclonal)	1/1000
Anti-Flag	Sigma-Aldrich (F7425)	Rabbit (polyclonal)	1/1000
Anti-Mouse HRP	Jackson ImmunoResearch (115-035-166)	Goat (polyclonal)	1/5000
Anti-Rabbit HRP	Jackson ImmunoResearch (115-035-044)	Goat (polyclonal)	1/5000
Anti-Mouse Alexa Fluor 488	Invitrogen (A-11029)	Goat (polyclonal)	1/1000
Anti-Mouse Alexa Fluor 594	Invitrogen (A-11032)	Goat (polyclonal)	1/1000
Anti-Mouse Cy5	Jackson ImmunoResearch (115-175-146)	Goat (polyclonal)	1/500
Anti-Rabbit Alexa Fluor 488	Invitrogen (A-11034)	Goat (polyclonal)	1/1000
Anti-Rabbit Alexa Fluor 594	Invitrogen (A-11037)	Goat (polyclonal)	1/1000
Anti-Rabbit Cy5	Jackson ImmunoResearch (111-175-144)	Goat (polyclonal)	1/1000

Animals

Animals were handled and killed in conformity with European law and internal regulations of INSERM. Pregnant Oncins France souche A (OFA; a substrain of Sprague Dawley) rats (Charles River) were used for rat neuronal cultures. Alix ko C57BL/6 mouse pups (Laporte et al., 2017;

Mercier et al., 2016) and their control littermates referred to thereafter as Alix wt, were also used for primary neuronal culture. Transgenic mice were held at the animal facility of the Grenoble Institute for Neurosciences and fed *ad libitum*. All animals were held at a twelve-hour light/dark cycle. One to two month-old Alix wt and Alix ko mice were used for electrophysiological recordings, histochemistry and electron microscopy studies.

Mice were anesthetized by intraperitoneal injection of 0.1 ml sodium pentobarbital (5.6% w/v; CEVA Santé Animale) and treated as described in the corresponding sub-headings of the material and methods section.

$Emx1^{IREScre}/Alix^{fl/fl}$ and $Emx1^{IREScre}$ control mice were used for the kainate injection epilepsy model. 8-11 weeks old mice were anesthetized under a mixture of 2 % isoflurane, 47.5 % O_2 and 47.5 % N_2O. Transgenic mice were held at the animal facility of the CIPMM and fed *ad libitum*. All animals were held at a twelve-hour light/dark cycle. This study was carried out in strict accordance with the European and German guidelines for the welfare of experimental animals. Animal experiments were approved by the Saarland state's "Landesamt für Gesundheit und Verbraucherschutz" animal licence number 36/2016.

<u>Golgi staining</u>

2-month-old anesthetized mice were dislocated prior to brain dissection and 100 µm thick coronal brain sections were cut on a vibratome in the hippocampal region. The dendritic spines of hippocampal neurons from the CA1 *stratum radiatum* were visualized by the Golgi impregnation technique. For this, we used the FD Rapid GolgiStain kit (FD NeuroTechnologies). Brain sections were immersed in equal volumes of solutions A and B for 7 d and impregnated with solution C for 48 h at 4°C. Then, the sections were washed twice in double-distilled water and incubated for 10 min in a mixture of one part of solution D, one part of solution E, and two parts of double-distilled water. Sections were washed twice, dehydrated with increasing concentrations of ethanol, and mounted with epoxy resin (Fluka) between two coverslips. Stacks of bright-field images with 0.3 µm spacing were acquired with a Zeiss Axioskop 50 microscope with 63x oil objective (NA 1.4; Plan-Apochromat) coupled to a CCD camera (CoolSnap ES; Roper Scientific) operated by Metaview software (Molecular Devices). Images were analysed with ImageJ. The number of dendritic spines (>1 µm protrusion of 100 µm portions of dendrite) per unit dendritic length was counted with ImageJ.

Transmission electron microscopy of the CA1 hippocampus

2-month-old, anesthetized mice were intracardially perfused with phosphate-buffered 0.9% NaCl (PBS), followed by 0.1 M phosphate buffered 4% paraformaldehyde, pH 7.4, supplemented with 0.05% glutaraldehyde (Sigma). The brains were carefully removed, postfixed for 4 h in the same fixative and 60 μm sections were cut with a vibratome. After several washes in PBS, the sections were postfixed in 1% glutaraldehyde in the same buffer for 10 min and processed for EM. This included treatment with osmium tetroxide (1% in 0.1 M PB), block staining with uranyl acetate, dehydration through a graded series of ethanol, and flat embedding on glass slides in Durcupan (Fluka) resin. Regions of interest were cut at 70 nm on an ultramicrotome (Reichert Ultracut E; Leica) and collected on one-slot copper grids. Staining was performed on drops of 1% aqueous uranyl acetate, followed by Reynolds's lead citrate. EM images were acquired in a JEOL-1200 electron microscope with a digital camera (Veleta, SIS; Olympus) and analysed with ImageJ. Twenty images per animal from 3 animals per genotype were used for quantification. The number of synapses per μm^2 was calculated. A synapse was considered if it met 3 criteria: a presynaptic bouton filled with at least 10 synaptic vesicles (1) juxtaposed to the head of a dendritic spine with a clearly visible PSD (2) and the presence of the neck in the section (3). Number of synaptic vesicles and areas of presynaptic boutons were quantified in each synapse using the free-shape tool and the cell counter plugins of ImageJ. We used the straight tool of ImageJ to measure the lengths of PSDs, and head and neck diameters. Note that the head diameter was taken parallel to the PSD and the neck diameter was perpendicular to the neck membranes.

Electrophysiological recordings in CA1

Ex vivo slice preparation: Brain slices were prepared from 2-month-old C57BL/6 wt and Alix ko mice. The brains were removed quickly and 350 µm-thick sagittal slices containing both cortex and hippocampus were cut in ice-cold sucrose solution (2.5 mM KCl, 1.25 mM NaH_2PO_4, 10 mM $MgSO_4$, 0.5 mM $CaCl_2$, 26 mM NaHCO3, 234 mM sucrose, 11 mM glucose, saturated with 95% O_2 and 5% CO_2) with a Leica VT1200 blade microtome (Leica Microsystemes, Nanterre, France). After cutting, hippocampi were extracted from the slice and transferred to oxygenated Artificial Cerebro-Spinal Fluid (ACSF: 119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 11 mM glucose) at 37± 1°C for 30 min and then kept at room temperature for at least 1 h before recordings. Each slice was individually transferred to a submersion-type recording chamber and continuously superfused (2 ml/min) with oxygenated ACSF. Extracellular recordings were obtained at 28°C from the apical dendritic layers of the hippocampal CA1 area, using glass micropipettes filled with ACSF. Field excitatory postsynaptic potentials (fEPSPs) were evoked by the electrical stimulation of Schaffer collaterals afferent to CA1. The magnitude of the fEPSPs was determined by measuring their slope. Signals were acquired using a double EPC 10 Amplifier (HEKA Elektronik Dr. Schulze GmbH, Germany) and analysed with Patchmaster software (HEKA Elektronik Dr. Schulze GmbH, Germany).

Input/output: The slope of fEPSPs was plotted as a function of stimulation intensity (30 to 60 µA).

Paired-pulse facilitation (PPF): PPF of synaptic transmission was induced by paired-pulse stimulation with an inter-stimulus interval of 50 ms. PPF was quantified by normalising the second response to the first one.

Frequency facilitation: Schaffer collaterals were stimulated repetitively by 25 stimuli of the same intensity at 25Hz.

Long-term potentiation: Test stimuli were delivered once every 15 s. Stimulus intensities were adjusted to produce 40-50% of the maximal response. A stable baseline was recorded for at least 15 min. LTP was induced by high frequency stimulation (4 trains delivered at 100 Hz with 5 min between each train). Average value of fEPSP slope was expressed as a percentage of the baseline response.

<u>EEG telemetry and unilateral intracortical kainate injection</u>

We took advantage of the unilateral intracortical kainate injection model for human temporal lobe epilepsy. Telemetric EEG transmitter implantation, kainate injection and data analysis was adapted from Bedner and colleagues (Bedner et al., 2015).

Alix$^{fl/fl}$ mice (Laporte et al., 2017) with heterozygous cre expression (Emx1^{IREScre} (Emx$^{tm1(cre)Krj}$, MGI: 2684610)(Gorski et al., 2002)) as well as control mice (Alix$^{fl/fl}$ x Emx^{wt}) were implanted with telemetric EEG transmitters (DSI PhysioTel® ETA-F10, Harvard Bioscences, Inc. Holliston, Massachusetts, USA) between 8 and 11 weeks of age. The animals were placed in a stereotaxic frame (Robot stereotaxic, Neurostar, Tübingen, Germany) for implantation of depth electrodes at 3.4 mm posterior to bregma and bilaterally 1.6 mm from the sagittal suture. After post-surgical care and recovery, mice were again placed in the stereotaxic frame and injected with 70 nl of a 20 mM solution of kainate (Tocris, Wiesbaden-Nordenstadt, Germany) in 0.9% NaCl, above the right dorsal hippocampus (1.9 mm posterior to bregma, 1.5 mm from sagittal suture and 1.3 mm from skull surface). Kainate was injected at a rate of 70 nl/ min with a 10 µl Nanofil syringe (34 GA blunt needle, World Precision Instruments, Sarasota, FL, USA). The syringe was kept in place for 2 min after the injection was completed to avoid liquid reflux.

Cages were placed on individual radio receiving plates (DSI PhysioTel® RPC-1, Data Sciences International, St. Paul, USA), which record EEG signals and sent them, together with the video recording (MediaRecorder Software, Noldus Information Technology, Wageningen, Netherlands), to an input exchange matrix (DSI PhysioTel® Matrix 2.0 (MX2), Ponemah software, DSI, Data Sciences International, St. Paul, USA). The animals were monitored for at least 20 h post kainate injection. In our model the mortality rate associated to *status epilepticus* is less than 5% in more than 50 mice with different genetic backgrounds over the last 12 months.

Immunohistochemistry

Free-floating vibratome sections (40 µm) were incubated for one hour in blocking buffer (5 % HS, 0.3 % Triton X in 1x PBS) at room temperature. Sections were incubated with primary antibodies (rabbit@Iba1 [1:500], Wako; goat@GFAP [1:1000], Abcam) diluted in blocking solution, overnight at 4°C. Secondary antibodies (Alexa546@rabbit [1:1000]; Alexa488@goat [1:1000], Invitrogen) and DAPI (0.025 µg/ml, Sigma) were diluted in blocking buffer and incubated for 2 h at room temperature.

Data analysis: EEG traces were analysed with the Neuroscore software (Version 3.3.1., Data Sciences International, St. Paul, USA). Electrographic seizures were detected with the spike detection protocol. Subsequently, an additional manual screen was employed to remove artifacts that were eventually picked up. Seizures were characterized by high frequency spiking and ceased with a postictal depression (flattening of EEG). Seizure detection was complemented by synchronized video monitoring. Electrographic seizures were associated with behavioural analogues of Racine stages II-V (Racine, 1972). The total duration of *status epilepticus* was defined from the first electrographic seizure to the first seizure free period lasting 1 h. Statistical analysis was performed with GraphPad Prism.

