Presynaptic inhibition upon CB1 and mGluR2/3 receptor activation requires ERK/MAPK phosphorylation of Munc18-1

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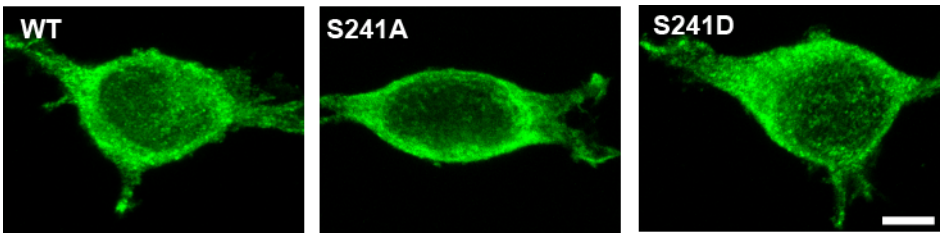
Appendix Supplementary Information

APPENDIX FIGURE S1

Figure S1 related to Figure 5. Munc18$_{S241D}$ localization in HEK cells



M18$_{S241D}$ localization in HEK293T cells is similar to M18$_{WT}$.

Typical example images of HEK293T cells infected with Lenti viruses expressing M18$_{WT}$, M18$_{S241A}$ or M18$_{S241D}$. Cells were stained 2 days after infection for Munc18-1 (green). Scale bar represents 5 μm.

APPENDIX SUPPLEMENTARY METHODS

Antibodies and chemicals

Primary antibodies were used at the following concentrations: Bassoon (Stressgene, 1:500), ERK (p42/p44 MAPK, Cell Signaling, 1:1000), pERK (phospho p42/44, Cell Signaling, 1:1000), Munc18-1 (SySy, 1:1000), pMunc18(S241), phospho Munc18-1 S241 (Phosphosolutions, 1:500), synapsin (SySy, 1:1000), pSynapsin (Millipore, 1:1000), Tubulin (SySy, 1:1000). PD98059 (10 μM), Tetrodotoxin (TTX, 2 μM), BDNF (100 ng/ml), LY379268 (1 μM), WIN55,212-2 (1 and 5 μM) and MG132 (10 μM) were from Sigma. Bicuculline (40 μM), 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM), and (2R)-amino-5-phosphonovaleric acid (D-AP5, 50 μM) from Ascent and U0126 (1 μM) from Tocris.

DNA constructs and Lentivirus production

Different Munc18 variants were generated using Quickchange (Stratagene). We substituted amino acids at potential phosphorylation sites for alanines (A) to prevent phosphorylation or to aspartate (D) to mimic the phosphorylated state of Munc18-1. All DNA constructs were verified by sequencing and subcloned into pLenti vectors, and viral particles were produced as described (Naldini et al, 1996). Viral particles were titrated in HEK 293 (ATCC) cells and equal titers of $M18_{WT,}$ $M18_{S241A}$ and $M18_{S241D}$ were used to infect neurons at DIV0 (for Munc18-1 rescue) or DIV9 (wild-type mouse or rat neurons). For flag-tagged MAPK1 (ERK2) constructs, MAPK1 was amplified by PCR from clone IRAVp968B02100D6 to create restriction sites for subcloning into pCMV3TAG1C plasmid (Invitrogen). The HA-Ubi expression plasmid was a gift from dr. J. de Winter (VU University Amsterdam).

Fear conditioning training

Male 10-week old C57BL/6J mice (Charles River) were divided randomly into 3 groups (no shock control (NS), immediate shock (IS) and delayed shock (DS); n=5 per group). Mice of the NS control group were placed in the conditioning chamber for 212 s without being subjected to an unconditioned stimulus (US; electrical stimulation). Mice receiving an immediate shock were placed in the conditioning chamber with immediate onset of the US (0.7 mA, 2-s duration, constant current) and then remained for another 210 s in the conditioning chamber. For delayed shock, mice were placed in the conditioning chamber for 180 s before the 2-s US and thereafter remained in the conditioning chamber for another 30 s. After 212 s all mice were returned to their home cage for 30 min before being sacrificed by cervical dislocation.

