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M-current inhibition in hippocampal excitatory neurons triggers intrinsic and synaptic homeostatic responses at different temporal scales

Abbreviated title: Distinct homeostatic responses at different timescales

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Abstract

Persistent alterations in neuronal activity elicit homeostatic plastic changes in synaptic transmission and/or intrinsic excitability. However, it is unknown whether these homeostatic processes operate in concert or at different temporal scales to maintain network activity around a set-point value. Here we show that chronic neuronal hyperactivity, induced by M-channel inhibition, triggered intrinsic and synaptic homeostatic plasticity at different timescales in cultured hippocampal pyramidal neurons from mice of either sex. Homeostatic changes of intrinsic excitability occurred at a fast timescale (1-4 hours) and depended on ongoing spiking activity. This fast intrinsic adaptation included plastic changes in the threshold current and a distal relocation of FGF14, a protein physically bridging Nav1.6 and Kv7.2 channels along the axon initial segment. In contrast, synaptic adaptations occurred at a slower timescale (≈ 2 days) and involved decreases in mEPSC amplitude. To examine how these temporally distinct homeostatic responses influenced hippocampal network activity, we quantified the rate of spontaneous spiking measured by multielectrode arrays at extended timescales. M-channel blockade triggered slow homeostatic renormalization of the mean firing rate (MFR), concomitantly accompanied by a slow synaptic adaptation. Thus, the fast intrinsic adaptation of excitatory neurons is not sufficient to account for the homeostatic normalization of the MFR. In striking contrast, homeostatic adaptations of intrinsic excitability and spontaneous MFR failed in hippocampal GABAergic inhibitory neurons, which remained hyperexcitable following chronic M-channel blockage. Our results indicate that a single perturbation such as M-channel inhibition triggers multiple homeostatic mechanisms that operate at different timescales to maintain network mean firing rate.
Significance Statement

Persistent alterations in synaptic input elicit homeostatic plastic changes in neuronal activity. Here we show that chronic neuronal hyperexcitability, induced by M-type potassium channel inhibition, triggered intrinsic and synaptic homeostatic plasticity at different time scales in hippocampal excitatory neurons. The data indicate that the fast adaptation of intrinsic excitability depends on ongoing spiking activity but is not sufficient to provide homeostasis of the mean firing rate. Our results show that a single perturbation such as M-channel inhibition can trigger multiple homeostatic processes that operate at different time scales to maintain network mean firing rate.
Preserving the delicate balance between stability and adaptability, neurons use various powerful mechanisms to stabilize firing rates at defined set-point value in response to bidirectional perturbations of the network activity (Turrigiano et al., 1998; Abbott and Nelson, 2000; Burrone et al., 2002; Desai et al., 2002; Turrigiano and Nelson, 2004; Davis, 2006; Marder and Goaillard, 2006; Turrigiano, 2011; Slomowitz et al., 2015; Styr and Slutsky, 2018). These homeostatic changes include synaptic adaptations and/or changes in intrinsic neuronal excitability. The homeostatic regulation of synaptic function includes modifications of synaptic strength, excitation-inhibition (E/I) balance, synapse number and structure (Turrigiano, 2012; Yin and Yuan, 2014; Wefelmeyer et al., 2016). The homeostatic regulation of intrinsic excitability involves alterations in ion channels expression and in the structural organization of the axon initial segment (AIS) (Turrigiano, 2011, 2012; Yoshimura and Rasband, 2014; Kole and Brette, 2018).

Little is known about the interaction between the synaptic and intrinsic homeostatic plasticity. The occurrence of intrinsic and synaptic adaptations were in some cases dependent on the developmental stage, brain region and type of neurons. For example, in the primary sensory cortex, visual deprivation during the pre-critical period induced synaptic upscaling of excitatory synapses onto layer 4 pyramidal neurons and reduction of inhibition, but no changes in intrinsic excitability (Desai et al., 2002; Maffei et al., 2004). In contrast, lid suture during the classical critical period triggered intrinsic, but not synaptic homeostatic plasticity in the layer 2/3 pyramidal neurons (Desai et al., 2002; Goel and Lee, 2007; Maffei and Turrigiano, 2008). In the hippocampus, long-term whisker deprivation triggered synaptic and intrinsic adaptations during early development, while only intrinsic excitability was affected in adult mice (Milshtein-Parush et al., 2017). In primary neuronal cultures, chronic inactivity triggered both, synaptic and intrinsic adaptive responses (Desai, 2003; Slomowitz et al., 2015; Joseph and Turrigiano, 2017). Interestingly, in hippocampal organotypic slices, changes in...
intrinsic excitability preceded those in synaptic strength (Karmarkar and Buonomano, 2006). More recently, it was shown in cultured cortical neurons that excitatory synaptic scaling and intrinsic excitability are tightly coordinated through bidirectional changes in the same signaling pathway involving Ca\textsuperscript{2+}-calmodulin-dependent protein kinase type IV (CaMKIV) (Joseph and Turrigiano, 2017).

Despite the compelling evidence for the existence of various synaptic and intrinsic homeostatic plasticity mechanisms, crucial questions remain unaddressed. When both intrinsic and synaptic adaptations occur, do they operate at similar timescales? Do intrinsic adaptations require ongoing spiking activity, or are they spike-independent? Are inhibitory neurons undergoing similar homeostatic plasticity changes as excitatory neurons following a similar hyperexcitability trigger? To address these questions, we examined the intrinsic and synaptic homeostatic adaptations induced by chronic M-channel blockade in cultured mouse hippocampal neurons using whole-cell patch-clamp electrophysiology and microelectrode arrays (MEAs) for extracellular spike recordings, allowing the same neurons to be recorded over long timescales (Slomowitz et al., 2015). Here, we show that chronic M-channel inhibition by the specific blocker XE991 triggered a fast, activity-dependent homeostatic intrinsic adaptation and a slow synaptic homeostatic plasticity in pyramidal excitatory neurons. Using an adeno-associated (AAV)-viral vector driving the expression of the fluorescent protein mCherry under the control of the specific GABAergic hDlx promoter (Dimidschstein et al., 2016), patch-clamp recording of inhibitory hippocampal neurons showed that following chronic M-channel block, no homeostatic plasticity of intrinsic excitability and spontaneous mean firing rate (MFR) was observed. In sharp contrast to excitatory neurons, hippocampal GABAergic neurons remained hyperexcitable. We conclude that the fast intrinsic adaptation of excitatory neurons is not sufficient to account for the homeostatic normalization of the MFR and that the same perturbation such as M-channel inhibition can
trigger multiple homeostatic mechanisms that operate at different timescales to maintain network activity.
Materials and Methods