Cell culture and transfection

Cortical and hippocampal neurons from rat E18 embryos and P0 Alix wt and ko mice were prepared as previously described (Faure et al., 2006). Briefly, cortices and hippocampi were dissected from E18 rat embryos or P0 mouse pups, treated with trypsin, and mechanically dissociated. Dissociated cells were seeded at a density of 5 × $10^4/cm^2$ in 100 mm dishes for cortical neurons and 1.5 × $10^4/cm^2$ onto acid-washed coverslips (either 14 mm diameter or 25 mm; Marienfeld) in either 4-well plates or P35 dishes (ThermoScientific) precoated for 4 h with 50 µg/ml poly-D-lysine (Sigma). Neurons were maintained in neuronal culture medium (Neurobasal medium containing 2% B27 supplement, 10 unit/mL penicillin, 10 µg/mL streptomycin, and 0.5 mM L-glutamine; Invitrogen) supplemented with 10% heat-inactivated horse serum (Invitrogen). Neurons were maintained in water-saturated 95% air/5% CO_2 at 37°C. The seeding medium was replaced after 20 h with serum-free neuronal culture medium.

Neurons were transfected at 10 DIV as previously described (Chassefeyre et al., 2015). Briefly, for each P35 dish, 2 µg plasmid DNA, 250 mM $CaCl_2$ were mixed with an equal volume of 2x BES-buffered saline and left to precipitate for 20 min at room temperature. Neurons were placed in transfection medium (Minimum Essential Medium containing 0.22% $NaHCO_3$, 20 mM D-glucose and 0.5 mM L-glutamine) supplemented with 2% B27, before the DNA precipitate was added. They were then incubated for 1.5 h at 37°C and 5% CO_2. Neurons were then washed by being placed in transfection medium (pre-warmed to 37°C in 10% CO_2) for 20 min at 37°C and 5% CO_2. Finally, they were transferred back into their conditioned medium.

Primary cultures of cerebellar granule neurons (CGN) were prepared from 6-day-old C57BL/6 Alix wt and Alix ko pups as described previously (Trioulier et al., 2004), with some modifications. The cerebella were removed, cleared of their meninges, and cut into 1-mm pieces. They were then incubated at 37°C for 10 min in 0.25% trypsin-EDTA and DNAse (1500 U/mL). Trypsin was inactivated and cells were dissociated in culture medium (DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 25 mM KCl, 10 mM HEPES and 10 unit/ml penicillin, 10 μg/ml streptomycin). After filtration on 70 μm cell strainers, neurons were plated at 5.10^5 cell/cm^2 onto poly-D-lysine (10 μg/ml, Sigma) precoated coverslips. Cytosine-β-D-arabinoside (10 μM, Sigma) was added after 1 day in vitro (DIV), to prevent the growth of non-neuronal cells, until 8 DIV when neurons were used for activity dependent bulk endocytosis experiment (see below).

<u>Multi-electrode array</u>

Dissociated hippocampal neurons were resuspended in neuronal medium and plated at a 10^6 cells/cm^2 on poly-L-lysine-coated multi-electrode arrays comprising 59 extracellular recording electrodes and one reference electrode (MEA-60 Multichannel Systems, MCS). Electrodes were 30 μm in diameter and separated by a distance of 200 μm. MEA-60 plates were connected to a 60-channel data acquisition system (USB-MEA64) and associated amplifier (MEA1060-Inv-BC) powered by a PS40W power supply system (MCS, Germany). The recording system was then placed in a humidified incubation chamber at 37°C and 5% CO_2. Neurons were left for 5 min to equilibrate before recording. Basal spontaneous activity was recorded for 3 min prior addition of drugs. Bicuculline (100 μM) and 4-aminopyridine (5 mM) were then added and neuronal activity recorded for another 10 min. Signals were recorded with a 1100 gain, sampled at 20 kHz and analyzed with MC Rack software (MCS, Germany). Raw signals were first filtered with a Butterworth band pass filter (2nd order) between 200 and 800 Hz to remove electrical noise and low frequency signals. Spike detection analysis was performed on filtered signals using a threshold of 6 standard deviations to mark out action potentials. Burst events were identified as a minimum of 5 consecutive spikes with an interspike interval lower than 100 ms. The minimum interval time between two bursts was fixed at 1000 ms. Representative traces were exported using custom-made Matlab functions (Matlab 2014b).

<u>Imaging of synaptophysin-pHluorin upon electrical stimulation</u>

Hippocampal neurons from Alix wt and ko mice were transfected with synaptophysin-pHluorin (Syp-pH) at 6 DIV by a calcium phosphate transfection procedure. The recycling of synaptic vesicles was imaged in a buffer solution containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES adjusted to pH 7.4 and 270 mOsm/l. Experiments were carried out at 34°C. Neurons were stimulated by electric field stimulation (platinum electrodes, 10 mm spacing, 1 ms pulses of 50 mA and alternating polarity at 5-40 Hz) applied by a constant current stimulus isolator (SIU-102, Warner Instruments). The presence of 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 µM D,L-2-amino-5-phosphonovaleric acid (AP5) prevented recurrent activity.

Experiments were performed on an inverted microscope (IX83, Olympus) equipped with an Apochromat N oil 100× objective (NA 1.49). Images were acquired with an electron multiplying charge coupled device camera (QuantEM:512SC; Roper Scientific) controlled by MetaVue7.1 (Roper Scientific). Samples were illuminated by a 473-nm laser (Cobolt). Emitted fluorescence was detected after passing a 525/50 nm filter (Chroma Technology Corp.). Time-lapse images were acquired at 1 or 2 Hz with integration times from 50 to 100 ms.

Image analysis was performed with custom macros in Igor Pro (Wavemetrics) using an automated detection algorithm as described previously (Martineau et al., 2017). The image from the time series showing maximum response during stimulation was subjected to an “à trous” wavelet transformation. All identified masks and calculated time courses were visually inspected for correspondence to individual functional boutons. The intensity values were normalized to the ten frames before stimulation.

Live Fluorescence Imaging of Alix and endophilin recruitment to synapses

For live imaging of protein recruitment, cultured hippocampal neurons were co-transfected with Alix-mCherry or endophilin-A2-mCherry together with Syp-pH expression vectors 2 to 4 days prior to imaging. All live imaging experiments were performed at 37°C and images were acquired using a spinning disk confocal microscope (AxioObserver Z1) with a 63x oil objective (NA 1.46, Zeiss) at 488 nm and 561 nm excitation. Transfected neurons were placed in basal medium for 10 min and then mounted in an imaging chamber (POC-R2 Cell cultivation system, Zeiss). Imaging lasted 12 min and consisted of 2 min in basal medium, 5 min in bicuculline/4AP solution and 5 min in basal medium. A 5x bicuculline/4AP solution was added (final concentration, 100 µM and 5 mM, respectively) for stimulation which lasted 5 min and the chamber was perfused with basal medium at 3 ml/min for washing. ROI were drawn on ‘presynapses’ defined by spots of synaptophysin-pHluorin that increased during stimulation.

For both synaptophysin-pHluorin and mCherry-Alix, fluorescence values were measured in these ROI and then normalised to the initial fluorescence values (fluorescence values prior to stimulation) using ImageJ.

Immunofluorescence

Cultured hippocampal neurons were fixed for 20 min at room temperature in phosphate-buffered 4% paraformaldehyde supplemented with 4% sucrose. After three washes in PBS, cells were permeabilized and blocked in PBS containing 0.3% Triton X-100 and 3% BSA for 15 min at room temperature. Coverslips were incubated for 1–2 h at room temperature with primary antibodies diluted in the blocking solution. After washing in PBS, cells were incubated for 1 h with secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 594, or Cyanine 5 (Cy5), diluted in the blocking solution. Coverslips were rinsed and mounted in Mowiol. Images were acquired on a Leica SPE microscope using a 40x dry objective (NA 0.75, Leica) or a 100x oil immersion objective (NA 1.4, Leica) at 488 nm, 532 nm or 635 nm.

Synapse density: Cultured hippocampal neurons were fixed and stained with antibodies at 14-16 DIV as described above. Presynaptic boutons (synapsin-1-positive) and postsynaptic terminals (PSD95-positive) were selected using the Spot Detector plugin in Icy software (de Chaumont et al., 2012) (wavelet detection with size filtering between 0.4 µm and 2 µm in diameter) on max image projections. Synapses were defined as spots of colocalization between the detected presynaptic and postsynaptic terminals that were within 3 µm of each other. Synapses were counted using the Colocalization Studio in ICY software (Lagache et al., 2018)

Protein recruitment assays: Cultured hippocampal neurons were transfected with expression vectors 24~48 h prior to stimulation. The transfected neurons were incubated in basal medium (150 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 10 mM HEPES and 33 mM D-glucose at pH 7.4) for 10 min prior to stimulation. Coverslips were either treated with basal medium alone or bicuculline/4AP solution (basal medium supplemented with 100 µM bicuculline and 5 mM 4-aminopyridine) for 5 min at 37°C and 5% CO_2. Coverslips were fixed and stained with antibodies against synapsin-1 and PSD95 as described above. Images were acquired on a Leica SPE microscope using a 100x oil immersion objective (NA 1.4, Leica) at 488 nm, 532 nm or 635 nm. The acquired images were analysed using ImageJ. Regions of interest (ROI) were drawn on areas of axons which colocalize with anti-synapsin-1 spots, but not with anti-PSD95 spots to ensure presynaptic measurement. Fluorescence intensities were then measured in these ROI and normalised to fluorescence intensities measured on other regions of axons to give the relative fluorescence intensity at presynaptic boutons.

Quantification of calcium increase during stimulation

Hippocampal neurons cultured on 24-well plates were placed in basal medium and incubated with 1 µM Fluo-4-AM for 1 h at 37°C. After washing, neurons were left in 250 µl basal medium for 5 min. Plates were then transferred to a Pherastar automatic plate reader (BMG Labtech, Germany) set to 37 °C, to record fluorescence intensity at 0.1 Hz during 590 s, for each well, and with fluorescence measurement settings as described on http://www.bmglabtech.com/media/35216/1043854.pdf. Neurons were stimulated with 50 µl 6x bicuculline/4AP solution (final concentration, 100 µM and 5 mM, respectively) after 290 s with automatic injection.