Hippocampi were extracted on ice and immediately frozen to -80°C until further use. On the basis of this temporal training sequence, only DS mice acquire conditioned contextual fear whereas IS mice serve as control for US effects (Stiedl et al, 2004; Wiltgen et al, 2001).

Electrophysiology on autaptic neurons

Hippocampi were dissected in HBBS (Invitrogen) supplemented with 7 mM HEPES and digested with 0.25% trypsin (Invitrogen) at 37 ˚C for 20 min. After trituration with a fire-polished glass pipette, cells were plated at a density of 1000 cells / 4 mm^2 well on top of pre-grown rat glia islands on 18 mm cover slips. Cultures were grown in Neurobasal supplemented with 2% B27, 18 mM HEPES, 0.5 mM Glutamax and penicillin/streptomycin (Invitrogen). Cells were whole-cell voltage clamped at -70 mV using Axopatch 200A. Recordings were performed at DIV14-17 with boro-cillicate glass pipettes (2.5-4 mOhm) containing (in mM): 125 K^+-gluconate, 10 NaCl, 4.6 $MgCl_2$, 4 K_2-ATP, 1 EGTA, 15 Creatine Phosphate, 20 U/ml phosphocreatine kinase (pH 7.30, 300 mOsm). Action potentials were induced by 0.5 ms depolarizing steps to +30 mV. Digidata 1322A and Clampex 9.0 were used for signal acquisition. All recordings were performed at room temperature. Custom made Matlab routines, Clampfit 9.0 and Minianalysis software were used for offline analysis.

Hippocampal slice preparation and electrophysiology

Mouse hippocampal coronal slices (300-350 µm) were prepared from P22-26 male and female C57BL/6 mice. Following decapitation, brains were quickly removed from the skull and placed in ice-cold slicing solution containing (in mM): 125 NaCl, 3 KCl, 1.2 NaH2PO4, 7 MgSO4, 0.5 CaCl2, 26 NaHCO3, and

10 glucose. Coronal slices containing the CA1 region of the dorsal hippocampus were obtained, using a Microm HM 650 V vibratome (Thermo Scientific, MA, USA), then cut in half along the midline and each half transferred into a holding chamber containing aCSF solution with 0.1% DMSO or 10 μM of PD98059 (PD) and allowed to recover for at least an hour at room temperature. aCSF solution contained (in mM): 125 NaCl, 3 KCl, 1.2 NaH2PO4, 1 MgSO4, 2 CaCl, 26 NaHCO3 and 10 glucose (300 mOsm) and was continuously bubbled with carbogen (95% O_2, 5% CO_2) and had an osmolality of ±300 mOsm. Solutions were heated to obtain a bath temperature of 32 ± 1°C using a TC02 heater (Multichannel Systems, Germany). Hippocampal CA1 pyramidal neurons were visually identified using a differential interference contrast microscope (BX51WI; Olympus, Germany) and a VX45 camera (Optronics, CA, USA). They were patched using standard borosilicate glass pipettes (3-5 MOhm tip resistance) filled with intracellular solution containing (mM): 110 K-gluconate; 10 KCl; 10 HEPES; 10 K_2Phosphocreatine; 4 ATP-Mg; 0.4 GTP, pH adjusted with KOH to 7.3; 280-290 mOsm) and kept in whole cell voltage clamp mode using a Multiclamp 700B patch-clamp amplifier (Axon Instruments, CA, USA) and maintained at -70 mV. Recordings were digitized with an Axon Digidata 1440A and acquired using pClamp 10 software (both Axon Instruments); sample rate was 10 kHz and low-pass filtering was set at 2-3 kHz. The whole cell parameters were monitored throughout the experiment and any change over 25% in either the cell's capacitance or the access resistance led to the recording being discarded. EPSCs were evoked using an aCSF filled glass pipette stimulating the CA3 to CA1 Schaffer Collateral with maximal stimulus duration of 50 μs.