Animals

Balb/c mice of either sex were used for generating the primary cultures of hippocampal neurons. All experimental protocols conformed to the guidelines of the Institutional Animal Care and Use Committee of Tel-Aviv University, Israel, and to the guidelines of the NIH (animal welfare authorization number 01-16-012).

Drugs

XE991 dihydrochloride (Tocris; Cat. No.2000/10), 1(S),9(R)-(−)-Bicuculline methiodide (Sigma; Cat.No: 14343), picrotoxin (Sigma; Cat.No. P1675), tetrodotoxin citrate (Alomone; Cat.No.T-550), NBQX hydrate (Sigma; Cat. No.N171), AP5-DL-2-Amino-5-phosphonopentanoic acid (Sigma; Cat.No: A5282) and QX314Br (Alomone).

Recombinant AAV-Dlx-mCherry plasmid and infection

Recombinant AAV-virus-Dlx-mCherry plasmid was prepared by inserting the hDlx promoter sequence (541 bp) upstream the coding sequence in the backbone of the pAAV2-mCherry plasmid. Sequence identity was verified by routine DNA sequencing. The Dlx promoter was shown to restrict reporter expression in vivo to all GABAergic interneurons in the forebrain, including hippocampus, as well as in cultured neurons in vitro (Dimidschstein et al., 2016). The Recombinant AAV2-virus-Dlx-mCherry was produced using standard production methods in HEK 293 cells. All batches produced were in the range of $10^9$ to $10^{10}$ viral particles per ml. Infections of hippocampal cultures were performed at 9 DIV and recording were carried out at 14-16 DIV.

Primary cultures of hippocampal neurons
Hippocampi were dissected out from neonate Balb/c mice brains of either sex (0-1 days old). Hippocampi were washed three times in a HBSS-based solution containing: 4 mM NaHCO3, 5 mM HEPES and Hank’s balanced salt solution (Sigma), pH adjusted to 7.3-7.4 at 4 ºC. Tissues were digested in a solution including: 137 mM NaCl, 5 mM KCl, 7 mM Na2HPO4, 25 mM HEPES, 4.45mg/ml trypsin type XI (Sigma) and 1614 U/ml DNase type IV (Sigma), pH adjusted to 7.2 at 4 ºC. Hippocampal tissues were incubated 15 minutes in 37 ºC and washed again once with 5ml HBSS/20% FBS (fetal bovine serum) and once with HBSS. The cells were dissociated in a HBSS solution including, 13.15 mM MgSO4 and 1772 U/ml DNase type IV (Sigma). Next, the cells were mechanically triturated with fire-polished Pasteur pipettes. HBSS/20% FBS was added to the dissociated cells and the mixture was centrifuged at 1000 x g, at 4 ºC for 10 minutes. The supernatant was discarded and a plating medium including MEM (Gibco), 24.7 mM glucose, 0.089mg/ml transferrin (Calbiochem), glutamax (Sigma), 0.75U/ml insulin (Sigma), 10 % FBS (Biological Industries) and B-27 (Gibco) was added to the pellet. The cells were resuspended in the plating medium with fire-polished Pasteur pipette and drops were added to glass coverslips coated with Matrigel in a 24-wells plate. After one hour of incubation at 37 ºC, the plating medium was added to the wells. The day after plating and twice a week, half of the medium was removed from the wells and replaced with the same volume of a feeding medium (MEM, 26.92mM glucose, 0.097 mg/ml transferrin, glutamax, B-27 and 3µM Ara-C (Sigma)).

**Acute hippocampal slices**

Three weeks old male Balb/c mice were anesthetized, decapitated and the brains were removed out, using procedures approved by the guidelines of the Institutional Animal Care and Use Committee of Tel-Aviv University, Israel. Horizontal slices (400 µM) were cut with a Vibratome (VT1200, Leica) in an oxygenated solution at 4 ºC containing: 1.25 mM NaH2PO4, 2.5 mM KCl, 7 mM MgCl2, 25 mM
NaHCO₃, 25 mM glucose, 87 mM NaCl, 75 mM sucrose and 0.5 mM CaCl₂. The slices were transferred to an oxygenated ACSF solution at 37 ºC for 45 minutes and then kept at room temperature.

**Patch-clamp electrophysiology**

Patch clamp was performed in the whole-cell configuration. Signals were filtered at 2 kHz and digitized at 10 kHz for mEPSCs or 5kHz for the other recordings. Signals were amplified using Axopatch 200B (Molecular Devices) for whole-cell patch recordings of cultured hippocampal neurons and Axopatch 700B for hippocampal slices recordings. In the experiments of chronic XE991 exposure involving recordings at different time points (1-48 hrs), untreated cells were also recorded to survey any possible variability, but no particular variance was observed in control untreated cells within the recording time window (1-48 hrs). For recordings in the current-clamp configuration, the extracellular solution contained 160 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM glucose, 1.2 mM Mg²⁺ and 1.8 mM Ca²⁺ (pH was adjusted to 7.3 with NaOH). Microelectrodes with resistances of 5–8 MΩ were pulled from borosilicate glass capillaries (Harvard Apparatus) and filled with an intracellular solution.