Quantification of ADBE by dextran uptake

The protocol for dextran uptake was adapted from (Clayton and Cousin, 2009b). 15-17 DIV hippocampal neurons were stimulated with bicuculline/4AP solution for 5 min, at 37°C and 5% CO_2, in the presence of 10 kDa tetramethylrhodamine-dextran (50 µM). Coverslips were immediately washed several times in washing solution (basal medium supplemented with 0.2% BSA and warmed to 37°C) to remove excess dextran. For ADBE inhibition, neurons were stimulated in the presence of 2 µM GSK3 inhibitor (CT99021, Tocris) and placed in fresh basal medium containing 2 µM GSK3 inhibitor for 10 min at 37°C and 5% CO_2. Neurons were then fixed as previously described and imaged with a Leica SPE microscope using a 40x dry immersion objective (NA 0.75, Leica) at 532 mm excitation. Analysis was performed on ImageJ. The number of fluorescent spots was counted in a defined field of view (130µm x 130 µm) in thresholding analysis with a diameter limit between 300 nm and 2 µm (resolution limit for the microscope and maximum size of a nerve terminal).

Rescue experiments: Cultured hippocampal neurons were transfected 2 to 4 d prior to the day of experiment. Dextran uptake assay was performed as described above. Images were acquired on a Leica SPE microscope using a 40x oil immersion objective (NA 1.25, Leica) at 488 nm and 532 mm excitation. The analysis was performed on ICY software. ROI were generated closely around axons by using 'thresholder'. Then dextran spots within these ROI were counted by using 'spot detector' with size limit between 300 nm and 2 µm. The length of axon per field of view was estimated by manually drawing 'Polyline type ROI' over the axon images. The number of dextran spots per µm of axon was calculated and expressed as ratio to control values.

EM examination of ADBE in culture neurons

Analysis of ADBE from cerebellar granule neurons was performed as described previously with some modifications (Cheung et al., 2010). 8 DIV CGN were pre-incubated in hyperpolarizing medium (170 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM TES, 5 mM $NaHCO_3$, 5 mM D-glucose, 1.2 mM Na_2SO_4, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, pH7.4) for 10 min prior to stimulation. Neurons were then incubated for 2 min with 10 mg/ml HRP (Sigma P8250) in either the hyperpolarizing medium or a high-potassic solution containing 50 mM KCl (123.5 mM NaCl, 50 mM KCl, 0.4 mM KH_2PO_4, 20 mM TES, 5 mM $NaHCO_3$, 5 mM D-glucose, 1.2 mM Na_2SO_4, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, pH7.4) before rapid washing in PBS and fixation in PBS-glutaraldehyde 2% for 30 min. After three washes in Tris buffer 100 mM, endocytosed HRP was revealed by incubation in Tris 100 mM containing 0.1% diaminobenzidine and 0.2% H_2O_2. The cultures were then post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon. Synapses were photographed with a JEOL-1200 electron microscope. Quantification was performed as follows: HRP-positive structures were quantified per synapse and classified as synaptic vesicles when the diameter was less than 100 nm and as bulk endosome when the diameter was more than 100 nm.

For focused ion beam scanning electron microscopy (FIB-SEM), the block was mounted on a pin, coated with gold, and inserted into the chamber of a HELIOS 660 Nanolab DualBeam SEM/FIB microscope (FEI Co., Eindhoven, The Netherlands). Region of interest (ROI) was prepared using focused ion beam (FIB) and ROI set to be approximatively 15 µm wide. During the acquisition process, the image acquisition parameters of the electron beam were 2 kV and 0.4 nA and the thickness of the FIB slice between each image acquisition was 10 nm. The segmentation of the synapse and ABDE was done with Amira software (Thermo Fisher Scientist) and the movie obtained using Imaris (Oxford Instruments).

Synaptosomal preparation from cortical neurons

Synaptosome-enriched membranes from 15 DIV cortical neurons were prepared as described previously with some modifications (Frandemiche et al., 2014). Briefly, cultured neurons were stimulated for 15 min with a mixture of bicuculline/4AP (50 μM/2.5 mM). After a wash in HBSS, neurons were homogenized by passing 15-20 times through 0.25G needle in cold buffer containing 0.32 M sucrose, 10 mM HEPES, 15mM NaF, 15mM β-glycerophosphate and protease inhibitors (Roche), pH 7.4. Samples were maintained at 4°C during all steps of the experiment. Homogenates were cleared at 1000g for 10 min to remove nuclei and large debris. The resulting supernatants were spun down at 12,000 g for 20 min to obtain a crude membrane fraction and washed twice in HEPES buffer 4 mM containing 1 mM EDTA, 15 mM NaF, 15 mM β-glycerophosphate and protease inhibitors (Roche), pH 7.4. The resulting pellet was solubilized in 0.5% Triton X-100, 20 mM HEPES, 100 mM NaCl, 15mM NaF, 15 mM β-glycerophosphate, pH 7.2, containing protease inhibitors (Roche) for 20 min at 4°C with mild agitation and analysed by Western blot.

Western blot

Cells lysates were resuspended in Laemmli buffer and resolved by SDS-PAGE in 10% polyacrylamide gels. Proteins were electro-transferred onto PVDF membranes that were then blocked for 30 min in TBS containing 0.1% Tween 20 and 5% dry milk and incubated for 1 h to overnight with primary antibodies diluted in the blocking solution. After washes in TBS–Tween, the membranes were further incubated for 1 h with secondary antibodies coupled to HRP, washed as before and incubated with luminescence-generating HRP substrate. Bound antibodies were revealed by luminography on film.

Statistical analysis

The comparison of two groups was performed using Student's t-test or its non-parametric correspondent, the Mann-Whitney test, if normality was not granted either because not checked or because rejected (Shapiro-Wilks test). The comparisons of more than two groups were made using one or two way ANOVAs followed by post-hoc tests (Holm Sidak's or Tukey's HSD, see table 1 for details) to identify all the significant group differences. N indicates independent biological replicates. The graphs with error bars indicate 1 SEM (+/-) except for figure 1C-H where we used box plots showing distribution of medians (whiskers = min and max values). The significance level is denoted as usual (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All the statistical analyses were performed using Prism7 (Graphpad version 7.0a, April 2, 2016).

Means, confidence intervals, degrees of freedom and p values were calculated using Prism7. Effect sizes are either those reported by the software or Cohen's D calculated as the difference of means divided by the pooled weighted standard deviation:

$$(|m1 - m2|)/\sqrt{((SD1^2 \times \frac{n1}{(n1+n2)}) + (SD2^2 \times \frac{n2}{(n1+n2)}))}.$$

The results of the statistical analyses are listed in table 1.

Results

Alix is required for synaptic plasticity

To study the effect of Alix on the number of synapses, we first compared brains of Alix ko (Laporte et al., 2017) and wt mice and found that the density of dendritic spines in the CA1 hippocampal region of adult brains stained with Golgi-Cox was not different (Supplementary figure 1). Similarly, EM examination of the same CA1 hippocampal region revealed no difference in the number of synaptic contacts between wt and Alix ko neurons (Figure 1A). In cultured hippocampal neurons, the number of synapses revealed by co-immunostaining with synapsin-1 and PSD95, was similar between wt and Alix ko (Figure 1B) indicating that Alix is not required for synaptogenesis. However, EM of the CA1 *stratum radiatum* of adult mice showed that Alix ko synapses contained significantly fewer synaptic vesicles (SV) than wt (Figure 1C, D). Furthermore, the size of postsynaptic densities, known to be strictly correlated with the number of SV (Harris and Stevens, 1989), was similarly reduced in Alix ko synapses (Figure 1F, G). Importantly, the surface of Alix ko synaptic boutons was also significantly increased suggesting plasma membrane accumulation in these synapses (Figure 1C, E). Finally, at the postsynaptic level, we also noticed that the ratio between the diameter of the spine head and that of the neck was changed in Alix ko animals suggesting possible defects in maturation or plasticity of adult synapses lacking Alix (Figure 1F, H).

Using electrophysiological recordings of acute hippocampal slices from adult animals, we next examined if the morphological differences detected in Alix ko synapses might translate into alterations of their function. For this, we performed field recordings in the CA1 *stratum radiatum* of hippocampal slices during stimulation of Schaffer collaterals. As shown on figure 2A the input-output curves were not significantly different between Alix ko and wt animals indicating that, in this region, both the connectivity and the basal synaptic transmission are not grossly affected by the lack of Alix. However, in the same slices, long-term potentiation (LTP) induced by tetanic stimulations was significantly impaired in Alix ko, compared to wt hippocampal slices (average recordings between 25 and 35 min post-high-frequency stimulation: 105.02 ± 13.86 % in Alix ko compared to 180.70 ± 11.19 % in wt, Figure 2B). In the same CA3/CA1 synapses, the paired-pulse facilitation ratio was significantly reduced in ko animals (1.43 ± 0.04 in ko vs 1.62 ± 0.07 in wt, Figure 2C). Furthermore, synaptic fatigue, induced by repeated stimulations at 25 Hz, was significantly increased in absence of Alix

(Figure 2D). Thus, electrophysiological recordings reveal presynaptic anomalies, which may be related to impairments in high frequency-induced LTP.

Alix is recruited to synapses upon synaptic activation.

We next used dissociated cortical neuron cultures to decipher the role of Alix at synapses. Western blot analysis during *in vitro* differentiation revealed that Alix expression strongly increases during synaptogenesis as indicated by the parallel rise in postsynaptic density protein 95 (PSD95) expression (Figure 3A). Moreover, synaptosome-enriched membranes prepared from cortical neurons (15 DIV) contained Alix (Figure 3B). This synaptic pool increased when neuron cultures were incubated 15 min with the $GABA_A$ receptor antagonist bicuculline, together with a weak potassium channel blocker 4-aminopyridine (4AP)(Figure 3B), a treatment known to increase the frequency of action potential bursts and thereby induce sustained intracellular calcium elevation (Supplementary figure 2A) (Hardingham et al., 2001; Hardingham et al., 2002). Therefore, this observation suggests that Alix tends to concentrate at synapses undergoing prolonged stimulations. To show that this is indeed the case, we used live imaging to follow mCherry-Alix relocalization to synapses in 15 DIV hippocampal neurons. While the fluorescent signal was homogeneously distributed throughout the entire neuronal cytoplasm in resting conditions (Figure 3C, t= 0 min), it almost doubled in discrete spots within neuronal processes during synaptic activation corresponding to active presynaptic boutons labelled with Synaptophysin-pHluorin (Syp-pH) (Granseth et al., 2006) (arrowheads Figure 3C, t= 2:20, 4 and 6 min; Movie 1). Alix recruitment to these sites is concomitant with the activation of glutamatergic synapses, as it became detectable a few seconds after addition of bicuculline/4AP to the culture medium and decreased soon after washing the cells (Figure 3 C, D). Thus, we demonstrate that mCherry-Alix recruitment occurs at presynaptic sites that display transient increase of Syp-pH fluorescence induced by stimulation (arrowheads Figure 3C, Figure 3D; Movie 1). Alix localization at axonal boutons was confirmed in bicuculline/4AP stimulated and fixed-neurons expressing Alix-YFP and immunolabelled for synapsin-1 to reveal presynaptic boutons (Figure 3E, F). Similar experiments using anti-PSD95 immunostaining showed that Alix-positive spots were juxtaposed to but did not overlap with the postsynaptic density marker (Figure 3G). Measuring Alix-YFP fluorescence intensities in dendritic spines before and during stimulation showed no accumulation of Alix at the post-synaptic level

(Supplementary figure 2B, C), further demonstrating that synaptic activation leads to Alix recruitment only at pre-synaptic parts.