For depolarization induced suppression of excitation (DSE) experiments, slices were perfused with aCSF with either 0.1% DMSO or 10 μM of PD98059. In both solutions, 2 μM of SR95531 (Gabazine) was added to block GABA-A mediated inhibitory responses. EPSCs were evoked at 0.2 Hz until an identifiable stable mean amplitude of the response appeared. When 5 successive EPSCs of similar and stable amplitude around this mean had occurred, the cell was depolarized to 0 mV for 10 seconds. Immediately after, EPSCs were evoked again at 0.2 Hz at -70mV and the responses were recorded for an additional 75 seconds (15 EPSCs). This experiment was repeated once after a minimum of 3 minutes post-depolarization, when the EPSCs had re-stabilized in order to obtain 2 DSE experiments from each cell whenever possible. The EPSC amplitude was normalized to the baseline of each individual experiment and averaged over each two experiments to result in one averaged and normalized result per cell.

For experiments evaluating the effect of the CB1 receptor agonist WIN55, 212-2 (WIN), slices were perfused with aCSF with either 0.1% DMSO or 10 μM of PD98059. In both solutions, 2 μM of SR95531 (Gabazine) was added to block GABA-A mediated inhibitory responses, as well as 200 nM of the A1 adenosine receptor antagonist 8-cyclopentyl-1, 3-dipropylxantine (DPCPX). EPSCs were evoked continuously at 0.1 Hz and a minimum of 5 minutes baseline was obtained, before WIN (2 μM) application was started. WIN was applied for a total of 30 min. Cells that did not undergo the entire 30 min of WIN application were discarded. All drugs used in slice electrophysiology were diluted in DMSO. 8-cyclopentyl-1,3-dipropylxantine (DPCPX; 200 nM) was purchased from Tocris Bioscience (UK), SR95531 hydrobromide (Gabazine; 2 μM) was obtained from Hello Bio (UK), PD 98059 (PD; 10 μM)

was obtained from Sigma (NL) and WIN55,212-2 (WIN; 2 μM) from Focus Biomolecules (PA, USA).

Acute brain slices

After decapitation of adult (>8 weeks) wild-type mice, coronal brain slices (300 μm) were sectioned in ice-cold artificial cerebrospinal fluid (ACSF) containing: 110 mM choline chloride, 11.6 mM Na-ascorbate, 7 mM $MgCl_2$, 3.1 mM Na-pyruvate, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose (carboxygenated with 5% CO_2/95% O_2). Slices were allowed to recover for 1 h in ACSF (125 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose; carboxygenated with 5% CO_2/95% O_2) at 37°C before application of the drugs. Drugs were diluted in ACSF and applied for 4 h (unless otherwise stated) at 37°C. Slices were transferred on ice for further biochemical processing.

Brain extracts and fractionations

For preparations of brain lysate to be used for immunoprecipitation, brains were pottered in hypotonic PKB buffer (50 mM Tris pH7.5, 1.5 mM $MgCl_2$, 5.0 mM EDTA, 100 mM NaCl; 2 embryonic brains in 3 ml or 1 adult brain in 6 ml) containing protease inhibitor cocktail according to manufacturer's recommendation (S8820; Sigma). Next, Triton-X100 (final concentration 1%) and NaCl (final concentration 150 mM) were added and samples tumbled for 30min at 4ºC. For whole cell protein level analysis, brains were pottered in PBS (pH 7.40) and subsequently centrifuged for 1min. The obtained pellet was re-suspended in 1x Laemmli Sample Buffer (LSB; containing 2% SDS, 10% glycerol, 0.26M β-mercaptoethanol, 60 mM Tris pH6.8) by vortexing and boiling. DNA was sheared using a syringe needle until sample was liquid. For

preparation of crude synaptosomal fraction (P2; including synaptosomes, mitochondria and myelin), hippocampi were homogenized with an electrical potter (12 strokes at 900 rpm) in homogenization buffer (5 mM HEPES/NaOH pH 7.4, including 350 mM sucrose, protease and phosphatase inhibitor cocktail (S8820; Sigma). To separate cell debris and nuclei, homogenates were pelleted at 1000g for 10 min at 4ºC. Supernatants were pelleted again at 20000g for 30 min at 4ºC to obtain P2 fractions.