The intracellular solution contained: 135 mM KCl, 1 mM KATP, 1 mM MgATP, 2 mM EGTA, 1.1 mM CaCl₂, 10 mM HEPES and 5 mM glucose (pH adjusted to 7.25 with KOH). For the experiments inspecting the threshold current and the RMP of dissociated neurons, synaptic blockers were added to the extracellular solution to prevent spontaneous spikes: 30 µM picrotoxin, 10 µM Bicuculline, 10 µM NBQX and 10 µM AP5. For recording of sEPSCs and sIPSCs, the same extracellular solution was used and contained, respectively, either 30 µM picrotoxin + 10 µM Bicuculline or 10 µM NBQX + 10 µM AP5. The intracellular solution contained: 0.4 mM GTP, 4 mM Na2ATP, 127 mM CsOH, 127 mM D-gluconic acid, 4 mM CsCl, 10 mM HEPES, 8 mM NaCl and 0.4 mM EGTA (pH adjusted to 7.25 with CsOH). 5 mM QX314Br was added to the intracellular solution prior to recordings. To isolate sEPSCs and sIPSCs on the same cell, the voltage was held on the reversal potential of GABAA (-45 mV) and AMPA (+5 mV) receptors, respectively. The reversal potentials were calculated and experimentally
verified with the recordings solutions prior to the experiments with XE991. Liquid junction potential was calculated and substracted from the recorded voltage (+5 mV and +17 mV were substracted, respectively, for recordings of cultured hippocampal neurons and of CA1 pyramidal neurons from slices). The reversal potential of GABAA was notably depolarized because of the intracellular addition of bromide ions together with QX314 as GABAA was shown to be permeable to Br-. For mEPSCs recordings, the extracellular solution contained 160 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM glucose and 2 mM CaCl2 (pH adjusted to 7.3 with NaOH). 1µM tetrodotoxin, 30 µM picrotoxin and 10µM bicuculline were added to the extracellular solution prior to the experiments. The intracellular solution contained 125 mM CsMeSO3, 15 mM CsCl, 10 mM HEPES, 0.5 mM CaCl2, 3 mM MgCl2, 3 mM Na4BAPTA and 2 mM Na2ATP (pH adjusted to 7.2 with CsOH). mEPSCs recordings were performed while holding the voltage at -70 mV and the patch-pipettes were coated with Sylgard (Dow Corning). Recordings from hippocampal slices were performed in ACSF: 125 mM NaCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 3 mM KCl, 10 mM glucose, 2 mM MgCl2 and 2 mM CaCl2. Intracellular solution for slices recording contained: 145 mM K-Gluconate, 2 mM MgCl2, 0.5 mM EGTA, 2 mM ATP-Tris, 0.2 mM Na2GTP and 10 mM HEPES. CA1 pyramidal neurons from acute slices do not fire spontaneously in these conditions, therefore no synaptic blockers were added to the extracellular solution. For prolonged recordings, only patch-clamp cells with access resistance of less than 30 MΩ and that did not variate by more than 10 % throughout the experiment were recorded. All the electrophysiological experiments were performed at room temperature.

**Immunostaining**

XE991 was added to hippocampal neurons from primary culture for 4 or 48 hours prior staining, meaning that the cells (control and treated) were at the same age when fixed for immunostaining (16 DIV). For staining of hippocampal cultures, neurons were fixed in 4% paraformaldehyde for 10 minutes and washed three times in PBS. Permeabilization of the membrane was performed by adding
0.1% Triton X-100 (Sigma) in a blocking solution (PBS with 0.1% BSA and 5% goat serum) for 4 minutes. After washing once with PBS, blocking solution was added to the coverslips for 10 minutes. The primary antibodies were added to the neurons during one hour at room temperature. The coverslips were washed twice with PBS for 10 minutes and incubated at room temperature during one hour with the secondary antibodies. After washing twice with PBS, the coverslips were mounted in Fluoromount (Sigma). The primary antibodies used for immunostaining were: mouse α-FGF14 (1:500, Neuromab, clone N56/21. Cat.No. 75096, lot:413-8RR-61), rabbit α-MAP2 (1:1000, Millipore. Cat.No. AB5622, lot: 2795016), rabbit α-VGAT (1:500, Synaptic Systems, Cat.No. 131002). The secondary antibodies used against the primary antibodies were: goat α-mouse Alexa488 (1:1000, Invitrogen-ThermoFisher, RRID AB_2536161), donkey α-rabbit Cy3 (1:500, Jackson, Cat.No.711-165-152, lot:143460), and goat α-mouse Cy3 (1:500, Jackson, Cat.No.115-165-146, lot:98592).

Multiple electrode array recordings and analysis

Cultures were plated on MEA plates containing 120 TiN recording and 4 internal reference electrodes (Multi Channel Systems [MCS], Germany). Electrodes are 30 μm in diameter and spaced 200 μm apart. Data were acquired using a MEA2100-Standard amplifier (MCS) with a sampling rate of 10 kHz per electrode. MEA recordings were previously shown to stay stable during 48 hours under control conditions (Slomowitz et al., 2015). Recordings were carried out under constant 37°C and 5% CO2 conditions, identical to incubator conditions. XE991 was added on the MEA plates for up to 48 hours. Control recordings were performed for 3 hours prior to addition of the blocker. The baseline for analyses was defined as the mean of the 3 time-points prior to XE991 addition. Percentage of the baseline was calculated and plotted for each hour during XE991 exposure. Spike sorting was performed offline on the recordings of the first 20 minutes from each hour, as it was shown previously that it represents well the full data (see, (Slomowitz et al., 2015). In brief, the raw data was filtered offline at 200 Hz using a Butterworth high-pass filter. Spikes cutouts were then detected offline using MC Rack.
software (MCS) based on a fixed threshold set to between 5-6 standard deviations from noise levels.