<u>Recruitment of Alix partners, ALG-2 and endophilin-A, at activated synapses</u>

In non-neuronal cells, Alix recruitment to plasma membrane wounds was shown to be caused by calcium entry. Calcium binds ALG-2, a penta-EF-hand containing protein, which thereafter interacts with Alix allowing its recruitment to the plasma membrane (Scheffer et al., 2014). In neurons, action potential depolarization induces massive and transient calcium accumulation in the bouton, which triggers fusion of SV with the plasma membrane. Adding the intra-cellular calcium chelators BAPTA-AM or EGTA-AM completely abolished Alix-YFP recruitment (Supplementary figure 3). Interestingly, GFP-ALG-2 concentrated at presynaptic boutons upon synaptic activation (Figure 4A, B), in contrast to an ALG-2 point-mutant unable to bind calcium (ALG-2ΔCa) (Figure 4B). ALG-2 recruitment does not depend on Alix, as it concentrates at stimulated synapses of both wt and Alix ko neurons (Figure 4B, 4C left bars). On the contrary, Alix recruitment to active synapses is tightly dependent on its capacity to interact with ALG-2, as a mutated version of the protein unable to interact with ALG-2 (AlixΔALG-2) (Suzuki et al., 2008; Trioulier et al., 2004) does not accumulate at presynaptic boutons upon stimulation (Figure 4C, right bars).

Among other partners of Alix, endophilins-A are main regulators of endocytosis at synapses and impact the number of SVs (Milosevic et al., 2011; Schuske et al., 2003). We therefore tested if endophilins could be recruited to active synapses, similarly to Alix and ALG-2. Endophilin-A2-mCherry was mainly detected at presynaptic boutons but its fluorescence increased during bicuculline/4AP treatment (Figure 4D, E; Movie 2). Remarkably, no such increase could be seen in Alix ko neurons, whereas Alix deleted of its endophilin binding domain (AlixΔendo), was still able to be recruited to synapses upon activation (Figure 4F). This observation strongly suggests that the increase in endophilin concentration during sustained synaptic activation requires Alix expression.

<u>Alix is required for ADBE</u>

Altogether, our observations indicate that calcium increase at synapses undergoing sustained activity allows the transient recruitment of ALG-2/Alix and endophilin-A to pre-synaptic parts. Because of our EM observations showing that Alix ko axonal bouton are bigger and have significantly fewer SVs, we next tested which step of the turnover of presynaptic vesicles might be affected by the lack of the protein. We used Syp-pH expressing neurons and followed their fluorescence during electrical stimulation to trigger exocytosis. Recapture of synaptic proteins from the plasma membrane results in decay in fluorescence, reflecting SV retrieval and vesicle re-acidification (Soykan et al., 2017). As illustrated in figures 5A and B, fluorescence increases during stimulation (exocytosis) followed by a decrease thereafter (endocytosis), with no drastic difference between Alix ko and wt neurons for the two stimulation frequencies, 5 and 40 Hz. However, the time constant of the exponential decay was slightly decreased in Alix ko neurons stimulated at 40 Hz, suggesting a higher rate of Syp-pH endocytosis occurring at this frequency (Figure 5B).

We next tested if the protein might intervene in activity-dependent bulk endocytosis (ADBE) at synapses. Cultured cerebellar granular neurons (CGN) have been extensively used to study this process (Cheung et al., 2010). Here, neurons were depolarized with 50 mM KCl, in the presence of Horse Radish Peroxidase (HRP) that is endocytosed and fills vesicles and endosomes as they form. EM examination of wt synapses showed that depolarization dramatically increased the number of vacuoles decorated by HRP. We verified using FIB SEM and 3D reconstruction that these vacuoles had undergone fission from the plasma membrane and could therefore be identified as bulk endosomes (Figure 5C yellow arrowheads; Figure 5D). Other HRP positive vesicles having the size of neurotransmitter vesicles were also more numerous in depolarized synapses (Figure 5C blue arrowheads; Figure 5D, E). In Alix ko synapses, the number of depolarization-induced bulk endosomes was strongly reduced (Figure 5E, F). Consistent with the increased endocytic rate detected by Syp-pH in Alix ko neurons stimulated at 40 Hz (Figure 5B), the number of newly formed SV was significantly increased, suggesting a mechanism compensating for ADBE deficiency (Figure 5G).

Another way to assess for ADBE is by the use of fluorescent 10 kDa dextran, a fluid phase cargo that accumulates inside bulk endosomes but fails to label vesicles formed by clathrin-dependent mechanisms upon neuronal stimulation (Clayton and Cousin, 2009a). Hippocampal neurons incorporated dextran when incubated in presence of bicuculline/4AP. A GSK3 inhibitor, known to block ADBE but not other modes of SV endocytosis (Clayton et al., 2010), completely abolished the dextran labelling (Supplementary figure 4A, B). In contrast, Alix ko neurons failed to endocytose dextran (Figure 6A, B) even though bicuculline/4AP stimulation increased calcium entry (Supplementary figure 2A) and neuronal activity (Supplementary figure 4C, D) in both Alix ko and wt neurons. As already shown (Morton et al., 2015), neurons also failed to endocytose dextran when treated with calcium chelators BAPTA and EGTA (Supplementary figure 4E), both of which blocked Alix recruitment to presynaptic boutons (Supplementary figure 3). Finally, rescue experiments showed that the impairment in ADBE observed in Alix ko cells is due solely to the absence of Alix, since restoring Alix expression fully restored the capacity of Alix ko cells to endocytose 10 kDa dextran (Figure 6 C, D). In sharp contrast, expression of AlixΔALG-2 or AlixΔEndo was not able to rescue dextran endocytosis by Alix ko neurons (Figure 6I). Noteworthy, an Alix mutant (AlixΔ4B) unable to bind the ESCRT-III protein CHMP4B, was still able to rescue ADBE in Alix ko neuron. These results demonstrate that the capacity of Alix to drive ADBE requires interaction with both endophilin-A and ALG-2 but not with ESCRT-III.

<u>Alix conditional-ko mice undergo less kainate induced acute seizures.</u>

The physiological relevance of ADBE remains today unclear but it is thought that it allows synapses to sustain high frequency stimulations such as those seen during epilepsy. We tested this possibility by studying the acute phase of a mouse model for human temporal lobe epilepsy (Bedner et al., 2015). The unilateral intracortical kainate injection induces the acute phase, *status epilepticus*, characterized by high seizure frequency, during several hours. Seizure activity was determined by telemetric EEG recording and synchronized video monitoring (Figure 7A, B). Here we used Alix conditional-ko mice where Alix is deleted in neocortical and hippocampal excitatory neurons (Gorski et al., 2002) (Figure 7A). The total duration of *status epilepticus* (SE) was comparable in Alix ko (median 1.70 h; Interquartile range, IQR = 1.85) and control mice (1.63 h; IQR = 2.55, Figure 7C). However, Alix ko mice experienced about 66 % less seizures than control mice (8; IQR = 13 seizures in ko vs. 18; IQR = 23 in control, Figure 7D). The average seizure duration was only minimally reduced in Alix ko compared to control (25.7 s; IQR = 19.36 vs. 32.9 s; IQR = 19.54, Figure 7E). These results show that the absence of Alix selectively reduces the number of high frequency events (single seizures), without affecting the total duration of *status epilepticus*.

The intracortical kainate injection model of temporal lobe epilepsy employed here produces seizures originating from the hippocampus, which can secondarily generalize and thereby spread to other (contralateral) brain areas. After EEG recording, 24 h post kainate injection, the brains were processed for immunohistochemical analysis (Figure 8) to assess microglial

(Iba1) reactivity in cortex (Figure 8A, B, D) and hippocampus (Figure 8A, C, D). In both control and Alix ko animals, quantification of Iba1 fluorescence intensity revealed an enhanced microglia activation on the ipsilateral side, related to the kainate injection (Figure 8B, C, E). While the difference between ipsi-and contralateral side was non-significant in control animals (≈ 10%), Alix ko animals displayed a significantly reduced contralateral microglial activation (≈ 30%, p=0.009) (Figure 8E). As expected, this difference between Alix ko and wt animals was not seen using GFAP immunolabelling, reflecting the fact that astroglial activation peaks only 4-7 days after a CNS insult. (Supplementary figure 5). The reduced contralateral microglial activation seen in Alix ko animals is an indicator for reduced propagation of the seizure activity, corroborating the previous seizure quantification (Figure 7D).

In summary, Alix is specifically required for ADBE. Impairment in this process seen in Alix ko mice could be related to the lower number of SV and increase in the surface of presynaptic membranes observed *in vivo,* as well as to impairments in synaptic function and plasticity in normal and pathological settings.

Discussion

To sustain neurotransmission and prevent expansion of the presynaptic plasma membrane, synaptic vesicle (SV) fusion is coupled to the endocytic recycling and regeneration of SV proteins and lipids. Vesicle components can be retrieved from the plasma membrane via clathrin scaffolds, or via clathrin-independent processes mediating fast and ultrafast endocytosis and, in the case of high frequency stimulation, bulk endocytosis (Gan and Watanabe, 2018). Using Alix ko cells, we recently discovered that Alix drives clathrin-independent-endocytosis (CIE) during ligand-induced endophilin-dependent endocytosis as well as bulk endocytosis (Laporte et al., 2017; Mercier et al., 2016). Furthermore, we had previously reported that Alix immunoreactivity in the rat hippocampus is strongly upregulated in synapses undergoing high frequency activation during kainate-induced epileptic seizures. Interestingly, endophilin-A and synaptophysin stainings revealed that this increase was presynaptic and only transient, as it was reversed soon after cessation of the seizures (Hemming et al., 2004). In order to study the possible role of Alix recruitment to presynaptic parts seen during seizures, we used primary cultures of cortical and hippocampal neurons which make networks undergoing spontaneous activities. Adding 4-aminopyridine (4-AP), a weak potassium channel blocker, together with bicuculline to block $GABA_A$ receptors, greatly increases spike frequency (Sup. Figure 6 C, D) and results in elevated calcium entry (sup. Figure 3A)(Hardingham et al., 2001). Consistent with the kainate-induced Alix increase seen during epileptic seizures, bicuculline/4AP induced Alix to transiently concentrate at presynaptic parts of firing synapses. The use of dextran uptake to monitor bulk endocytosis revealed that the very same treatment triggered activity dependent bulk endocytosis (ADBE) which required Alix expression. Despite this, monitoring the decay of Syp-pH fluorescence did not reveal any obvious blockage of the endocytosis of the protein at 40 Hz. This is in good agreement with observations showing that at these frequencies, Syp-pH reveals synaptic CME or CIE but not ADBE (Nicholson-Fish et al., 2015; Soykan et al., 2017). In fact, Syp-pH fluorescent decay in Alix ko hippocampal neurons suggested an increase in endocytosis, confirmed by EM in cerebellar neurons, which could represent a compensatory process for the absence of Alix and concomitant ADBE impairment. Thus, the use of Alix ko neurons revealed that Alix is required for ADBE at synapses but does not seem to be involved in SV endocytosis.