For hippocampal P2 fractions after contextual fear conditioning training, the obtained pellets were re-suspended in buffer containing 5 mM HEPES/NaOH, pH 7.4, including 350 mM sucrose, 150 mM NaCl, protease and phosphatase inhibitor cocktail (S8820; Sigma). Protein concentration was determined by Bradford protein assay (BIO-RAD) and 20mg protein per condition was subjected to Western Blot analysis. For slice P2 fractions from drug application experiments, the obtained pellets were re-suspended in 1% deoxycholate (DOC) buffer (150 mM NaCl, 50 mM Tris, pH 8.8, 1% DOC) and lysed for 30 min. Subsequently, an equal volume of modified RIPA buffer was added (150 mM NaCl, 50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.1% SDS) (Kalia et al, 2006). All buffers were supplemented with protease and phosphatase inhibitor cocktail according to manufacturer's recommendation (S8820; Sigma). Protein concentration was determined by Bradford protein assay (BIO-RAD).

Immunoprecipitation and Western Blots

For immunoprecipitations from slice P2 fractions from drug application experiments 500 mg of protein was incubated together with primary antibody and 75 μl of 10% Protein A beads in PBS overnight. The next day, beads

were washed 3 times with a 1:1 mixture of DOC and RIPA buffer. Proteins were eluted by boiling in 2x LSB and analyzed by Western Blotting. For experiments in HEK cells or neuronal cultures, cells were lysed in either LSB (for Western blot or denatured immunoprecipitation, containing 2% SDS, 10% glycerol, 0.26M β-mercaptoethanol, 60 mM Tris pH 6.8) or PKB buffer (for co-immunoprecipitation, containing 50 mM Tris pH7.5, 1% Triton-X100, 1.5 mM $MgCl_2$, 5.0 mM EDTA, 100 mM NaCl). Immunoprecipitations were performed for at least 2 h or overnight at 4ºC using antibodies conjugated to Protein A Agarose beads (Bioconnect). Samples were washed 4 times with buffer, proteins were eluted by boiling in 1x LSB and analyzed by Western blotting.

For Western Blots, samples were loaded into 8-10% SDS-PAGE gels and run on 30 mA per gel until satisfactory mass separation. Proteins were then transferred to PVDF membranes or in case of phospho-proteins to nitrocellulose membrane at 350 mA. Blocking with 2% milk + 0.5% BSA or in case of phospho-proteins 2% BSA for at least 1 h was used to prevent unspecific binding. Primary antibodies were applied 2 h or over night at 4ºC. After substantial washing, alkaline phosphatase labeled secondary antibodies (polyclonal goat anti rabbit (D0487) or polyclonal goat anti mouse (D0486), both 1:1000, DAKO) were applied for 30 min at 4ºC. Blots were then washed and scanned using ECF substrate for Western Blot (GE Healthcare) on a Fujifilm FLA-5000 Reader. All solutions for blocking, staining or washing were prepared in PBS (pH 7.4) containing 0.1% Tween-20 or in case of phospho-proteins in Tris buffered saline (pH 7.4) containing 0.1% Tween-20. Western Blots were stripped using Re-blot Plus Strong Antibody Stripping Solution (Millipore). Results were analyzed using the GelAnalyzer tool in ImageJ (NIH; Bethesda, MD).

In vitro kinase assay

Different Munc18 constructs were transfected into HEK cells using calcium phosphate transfection. Cells were lysed in PKB buffer. After Munc18 immuno-isolation, samples were washed 3 times with PKB buffer and 2 times with kinase buffer (Cell Signaling). To each sample, 20 μl kinase buffer containing (radioactively labeled) ATP and kinase was added and incubated for 30 min at 37ºC. The reaction was stopped by addition of 5x LSB.