Spike cutouts were then transferred to Offline Sorter (Plexon Inc., Dallas, Texas, USA) for spike sorting. Spikes were plotted in 2-D or 3-D principal component (PC) space and unit clusters were semi-automatically detected using K-means clustering algorithm followed by template sorting. Clusters were then manually inspected to ensure stability throughout the experiment. Only clusters that fulfilled the following requirements were considered units and used for analysis: (1) There was no spiking during the absolute refractory period. (2) The clusters were well defined relative to other clusters from the same electrode throughout the entire experiment. (3) There were no sudden jumps in cluster location on PC axes. (4) The cluster is not centered on the origin of the PC axes. Analyses of MFR, were performed using custom-written scripts in MATLAB (Mathworks, Natick, Massachusetts, USA). All the parameters were calculated by averaging the mean firing rates of all units for a given time-point, per experiment. The eight experiments were then pooled and the averages were plotted on the graphs. Unless otherwise noticed, we opted for this form of presentation, which gives equal weight to each repeat of the experiment. We nevertheless observed similar results when we pooled all the units without discerning between experiments. For MFR, similar results were observed with the unsorted data and after spike sorting.

Data analysis and statistics

For all experiments, data were collected from at least three different batches or more. Control cells were collected from each batch to minimize possible variations between batches. All graphs were built with Prism 8.0 (GraphPad). Error bars represent SEM. In acute XE991 treatments that were carried out in the same neuron, statistical comparisons between untreated and treated cells were performed with two-tailed paired t test. In chronic treatments that were carried out in two independent groups of cells, statistical comparisons were performed with two-tailed unpaired t-test. When treatments involved more than two independent groups of cells without matching between measures, statistical comparisons were
performed with one-way ANOVA and Post-hoc Tukey’s Multiple Comparison Test. For F-I curves, when treatments involved more than two independent groups of cells with matching between measures, Data were analyzed with Bonferonni multiple comparison test, using SPSS. In acute XE991 treatments that were done on the same cell, statistical comparisons were performed with two-tailed paired test. In chronic treatments that were performed in two independent groups of cells, statistical comparisons were performed with two-tailed unpaired test. Multiple comparisons were corrected with Bonferonni correction. Analyses of patch-clamp recordings were performed with Clampfit 10.4 (Molecular Devices). To analyze the ADP size, we measured the ADP area. The ADP area was measured as the area under the signal confined between the lowest point separating the spike that precedes the ADP and the ADP itself until the return of the signal to RMP. For sEPSCs and sIPSCs analysis, the baseline of the traces was manually adjusted and the area under (for sEPSCs) or over (for sIPSCs) the baseline was defined as the charge transfer. For mEPSCs analysis, a template was created for each cell based on the shape of several mEPSCs merged together and events were detected based of this template. The data taken to plot the cumulative probability of mEPSCs consists of 100 mEPSCs randomly selected for each cell and pooled for each condition. Images from immunostaining experiments were obtained using confocal microscopy (Leica TCS SP5) with oil-immersion objectives of 63x. Fields containing hippocampal neurons from primary cultures were indiscriminately selected. Quantifications were achieved with ImageJ (NIH). Distances from the soma to the AIS were measured as described previously (Lezmy et al., 2017). For each experiment, the images were split in two groups and the measurements were performed in blind independently by two different individuals. The results were compared before pooling the data together. Images were converted to TIFF files and imported into MATLAB for analysis, using a self-written algorithm. Semi-automatically, the algorithm recognizes the soma and the axon. At each pixel along the recognized axon, fluorescence intensity is measured. AIS was defined to be between the proximal and distal points along the axons, in which the
fluorescence intensity was above 50% of the maximum. The AIS’s length and distance from the soma are calculated and exported to a Microsoft Excel file.

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Results

M-channel inhibition induces fast adaptive changes in intrinsic excitability of pyramidal excitatory neurons that depend on ongoing spiking activity

Recently, we have shown that sustained M-channel blockade by XE991 concomitantly triggers a fast (1-4h) adaptation of intrinsic excitability and a distal relocation of Kv7.3 and Nav channels along the AIS in cultured hippocampal neurons and in CA1 pyramidal neurons of acute hippocampal slices (Lezmy et al., 2017). Here, we asked whether this fast homeostatic adaptation of intrinsic excitability depends on ongoing spiking activity. To address this question, we exposed hippocampal neurons to the M-channel blocker XE991 (10 µM), in the presence of 1 µM tetrodotoxin (TTX), the Na⁺ channel blocker, to suppress spontaneous firing for 4 hours, the time necessary for intrinsic homeostatic plasticity to occur. Whole-cell patch-clamp recordings from pyramidal-like excitatory neurons of hippocampal cultures (14 to 16 days in vitro [DIV]) show that acute XE991 (10 µM) exposure induced neuronal hyperexcitability, as reflected by a significant decrease in the threshold current (n = 22, control: 514 ± 40 pA, acute XE991: 350 ± 31 pA; one-way ANOVA, ***P<0.000, F (4, 112) = 14.65; Fig. 1A,C) and a depolarized resting membrane potential (RMP) (n = 22, control: -60.2 ± 1.0 mV, acute XE991: -55.2 ± 1.6 mV) (Lezmy et al., 2017). Following four hours incubation with XE991, the threshold current and the RMP returned to values similar to those of untreated neurons (Fig. 1B,C; one-way ANOVA, Sidak’s multiple comparisons test, control vs 4h XE991, P = 0.4484). This fast adaptation of intrinsic excitability was paralleled by a distal relocation of FGF14, a protein that physically bridges Nav1.6 and Kv7.2 channels at the AIS (Pablo and Pitt, 2017). The distance between the soma and the proximal boundary of the FGF14 immunoreactive signal increased significantly and reached steady values after 4 hours of XE991 exposure (untreated: 5.96 ± 0.78 µm; n = 69, 4 h XE991: 12.16 ± 0.79 µm; n = 95; one-way ANOVA, F (3, 296) = 16.46, P<0.0001; Fig. 1D,E). No changes in the total length of the FGF14 segment were observed (Fig. 1F). The extent of this distal redistribution...
remained the same following longer XE991 exposure for up to two days (Lezmy et al., 2017). However, when 1 µM TTX was added together with 10 µM XE991 for four hours and washed out just prior to patch-clamp recording, it prevented the intrinsic homeostatic adaptations. Under these conditions, the acute effect of XE991 was preserved (Fig. 1B,C). Thus, 4 hours treatment with XE991+TTX decreased significantly the threshold current (339 ± 14 pA, n=26; one-way ANOVA, Sidak’s multiple comparisons test: control vs 4h XE991+TTX P = 0.0002) compared to 4 hours exposure of XE991 alone (578 ± 24 pA, n=30; control vs 4h XE991, P = 0.4484) or TTX only (521 ± 40 pA, n=17; control vs TTX, P = 0.9126) or to control neurons (514 ± 40 pA, n=22) (Fig. 1C). Similarly, TTX prevented the distal relocation of FGF14 along the AIS (Fig. 1D,E; one-way ANOVA, Sidak’s multiple comparisons, untreated vs 4h XE991+TTX, P = 0.8589). This result indicates that the intrinsic homeostatic plasticity achieved within 4 hours is clearly contingent on ongoing spiking activity.