ADBE is triggered by high [Ca^{2+}] in response to sustained activity (Paillart et al., 2003). Calcium and calmodulin-dependent phosphatase calcineurin were suggested to make a link between synaptic activity and formation of bulk endosomes through dynamin (Morton et al., 2015). Alix may represent another link as its recruitment and activity in ADBE seems dependent on calcium increase. EGTA, which binds calcium at an approximately 100 times slower on-rate than BAPTA (Adler et al., 1991; Schneggenburger and Neher, 2005), and was already known to block ADBE without affecting synaptic release, was equally efficient as BAPTA in inhibiting Alix recruitment. This recruitment required Alix capacity to bind the calcium-binding protein ALG-2, which also concentrated at hyperactive synapses. ALG-2 is a cytosolic penta-EF hand-containing protein with two Ca^{2+} binding sites (Kd= 1.2 μM), and its interaction with Alix strictly depends on calcium (Missotten et al., 1999; Shibata et al., 2004; Trioulier et al., 2004). Conformational change and exposure of hydrophobic patches occur at μM concentrations of Ca^{2+}, suggesting that ALG-2 functions as a calcium sensor (Maki et al., 2016). Indeed, in non-neuronal cells, calcium entry provoked by membrane wounds leads to the sequential recruitment to the membrane of ALG-2, Alix and ESCRT-III proteins necessary for membrane repair (Jimenez et al., 2014; Scheffer et al., 2014). In neurons, the activity-dependent accumulation of ALG-2 at synapses required Ca^{2+} binding as it was totally abolished by point-mutations within the two Ca^{2+} binding sites. Interestingly, ALG-2 recruitment at synapses did not require Alix, as it also occurred in Alix ko neuron. Thus, our observations highlight synaptic ALG-2 as an obvious candidate for sensing calcium elevation and suggest the following scenario: sustained high frequency depolarization leads to massive elevation of calcium in the synaptic bouton; Ca^{2+} binding to ALG-2 causes its accumulation at the synaptic membrane and binding to cytosolic Alix, whose recruitment to the membrane drives ADBE. It remains unclear how ALG-2 interacts with lipid bilayers (Maki et al., 2016; Scheffer et al., 2014). For Alix, a change in conformation induced by binding to ALG-2 allows its interaction with lysobisphosphatidic acid (LBPA), a phospholipid only present in late endosomes (Bissig and Gruenberg, 2014; Bissig et al., 2013; Sun et al., 2015). If they exist, equivalent lipids from the plasma membrane favouring Alix recruitment remain to be discovered.

Little is known about the molecular mechanisms underlying the plasma membrane deformation during ADBE. The proline rich domain of Alix interacts with the SH3 domains of endophilins-A1-3 which contain N-BAR domains and have been shown to regulate clathrin-dependent and -independent SV endocytosis at different synapses (Gad et al., 2000; Llobet et al., 2011; Ringstad et al., 1999). BAR domains are dimerization domains able to induce, stabilize and sense membrane curvature (Farsad et al., 2001; Kjaerulff et al., 2011).

Interestingly, a proteomic approach has recently revealed the presence of endophilin-A1, Alix and ALG-2 in bulk endosomes (Kokotos et al., 2018). Furthermore, endophilin-A2, actin and dynamin mediate a restricted type of CIE activated upon ligand binding to cargo receptors (Boucrot et al., 2014; Renard et al., 2014). Endophilin-A and Alix act in the same CIE pathway, even if Alix is more promiscuous for ligands, as it is also involved in fluid phase endocytosis. In fibroblasts, interaction of Alix with endophilins favours their presence at the membrane (Mercier et al., 2016). In hippocampal neurons, endophilin-A2 decorated presynapses of resting neurons but Alix was needed for its synaptic increase during stimulation. In turn, Alix recruitment did not need interaction with endophilins to be recruited. Concave cytoplasmic surfaces of the plasma membrane are thought to trigger the recruitment of endophilin-A through their BAR domains (Boucrot et al., 2014; Kjaerulff et al., 2011). At synapses, Alix might represent an adaptor concentrating endophilins onto the flat membranes of the peri-active zone to drive membrane bending in response to high calcium concentrations. Alix-endophilin complex is required for ADBE, as demonstrated by the lack of rescue of ADBE by an Alix point mutant unable to interact with endophilin, a finding reminiscent of that seen in the case of CIE of Cholera Toxin B (CTxB) (Mercier et al., 2016). Interestingly, the incapacity of Alix to interact with Chmp4B from ESCRT-III, known to alleviate its plasma membrane repair ability (Jimenez et al., 2014; Scheffer et al., 2014), did not alter rescue of ADBE. This result also discriminates Alix-driven ADBE from roles of the ESCRT system in the degradation of SV proteins (Sadoul et al., 2017; Sheehan et al., 2016).

The role of ADBE *in vivo* is not fully understood, in part due to a lack of models in which synaptic ABDE is selectively impaired. The severe LTP impairment and increased synaptic fatigue observed in Alix ko hippocampus could be related to the difficulty of synapses to deal with the repeated stimulations necessary to trigger potentiation. As such, they represent a first demonstration of the necessity of ADBE for normal synaptic plasticity. Excitatory, glutamatergic neurotransmission plays a central role in the generation of seizure activity (Albrecht and Zielińska, 2017; Barker-Haliski and White, 2015) and figures among the primary anti-epileptic drug targets (Rogawski and Löscher, 2004). Sustained, intense and synchronous neurotransmission as occurring during seizures requires replenishment of synaptic vesicle pools mainly through activity-dependent bulk endocytosis (ADBE) (Cheung and Cousin, 2012). Impairing ADBE *in vivo,* by deleting Alix in neocortical and hippocampal excitatory neurons (Gorski et al., 2002), led to a significantly reduced number of seizures during SE, without affecting the total duration of SE or the mean seizure duration. The present results suggest that excitatory networks lacking Alix have a reduced capacity to initiate sequential seizures, due to an impaired replenishment of synaptic vesicles through ADBE. Seizure termination and *status epilepticus* cessation however seem to be governed by distinct mechanisms, probably involving inhibitory transmission to re-establish the balance between excitation and inhibition in the brain. Our results highlight Alix ko mice as an invaluable tool for exploring and understanding more precisely the exact role of ADBE at synapses undergoing normal and pathological stimulations.

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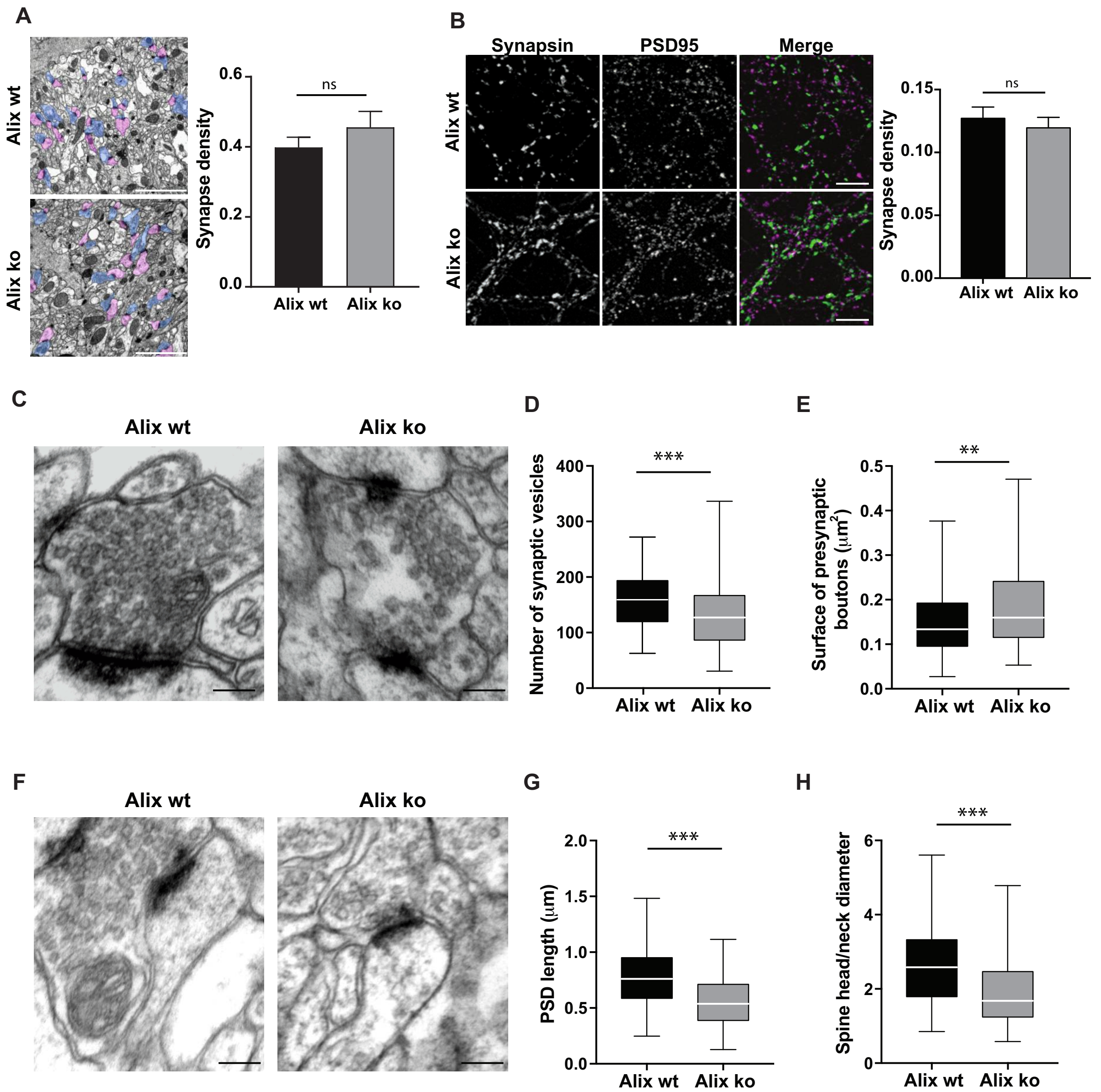

Figure 1. Alix is required for synaptic organisation. A, B) show no apparent effect of the lack of Alix on the number of synapses both in vivo or in vitro. A) Representative electron micrographs of the CA1 stratum radiatum from Alix wt and ko brains. Presynaptic profiles are highlighted in blue, and dendritic spines are in purple (scale bar: 2 µm). Graph shows the synaptic density per μm^2 (n=3 animals per genotype, p=0.1395, Unpaired t test). B) 15 DIV hippocampal neurons were stained by dual immunofluorescence with anti-PSD95 and anti-synapsin-1 antibodies. Immunolabelled objects were considered synapses when both stainings were juxtaposed (scale bar: 10 µm). The graph represents the number of synapses per µm2 (n=7 experiments, p=0.5501, Unpaired t test). C-G) Anomalies can be detected in synapses of Alix ko brains. C, F) Representative electron micrographs of CA1 from Alix wt and ko mice (scale bar: 200 nm). Graphs represent D) numbers of synaptic vesicles per µm2 (n=136 synapses from 3 animals, p=0.0009, Mann Whitney test), E) presynaptic bouton surface area (n=136 synapses from 3 animals, p=0.0036, Mann Whitney test), G) PSD length (n=136 synapses from 3 animals, p=0.0001, Mann Whitney test) and H) ratio between the diameter of the spine head and neck.