Electron Microscopy

Autaptic neurons were fixed at DIV14-16 for 1-2 h at room temperature with 0.1 M cacodylate buffer/0.25 mM $CaCl_2$/0.5 mM $MgCl_2$ (pH 7.4) (de Wit et al, 2006; Wierda et al, 2007). As for electrophysiology and imaging, only glia islands containing a single neuron were used for analysis. After fixation, cells were washed three times for 5 min with 0.1 M cacodylate buffer (pH 7.4), post-fixed for 2 h at room temperature with 1% osmium tetroxide/1% rutheniumcyanide in bidest, washed and stained with 1% uranyl acetate for 15 min in the dark. Following dehydration through a series of increasing ethanol concentrations, cells were embedded in Epon and polymerized for 24 h at 60°C. After polymerization of the Epon, the coverslip was removed by alternately dipping in liquid nitrogen and hot water. Cells of interest were selected by observing the flat Epon embedded cell monolayer under the light microscope, and mounted on pre-polymerized Epon blocks for thin sectioning. Ultrathin sections (~90 nm) were cut parallel to the cell monolayer and collected on single-slot, formvar-coated copper grids, and stained in uranyl acetate and lead citrate (Leica ultrastainer). Autaptic synapses were selected at low magnification using a JEOL 1010 electron microscope. All analyses

were performed on single ultrathin sections of randomly selected synapses. The distribution of synaptic vesicles, total synaptic vesicle number and active zone length were measured on digital images of synapses taken at 100.000x magnification using a custom-made Matlab routine. The observer was blinded for the genotype. For all morphological analyses we selected only synapses with intact synaptic plasma membranes with a recognizable pre- and postsynaptic density and clear synaptic vesicle membranes. Docked synaptic vesicles had a distance of 0 nm from the synaptic vesicle membrane to the active zone membrane. The active zone membrane was recognized as a specialized part of the presynaptic plasma membrane that contained a clear presynaptic density.

Mass spectrometry

For mass spectrometry of Munc18 interactors, brains were harvested from adult rats and P2 fractions were isolated as described (Li et al, 2007) and incubated with Munc18-1 antibodies for 2 h at 4ºC and washed with low salt wash buffer for three times. For protein elution, the beads were boiled in SDS sample buffer, separated, and supernatants were run on a 4-12% SDSPAGE gels. The gel was stained with the Colloidal Blue staining kit (Invitrogen). For mass spectrometry analysis, 1D SDS-PAGE gel lanes were cut into 2-mm slices and subjected to in gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with 5 μg trypsin (Promega, sequencing grade). Samples were separated in the first dimension by a polysulfoethyl A strong cation exchange column (PolyLC), and the second dimension on an analytical capillary C18 column (150 mM × 100 μm i.d. column). The eluate from the C18 column was mixed with matrix (7 mg α-cyano-hydroxycinnaminic acid in

1 ml 50% acetonitril, 0.1% trifluoroacetic acid, 10 mM dicitrate ammonium), delivered at 1.5 µl/min and deposited onto an Applied Biosystems matrix-assisted laser desorption ionization plate by means of a robot (Dionex) once every 15 s for a total of 384 spots.

MALDI plate analysis was performed on a 4800 Proteomics Analyzer (Applied Biosystems, Forster City, CA). Peptide collision-induced dissociation was performed at 1 kV with nitrogen collision gas. Tandem MS (MS/MS) spectra were collected from 5000 laser shots. Peptides with a signal to noise ratio over 50 at the MS mode were selected for MS/MS, at a maximum of 30 MS/MS per spot. The precursor mass window was set to a relative resolution of 180. Peaklists were extracted using GPS software (AB Sciex, version 3.6). MS/MS spectra search was performed against the mouse SwissProt (release 7 February 2007; ~15,000 sequences) and NCBInr (release October 2007; ~150,000 sequences) databases using Mascot (version 2.2, Matrix Science) and GPS Explorer (version 3.6, Applied Biosystems) software.

Supplemental Data References

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