**M-channel blockade triggers homeostasis of spike afterdepolarization**

M-channels are known to control spike afterdepolarization (ADP) and burst generation in hippocampal CA1 pyramidal neurons (Yue and Yaari, 2004). Inhibition of M-currents allows the spike ADP to grow, which can convert solitary spikes into bursts of action potentials (Chen and Yaari, 2008); therefore, large ADPs cause neurons to fire in bursting mode. Here, in CA1 pyramidal neurons from hippocampal slices, acute M-channel inhibition by XE991 (30 µM) promoted an increase in the spike ADP size (ADP area increased from 582 ± 135 mV*ms to 1223 ± 368 mV*ms; n = 6; paired two-tailed t-test P=0.047) (Fig. 2A-C). The increase in ADP size generated another spike in 50% of the cells. In these neurons, the increase in burst firing upon acute XE991 exposure was nullified following sustained M-channel inhibition, in conjunction with a decrease in ADP size, which returned to the baseline values within three hours of XE991 treatment (ADP area 380 ± 129 mV*ms; n=6) (Fig. 2A-C). In the other neurons, the increase in ADP size was not enough to induce an additional spike, but the
initial increase in ADP size was still followed by a decrease to the baseline ADP size after prolonged XE991 treatment (Fig. 2B). These results suggest that the fast intrinsic homeostatic adaptation triggered by sustained M-channel blockade includes the size of the ADP, which is meaningful for the bursting firing pattern.

**M-channel block triggers a slow compensation of the mean firing rate**

Next, we examined how sustained M-channel inhibition affects the spontaneous ongoing firing rate of hippocampal neurons. Under this experimental setting, we previously showed that M-current blockage by 10 µM XE-991 was maintained at the same level (≈ 70% inhibition) when neurons were chronically exposed to the drug for extended periods of 1–48 h (Lezmy et al., 2017). A significant increase in the ongoing firing rate was found after acute M-channel inhibition (≈ 1.6-fold increase, two tailed paired t-test \( P=0.0012, n=11 \)) and was still observed following 1–4 h of XE991 chronic treatment (≈ 1.5-fold increase, one-way ANOVA, \( P=0.0057, n=26-84 \)) (Fig. 3A-C). At longer XE991 exposure times (24 and 48 h), the ongoing firing rate progressively decreased and returned to the values of untreated neurons (Fig. 3C), reflecting an additional slow homeostatic plasticity process.

To test the time course of homeostatic compensation of the mean firing rate (MFR) to chronic M-channel blockade at the level of neuronal population, we tested the effect of XE991 on spontaneous firing properties of cultured hippocampal neurons grown on a multi-electrode array (MEA) for ~2-3 weeks. Each MEA contains 120 recording electrodes, whereas each electrode is capable of recording the activity of several adjacent neurons (Slomowitz et al., 2015; Styr et al., 2019). Under control conditions, the average firing rates of the hippocampal network showed long-term stability for up to 48 hours (Fig. 3F) (Slomowitz et al., 2015). Spiking network activity was continuously monitored in an incubator chamber during a baseline recording period and for 2 days following application of 10 µM XE991. XE991 application caused an immediate 2.14-fold increase in network MFR (baseline: 103.6 ±
2.9% and acute XE991: 219.9 ± 33.1%. One-way ANOVA, Friedman test with Dunn’s multiple comparisons test. P<0.0002), which remained elevated 2h after XE991 application (2h XE991: 172.3 ± 17.8%. P<0.0021). Then, MFR gradually returned to the baseline values (140.0 ± 13.5 % and 103.5 ± 13.7%, 1 and 2 days after XE991 application, respectively (Fig. 3D,E). These results demonstrate that similarly to other chronic pharmacological perturbations inducing network hyperactivity, such as GABA(A) receptor blockade (Vertkin et al., 2015) and glutamate uptake blockade (Styr et al., 2019), recovery of mean firing rates following M-type potassium channel blockade occurs at a slow timescale of 1-2 days.

**Chronic M-channel inhibition triggers a slow homeostasis of E/I ratio**

To examine the potential alterations in the synaptic output of the network, spontaneous excitatory post-synaptic currents (sEPSCs) and spontaneous inhibitory post-synaptic currents (sIPSCs) were recorded from pyramidal-like excitatory hippocampal neurons by holding the cells successively at the inhibitory and excitatory reversal potentials, respectively (see Methods). Acute XE991 treatment promoted a significant increase in the sEPSCs charge transfer (n = 17, control: 25.0 ± 4.3 pA*s, acute XE991: 43.5 ± 9.5 pA*s; two-tailed paired t test: *P = 0.043) (Fig. 4A,B) but did not have a significant effect on sIPSCs (Fig. 4C,D). Therefore, the excitatory/inhibitory (E/I) ratio was significantly increased by acute XE991 exposure (n = 17; control: 0.35 ± 0.06, acute XE991: 0.77 ± 0.15; two-tailed paired t test: *P = 0.0214) (Fig. 4E). After four hours of M-channel inhibition, the sEPSCs charge transfer was still higher than that of untreated neurons, then following 24 and 48 hours of XE991 exposure, the sEPSCs charge transfer returned to values similar to those of untreated neurons (Fig. 4B; one-way ANOVA: *P = 0.03, F (3, 193) = 3.044; post-hoc Tukey’s Multiple Comparison Test: significantly different untreated (n = 68) vs. 4 h XE991 (n = 50)). In contrast, the sIPSCs charge transfer values after 4, 24 and 48 hours of XE991 treatment were similar to those of the control neurons (Fig. 4D). Thus, after four hours of M-channel blockade we still observed an increase in the E/I ratio, similar to that of the acute XE991
exposure (untreated: 0.53 ± 0.04, n = 68; 4 h XE991: 0.74 ± 0.1, n = 50). Following 24 (n = 45) and 48
hours (n = 34) of XE991 treatment, the E/I ratio resumed back to initial values of untreated neurons
(Fig. 4E).