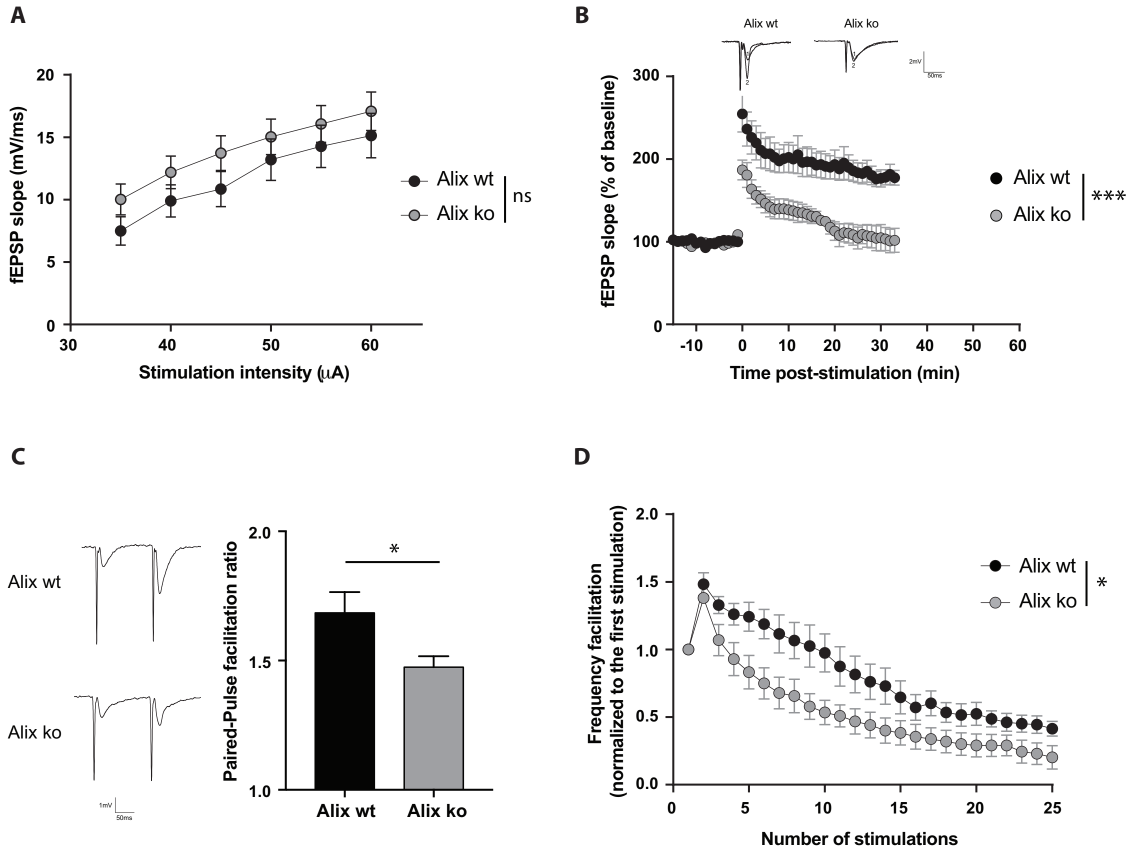

Figure 2. Synaptic plasticity in CA1 is altered in absence of Alix. A) Input-output relationship of fEPSP slope evoked by stimulation of Schaeffer collaterals at various intensities in slices from Alix wt and ko mice (n=16 wt slices and n=13 ko slices from 3 animals per genotype, p=0.9166, two-way ANOVA). B) Long-term potentiation of fEPSP slope evoked by high frequency stimulation of Schaeffer collaterals delivered at time 0. Inserts show representative EPSPs traces. (n=5 slices from 3 animals per genotype, p=0.0001, two-way ANOVA). C) Paired-pulse facilitation ratio. Facilitation is induced by two consecutive stimuli of Schaeffer collaterals separated by 50 ms (n=16 wt slices and n=13 ko slices from 3 animals per genotype, p=0.0187, Unpaired t test). Representative traces are shown on the left. D) Synaptic fatigue induced by stimulation of Schaffer collaterals at 25 Hz (n=5 slices from 3 animals per genotype, p=0.0001, two-way ANOVA). (n=136 synapses from 3 animals, p=0.0001, Mann Whitney test).

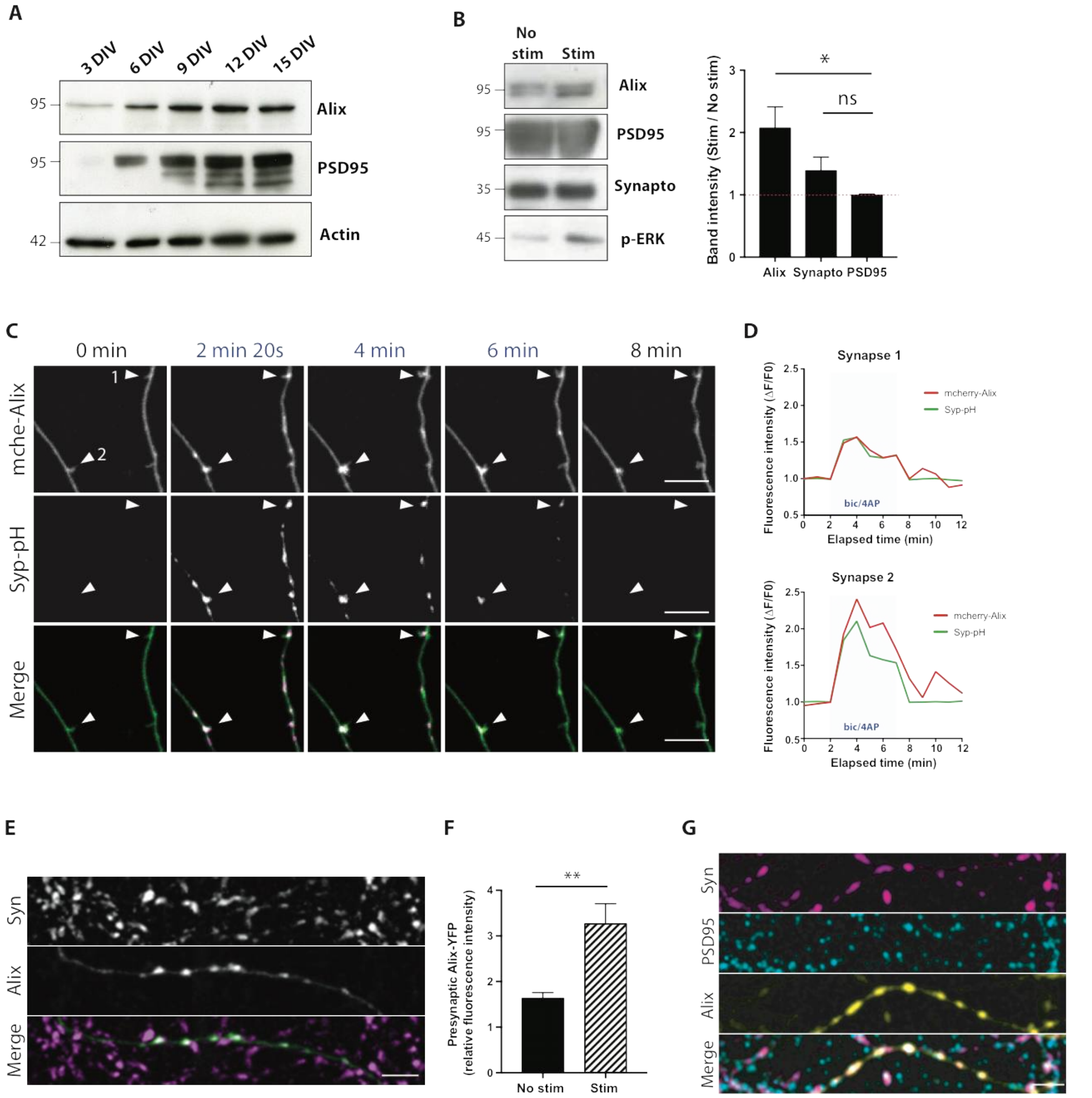

Figure 3. Alix is recruited presynaptically during synaptic activation. A) Western blot showing Alix expression during neuronal maturation in cortical cultures. PSD95 expression was used to monitor synaptogenesis with time in culture. B) Western blot showing increase of Alix in synaptosome-enriched neuronal membranes upon neuronal stimulation by bicuculline/4AP. Synaptophysin and PSD95 were used as pre- and postsynaptic markers, respectively. The phosphorylated form of ERK (p-ERK) was used to assess the efficiency of the stimulation. (n=4 experiments, Alix versus PSD95, p=0.0187, one-way ANOVA). C) Images from time-lapse video microscopy of 15 DIV hippocampal neurons expressing mCherry-Alix (mche-Alix) and synaptophysin-pHluorin (Syp-pH). White arrowheads indicate presynaptic boutons where Alix is recruited during a 5 min bicuculline/4AP stimulation starting at 2 min (images at 2:20, 4 and 6 min) (scale bar: 10 µm). D) Profile of mche-Alix recruitment to presynaptic boutons 1 and 2 of figure 3C and the corresponding Syp-pH profile. Blue box indicates the bicuculline/4AP incubation. E, F) 15 DIV hippocampal neurons expressing Alix-YFP (green) stimulated for 5 min, fixed and presynaptic boutons revealed with anti-synapsin-1 antibody (Syn, magenta) (scale bar: 5 µm). Graph shows the presynaptic increase in Alix-YFP upon stimulation. Presynaptic Alix-YFP corresponds to the ratio of YFP fluorescence between synapsin-positive and -negative axonal regions (n=12 neurons per condition from 4 experiments, p=0.0017, Unpaired t test). G) 15 DIV hippocampal neurons expressing Alix-YFP (yellow), stimulated for 5 min, fixed and stained with anti-PSD95 (cyan)

for postsynaptic densities and anti-synapsin-1 (magenta) (scale bar: 5 µm).