**Chronic M-channel inhibition triggers a slow homeostatic scaling**

To understand the mechanisms by which the MFR and the sEPSCs charge transfer slowly compensate
following chronic M-channel inhibition, we examined the miniature excitatory post-synaptic currents
(mEPSCs) properties. The frequency and amplitude of mEPSCs were not modified by acute XE991
exposure (Fig. 5A,C). Prolonged treatment with XE991 (4, 24, 48 hours) did not alter significantly the
frequency of mEPSCs (Fig. 5B). In contrast, the amplitude of mEPSCs significantly decreased
following 48 hours of M-channel block (Fig. 5D,E; n = 49-68; one-way ANOVA: P = 0.0117, F (3,
219) = 3.752; Tukey's multiple comparisons test, untreated vs 48h XE991, significantly different),
suggesting that slow modifications in excitatory transmission occur in a timescale of days.

**Lack of homeostatic plasticity at the level of intrinsic excitability and spontaneous MFR in
GABAergic inhibitory neurons following chronic XE991 treatment**

So far, our results indicate that the fast (≈ 4h) intrinsic homeostatic plasticity of excitatory neurons is
not sufficient for the homeostatic normalization of the MFR, a slow process that needs two days to
reach the baseline values. To explore the mechanisms underlying this mismatch in timescales, we set
out to examine the intrinsic excitability and the spontaneous mean firing rate of GABAergic inhibitory
neurons in the same hippocampal cultures following acute and extended exposure to the M-channel
blocker XE991 (10 µM). To identify GABAergic neurons, we infected hippocampal cultures with a
recombinant virus derived from an AAV-viral vector driving the expression of the fluorescent protein
mCherry under the control of the specific GABAergic hDlx promoter (Fig. 6A) (Dimidschstein et al.,
2016). The Dlx promoter was shown to restrict reporter expression *in vivo* to all GABAergic
interneurons in the forebrain, including hippocampus, as well as in cultured neurons in vitro (Dimidschstein et al., 2016). Based on this method, we estimate that GABAergic inhibitory neurons represent about 20% of the total neuronal population in our cultures. Whole-cell patch-clamp recordings from GABAergic neurons show that acute XE991 (10 µM) exposure induced hyperexcitability, as reflected by a significant decrease in the threshold current (Fig. 6B,C; n = 27, control: 480 ± 20 pA, acute XE991: 369 ± 17 pA; one-way ANOVA, Tukey’s multiple comparisons test: P=0.0013, F (2, 69) = 7.324). Following four hours incubation with XE991, the threshold current was not significantly different from that of the acute treatment (Fig. 6B,C; n = 18, 4 h XE991: 431 ± 31 pA; one-way ANOVA: P = 0.1441). Although the threshold current increased, it did not return to the values of untreated neurons. F-I curve experiments in GABAergic neurons confirmed this trend where spike discharges were evoked by incremental depolarizing current injections for 400 ms (Fig. 6D,E).

Acute XE991 (10 µM) exposure significantly increased the frequency of evoked spike discharge (n = 26; Bonferroni multiple comparison test, control vs acute: 250 pA, *P = 0.0036, t=3.209, df=25; 300 pA, **P = 0.0042, t=3.151, df=25; 350 pA, *P = 0.0168, t=2.562, df=25; 400 pA, *P = 0.0191, t=2.549 df=20). However, following four hours incubation with XE991, the F-I curve still showed increased frequency of spike discharge and was not significantly different from acute XE991 treatment (Fig. 6D,E; n = 20-26, two-tailed unpaired t test, where multiple comparisons were adjusted with multiple t-test bonferroni correction). For comparison, in pyramidal-like excitatory neurons, acute XE991 exposure significantly increased the frequency of evoked spike discharge but sustained block of M-channels (4h XE991) led to rapid adaptive changes that brought the values back to those of untreated neurons (Fig. 6F,G; n = 47-68; Bonferroni multiple comparison test, control vs. acute: 50 pA, *P = 0.0137 , t=2.985, df=10; 100 pA, *P=0.0166, t=2.863, df=10; 150 pA, **P = 0.0074, t=3.348, df=10; control vs 4h XE991: 50 pA, P = 0.3988, t=0.8470, df=113; 100 pA, P = 0.9400, t=0.07548, df=113; 150 pA, P = 0.6537, t=0.4498, df=112; 200 pA P = 0.5964, t=0.5311, df=113; 250 pA, P = 0.5722,
Along the same line, the lack of fast intrinsic adaptation of GABAergic neurons was paralleled by the lack of a distal relocation of FGF14. Immunocytochemical co-labeling of GABAergic inhibitory neurons by vGAT and FGF14 indicates that following 4h XE991 treatment no significant changes were observed in the distance between the soma and the proximal boundary of the FGF14 immunoreactive signal and in the total length of the FGF14 segment (Fig. 6H-J; n = 90-93; unpaired two-tailed t test: P = 0.7632, t=0.3017, df=181 and P = 0.2049, t=1.272, df=181).