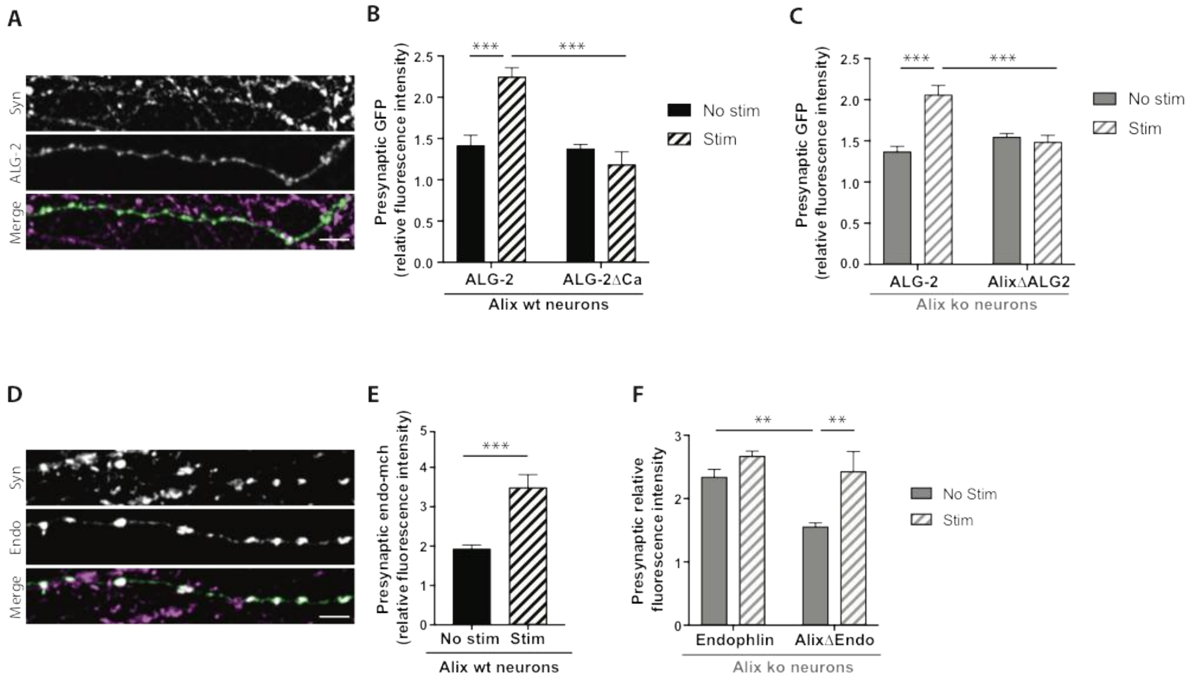

Figure 4. Interplay between Alix, ALG-2 and endophilin recruitments at activated synapses. All experiments made use of 15 DIV hippocampal neurons expressing the indicated constructs. Neurons were stimulated with bicuculline/4AP for 5 min before fixation and staining with antisynapsin-1. A) GFP-ALG-2 (green) is recruited presynaptically (scale bar: 5 µm). B) Quantification of synaptic recruitments of GFP-ALG-2 and GFP-ALG2ΔCa in Alix wt neurons shows that ALG-2 presynaptic increase depends on its capacity to bind calcium (n=10 and n=7 neurons for GFP-ALG2 stimulated and not stimulated, respectively and n=6 and n=12 neurons for GFP-ALG2ΔCa stimulated and not stimulated, respectively, in 3 experiments, p=0.0001, one-way ANOVA). C) In Alix ko neurons GFP-ALG2 is recruited pre-synaptically but GFP-AlixΔALG2 is not recruited indicating that Alix recruitment depends on its capacity to bind ALG-2 (n=17 neurons for both GFP-ALG2 stimulated and not stimulated, and n=15 and n=18 neurons for GFP-AlixΔALG2 stimulated and not stimulated, respectively, from at least 3 experiments, p=0.0001, one-way ANOVA). D, E) endophilin-mCherry (green) concentrates at presynaptic parts of stimulated wt, but not Alix ko, neurons (scale bar: 5 µm) (n=12 and n=10 neurons for stimulated and not stimulated, respectively from 3 experiments, p=0.0001, Mann Whitney test). F) Alix recruitment does not depend on its binding to endophilins as shown using GFP-AlixΔendo in Alix ko neurons (n=15 and n=13 neurons for endophilin-mCherry stimulated and not stimulated, respectively, and n=9 and n=10 neurons for GFP-AlixΔendo stimulated and not stimulated, respectively, from 3 experiments p=0.0031, one-way ANOVA).

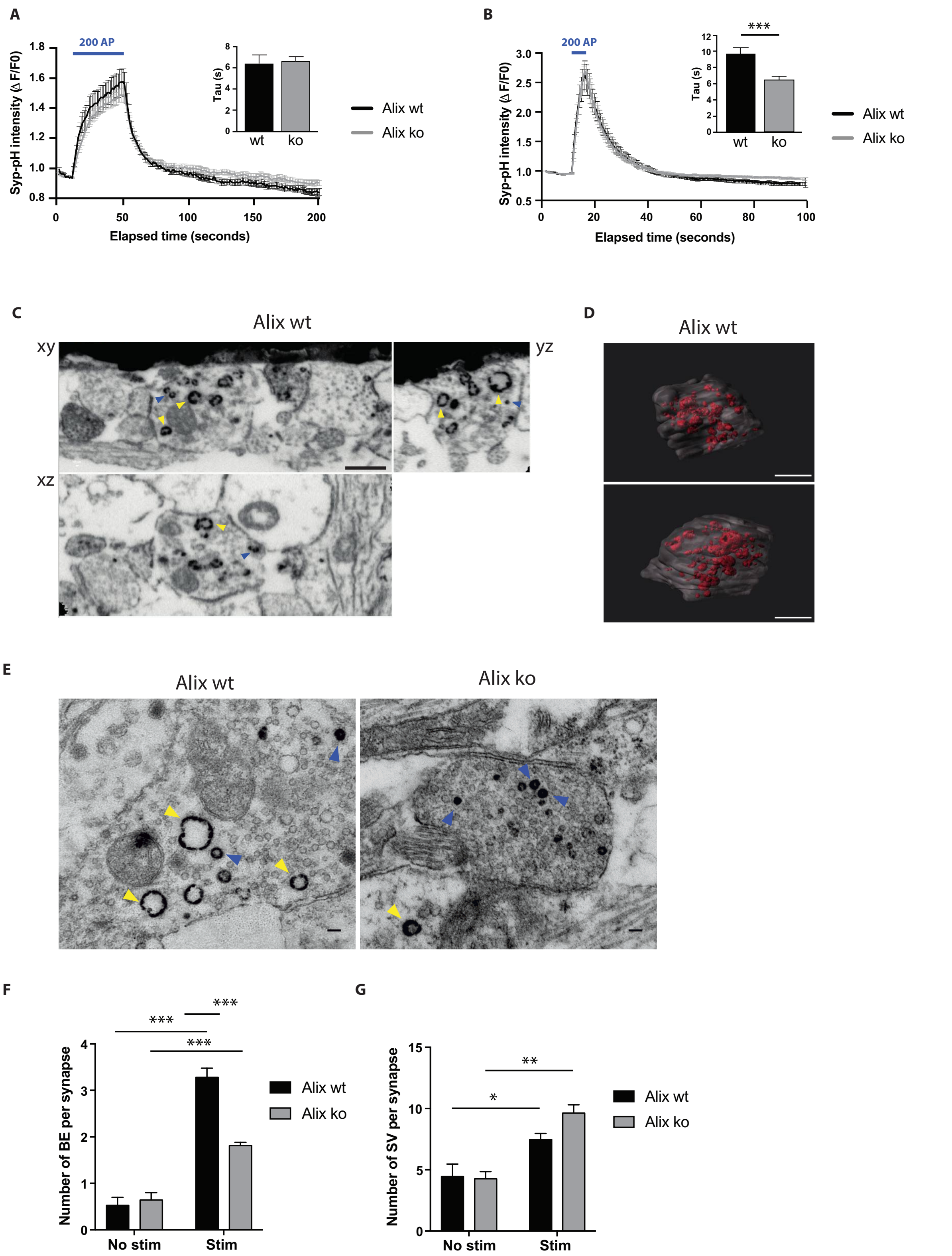

Figure 5. Alix is necessary for activity-dependent bulk endocytosis. A, B) Average traces of Syp-pH fluorescence in synaptic boutons of Alix wt and ko hippocampal neurons stimulated with 200 action potentials (AP) applied at 5 Hz (A) or 40 Hz (B) (16-46 fields of view per condition from 4 experiments for wt and 5 for ko mice). Insets show the exponential fit of fluorescence decay after stimulations in

the fields imaged. (A) p = 0.99; (B) p = 0.001 one-way ANOVA. C, E) Electron micrographs of cerebellar granule neurons stimulated in presence of free HRP to label newly-formed synaptic vesicle (blue arrowhead) and bulk endosomes (yellow arrowhead). C) Focused ion beam scanning electron microscopy (FIB-SEM); orthogonal views from different planes (xy, xz or yz) extracted from a stack used for the 3D reconstruction of WT pre-synaptic terminal shown in D). Scale bar: 500nm. D) Two different views of the reconstructed synapse are shown where the membrane is represented in transparent gray and HRP-positive structures in red (Scale bar: 500nm). E, F, G) transmission electron microscopy of HRP incubated CB neurons was used to quantify the number of bulk endosomes (F) and synaptic vesicles (G) in Alix wt and ko cerebellar neurons (F, n=4 experiments, p=0.0008, one-way ANOVA) or synaptic vesicles (G, n=4 experiments, p=0.0393 one-way ANOVA). (E, scale bar: 100 nm).