Next, we investigated the spontaneous MFR of GABAergic inhibitory neurons in the same hippocampal cultures following acute and 48 h exposure to the M-channel blocker XE991 (10 µM). A significant increase in the ongoing firing rate was found after acute M-channel inhibition, which was still observed following 48h of XE991 chronic treatment (Fig. 7A-C; n = 24; control: 1.86 ± 0.35 Hz and acute XE991: 3.72 ± 0.41 Hz; one-way ANOVA and Tukey's multiple comparisons test: P=0.0048, F (2, 72) = 5.754; control vs acute: P= 0.0122; control vs 48 h XE991: P= 0.0122). Taken together, these results indicate that in sharp contrast to pyramidal excitatory neurons, GABAergic neurons remained hyperexcitable due to the lack of homeostatic adaptation to M-channel blockade at the level of intrinsic excitability and spontaneous MFR (Figs. 6 and 7).
Discussion

In neural networks, spontaneous firing arises from the complex interplay between synaptic currents and intrinsic neuronal properties. Homeostasis is essential for normal brain function as it can prevent deleterious states of epileptic activity or quiescence by providing negative feedback control. Many lines of evidence indicate that neuronal networks use various types of regulatory mechanisms to achieve homeostasis over a wide range of temporal and spatial scales (Turrigiano and Nelson, 2004; Davis, 2006; Marder and Goaillard, 2006; Turrigiano, 2011; Turrigiano, 2017; Styr and Slutsky, 2018).

Homeostatic synaptic scaling and homeostatic modulation of intrinsic excitability regulate firing rates; however, it is not clear whether these two forms of homeostasis are redundant or are induced in parallel to fulfill distinct functions, or whether these two processes are interacting to achieve complementary tasks.

In the present work, homeostatic plasticity was triggered by chronic M-channel blockade in hippocampal cultures resulting in both intrinsic and synaptic homeostatic adaptations of pyramidal excitatory neurons. Acute M-channel inhibition increases neuronal excitability and causes hippocampal CA1 pyramidal neurons to increase the spike ADP (Brown and Adams, 1980; Halliwell and Adams, 1982; Jentsch, 2000; Shah et al., 2002). Assembled as heterotetramers of Kv7.2 and Kv7.3 subunits, M-channels generate subthreshold, non-inactivating voltage-gated K⁺ currents that play an important role in controlling neuronal excitability (Jentsch, 2000; Shah et al., 2002; Delmas and Brown, 2005). We found that few hours of M-current blockade led to a progressive decrease in intrinsic excitability of hippocampal excitatory neurons (Lezmy et al., 2017). This fast homeostatic adaptation was associated with a distal shift of the spike trigger zone and a distal relocation of FGF14, Na⁺ and Kv7 channels along the AIS (Lezmy et al., 2017). Here, we showed that the fast (1-4 hrs) homeostatic intrinsic adaptation preceded a slow (≈ 2 days) homeostatic process of synaptic downscaling contributing for the compensation of the increased MFR. This was reflected by a decrease in mEPSCs amplitude.
Importantly, TTX co-application with the M-channel blocker for 4 hours was able to prevent intrinsic homeostatic plasticity by precluding the AIS FGF14 distal relocation and by restoring the values of the threshold current, similar to those of neurons acutely exposed to XE991. Thus, ongoing spiking activity is required for the fast adaptive change in intrinsic excitability of hippocampal excitatory neurons.

Concomitant to this process, extended M-channel blockade with XE991 triggered a fast adaptation of the spike ADP. In CA1 pyramidal cells, the persistent Na+ current INaP (French et al., 1990) in the perisomatic region is the predominant inward current generating the spike ADP (Azouz et al., 1996; Su et al., 2001; Yue et al., 2005; Golomb et al., 2006). The depolarizing action of INaP is mainly counteracted by M-channels. The M-current prevents the escalation of the spike ADP into a spike burst (Yue and Yaari, 2004, 2006). Here in CA1 pyramidal neurons of acute hippocampal slices, we showed that the XE991-induced increase in spike ADP size and the trend to fire in burst, adapted within three hours of M-channel block and returned to values of untreated neurons. Our data indicate that the adaptation of the spike ADP (2-3 hours) occurred concomitantly with the fast homeostatic changes in intrinsic excitability (see also, (Lezmy et al., 2017)). The fast timescale of homeostatic intrinsic plasticity described in the present study significantly differs from that observed in cortical neurons following activity deprivation (TTX), which takes longer time (24-48h) to be induced (Desai et al., 1999; Bulow et al., 2019). Although we cannot exclude changes in ion channel conductances, these differences suggest that, depending on the nature of the plasticity trigger, different signaling pathways can be mobilized to produce multiple forms of intrinsic plasticity. In addition, we showed that the ongoing spiking potently affects the adaptive changes in intrinsic excitability. In light of our present data, we speculate that the transient rise in spiking activity of hippocampal excitatory neurons following M-channel block may trigger the fast adaptive changes in intrinsic excitability associated with the distal shift of the spike trigger zone and the distal relocation of Na+ and Kv7 channels along the AIS (Lezmy et al., 2017). In support of this suggestion, a previous study showed that optogenetic
stimulation of cultured hippocampal neurons produced AIS relocation of Ankyrin G immunoreactivity only when the flashes were grouped into bursts (5 flashes at 20 Hz every 5 s), keeping the overall 1 Hz stimulation frequency constant (Grubb and Burrone, 2010). Interestingly, the effects of 1 Hz burst photostimulation were partially blocked by TTX (Grubb and Burrone, 2010). It was therefore suggested that high-frequency bursts of activity in hippocampal excitatory neurons can produce AIS relocation while steady stimulation at the same overall frequency does not (Grubb and Burrone, 2010).