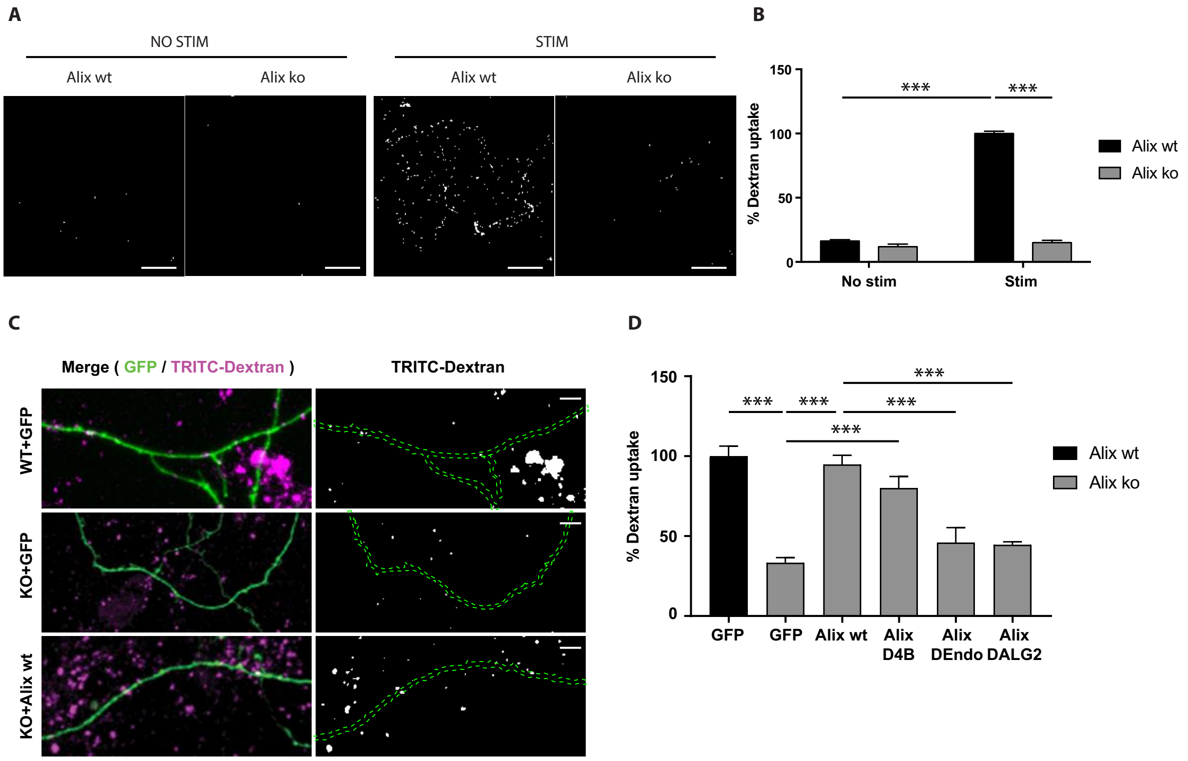

Figure 6. Alix binding to Alg-2 and endophilin but not CHMP4 is essential for bulk endocytosis. A, B) Dextran uptake is triggered by Bicucculine/4AP stimulation in Alix wt, but not in Alix ko, neurons. Confocal images of Alix wt and Alix ko hippocampal neurons stimulated in the presence of 10kDa dextran (scale bar: 50 µm) (n=4 experiments, p=0.0001, one-way ANOVA). C) Representative images of Dextran uptake by Alix wt neurons (top), Alix ko neurons expressing GFP (middle), or Alix ko neurons expressing GFP and Alix (bottom). D) Dextran uptake is rescued in Alix ko neurons expressing Alix wt and AlixΔChmp4B (AlixΔ4B), but not AlixΔALG2 or AlixΔendo. % of dextran uptake corresponds to the number of dextran spots per µm expressed as percentages of the positive control for each experiment (n=17 neurons for wt neurons, n=13 neurons for ko+GFP, n=11 for ko+Alix, n=12 neurons for ko+AlixΔendo, n=12 for ko+AlixΔChmp4B, and n=11 for ko+AlixΔALG2, all from at least 3 experiments, p=0.0001, one-way ANOVA).

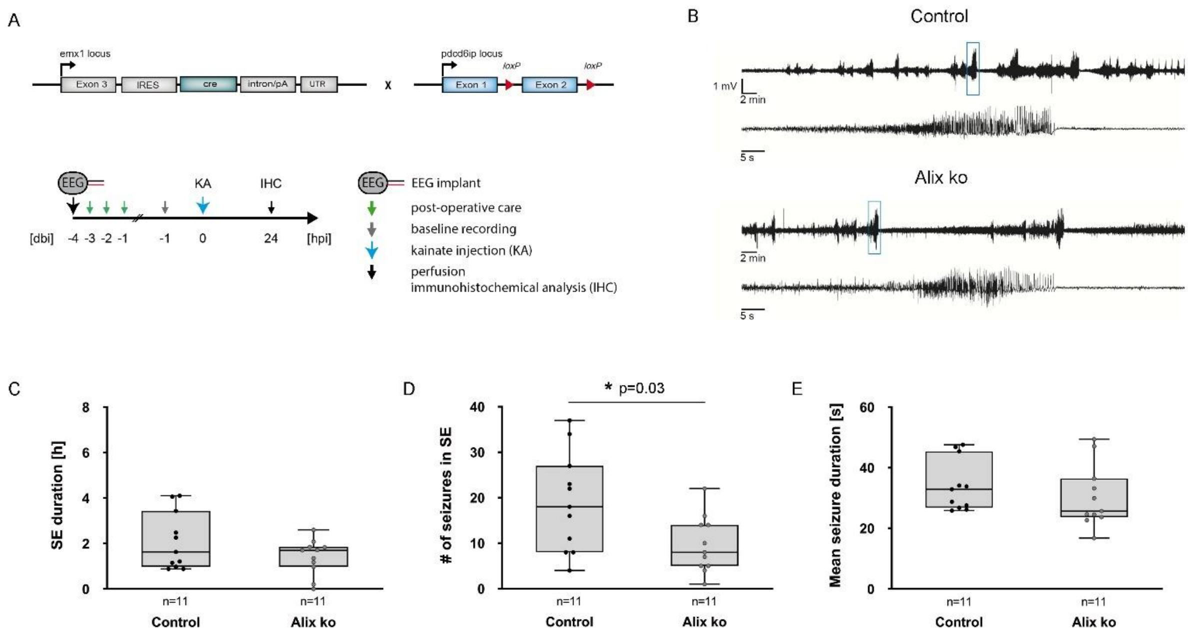

Figure 7 Alix ko mice develop less seizures during status epilepticus. A) Emx1 ^{IREScre} (Emx-Cre) and Alix $^{fl/fl}$ mouse lines were crossbred to delete Alix in neocortical and hippocampal excitatory neurons (Alix ko). Animals were implanted with telemetric EEG transmitters to assess seizure activity. EEG recording started one hour before *status epilepticus* (SE) induction via intracortical kainate (KA) injection and ended 24 hours post KA. Subsequently, the brains were processed for immunohistochemical staining. B) Representative EEG traces from Emx-Cre control and Alix ko animals. C) Total duration of SE did not differ between Alix ko and Emx-Cre control mice (p= 0.49). D) Alix ko animals experience about 66 % less seizures during SE than Emx-Cre controls (*p= 0.03). E) Mean seizure duration during SE was not affected in Alix ko mice compared to Emx-Cre controls (p= 0.19). N (control) = 11 animals, n (Alix ko) = 11 animals. Statistical analysis was performed with the Mann-Whitney test, *p< 0.05.

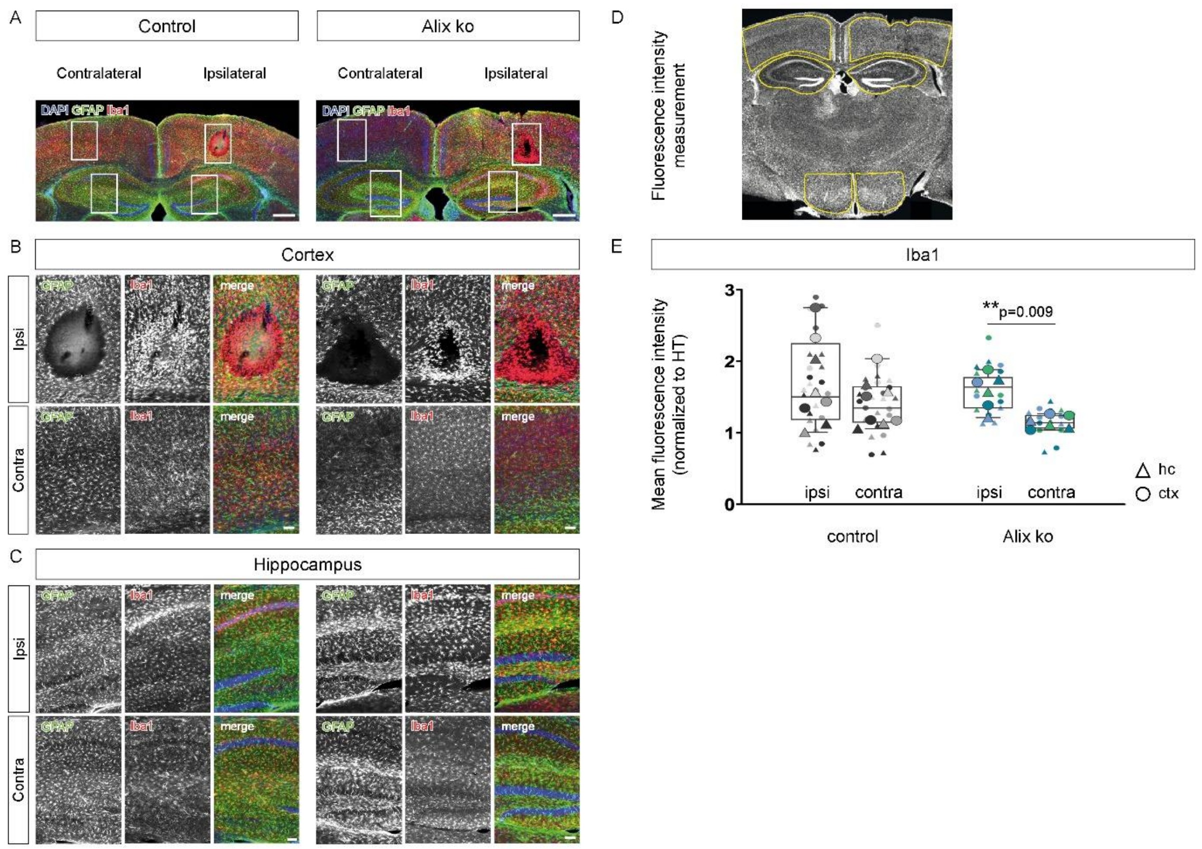

Figure 8 Reduced contralateral activation of microglia in Alix ko mice suggests reduced (contralateral) propagation of epileptiform activity. A) Coronal overviews of brain slices 24Scale bar= 500 µm. B) Magnification of highlighted cortical areas in (A) show an increased ipsilateral (ipsi) microglial activation (Iba1) compared to contralateral (contra), which was more pronounced in Alix ko mice. Astroglial reactivity (GFAP) was moderately increased adjacent to the injection site in both groups. Scale bar= 100 µm. C) Magnification of highlighted hippocampal areas in (A). Contralateral hippocampi of Alix ko mice displayed reduced microglial activation (Iba1) relative to the ipsilateral side, which was less apparent in control mice. Astroglial reactivity (GFAP) was comparable in hippocampi of control and Alix ko mice. Scale bar= 100 µm. D) Depiction of fluorescence intensity measurement areas in cortex, hippocampus and hypothalamus. E) In Alix ko mice, the contralateral Iba1 immunoreactivity was about 30 % reduced in comparison to ipsilateral (**p= 0.009), in contrast to control mice (p= 0645). Circles and triangles represent individual quantifications from cortex and hippocampus, respectively. One data point corresponds to the average of three slices from the same animal. Red and black symbols indicate ipsi- and contralateral sides. Fluorescence intensity values were normalized to the hypothalamic area (HT) of the respective hemisphere. N (control) = 4 animals, n (Alix ko) = 3 animals. Statistical analysis was performed on the average value of three slices per animal, with the Mann-Whitney test, *p< 0.05, **p <0.01.