The chronic M-channel inhibition also triggered a slow homeostatic adaptation of the E/I balance by inducing alterations in excitatory transmission. The homeostatic normalization of the E/I balance to chronic M-channel blockade occurred at a timescale of days, mainly due to compensations in sEPSCs rather than alterations in sIPSCs. The initial increase in synaptic excitatory output and its subsequent slow homeostatic adaptation is in line with the rise and the subsequent slow decrease of the MFR. The decrease in mEPSCs amplitude after 48 hours of XE991 exposure suggests that the homeostasis involves slow adaptive changes in excitatory transmission. The paradoxical mismatch of the different plasticity timescales, suggest that the fast (≈ 4h) intrinsic homeostatic plasticity is not sufficient to account for the slow (≈ 2 days) homeostatic normalization of the network MFR. Our results show that in sharp contrast to pyramidal excitatory neurons, homeostatic plasticity of intrinsic excitability and of spontaneous MFR failed in GABAergic neurons of the same hippocampal cultures. Hence, inhibitory neurons remained hyperexcitable following chronic exposure to the M-channel blocker XE991 (Figs. 6 and 7). Our data are in line with an in vivo study in the primary visual cortex showing homeostatic compensation at the level of spontaneous excitatory activity, but not in inhibitory neurons following enucleation (Barnes et al., 2015). Previous work suggests that loss of M-channel function in GABAergic interneurons elevates their excitability (Lawrence et al., 2006), in line with our current results. Importantly, a recent study showed that selective deletion of M-channels (Kv7.2/Kv7.3) in parvalbumin GABAergic neurons leads to a compensatory increase of the fast excitatory
transmission in pyramidal neurons (Soh et al., 2018). In addition, parvalbumin-Kv7.2 null-mice showed increased seizure susceptibility, suggesting that decreases of M-channel activity in inhibitory neurons remolds the excitatory networks (Soh et al., 2018). Importantly, a substantial subpopulation of inhibitory neurons (e.g., VIP interneurons) has long been recognized as a potential disinhibitory circuit motif in the hippocampus. Since the Dlx promoter used to detect inhibitory neurons in our hippocampal culture restricts reporter expression to all types of GABAergic neurons (Dimidschstein et al., 2016), future studies are needed to identify the specific interneuron type lacking homeostatic adaptation.

Taken together, the data of the present study show that M-channel blockade triggers at different temporal scales, synaptic and intrinsic homeostatic plasticity of hippocampal excitatory neurons and that the latter process requires ongoing spiking activity. Although the nature of the interactions between the two plasticity processes is unknown, we speculate that the transient increase in spiking activity of hippocampal pyramidal neurons may trigger the fast adaptive changes in intrinsic excitability. Remarkably, homeostatic plasticity of intrinsic excitability and of MFR failed in hippocampal GABAergic inhibitory neurons in response to M-channel inhibition, indicating that interneurons are vulnerable to this specific activity perturbation. In this context, the persistent hyperexcitability of GABAergic neurons may have a significant impact on network MFR. In all, our results indicate that the fast adaptation of intrinsic excitability in excitatory neurons is not sufficient to normalize the MFR, and that a single perturbation like M-channel inhibition can trigger multiple homeostatic mechanisms, which operate at different timescales to maintain network mean firing rate.
References


Figure Legends

Figure 1: M-channel inhibition triggers fast adaptive changes in intrinsic excitability and AIS

FGF14 distal relocation that tightly depend on ongoing spiking activity. (A, B) Representative traces of solitary spike discharge evoked by 2 ms step injection of depolarizing currents with increments of 25 pA: (A) in control conditions (black) and following acute XE991 exposure (red), (B) in control untreated conditions (black) and following 4 hours exposure to XE991, TTX and TTX+XE991 (red). In each case, the values of the threshold current are shown. (C) Acute XE991 exposure significantly decreased the threshold current (n = 22, control: 514 ± 40 pA, acute XE991: 350 ± 31 pA; one-way ANOVA, F (4, 112) = 14.65, ***P<0.001). The threshold currents of neurons exposed to 4h XE991 (n = 30, 578 ± 24 pA) or to 4h TTX (n = 17, 521 ± 40 pA) were not significantly different from control (one-way ANOVA, Sidak's multiple comparisons test; control vs 4h XE991, P = 0.4484 and control vs 4h TTX, P >0.9999). In contrast, treatment with XE991+TTX for 4 h (n = 26, 339 ± 14 pA) significantly decreased the threshold current compared with that TTX alone or to control (one-way ANOVA, control vs 4h XE991+TTX, P = 0.0002). (D) Representative FGF14 (green) and MAP2 (red) immunostaining label the AIS and the somatodendritic compartment, respectively. The AIS relocated distally away from the soma following 4 hours of exposure to XE991 but TTX prevents this effect. (E) The FGF14 immuno-reactive signal relocated away from soma following 4 hours of exposure to XE991 (untreated: 5.96 ± 0.78 µm; n = 69, 4 h XE991: 12.16 ± 0.79 µm; n = 95; one-way ANOVA, F (3, 296) = 16.46, ****P<0.001). Addition of TTX together with XE991 prevented the distal shift of the AIS. (F) No significant changes in the total length of the FGF14 segment were observed (one-way ANOVA, F (3, 298) = 0.3143, P=0.8151).

Figure 2: Afterdepolarization size readjust faster than the mean firing rate. (A) Representative traces of spikes discharges evoked by 5 ms step injection of depolarizing threshold currents in the same CA1 neuron from hippocampal slice before (black), after 5 minutes (red) and 150 minutes (blue) of exposure.
XE991 exposure. In this neuron, acute XE991 treatment increased the afterdepolarization (ADP) size and generated a doublet, which was reversed to a single spike following extended XE991 treatment. (B) Representative traces of spikes discharges evoked by 5 ms step injection of depolarizing threshold currents in a CA1 neuron in which the ADP size changed without leading to burst generation. The traces were superimposed and the RMPs were aligned to emphasize the initial increase and the adaptation in the ADP size throughout XE991 exposure. (C) Graph gathering the data from six different cells. Acute XE991 treatment significantly increased the ADP size (control: 582 ± 135 mV*ms, acute: 1223 ± 368 mV*ms, two-tailed paired t test, control vs. acute: *P = 0.0474, n = 6, t = 2.614, df = 5) and decreased it back following prolonged exposure (1-3 h: 380 ± 129 mV*ms, two-tailed paired t test, acute vs. 1–3 h: *P = 0.0218, n = 6, t = 3.288, df = 5).

**Figure 3**: The fast homeostatic adaptation in intrinsic excitability is followed by a slow compensation of the mean firing rate. (A) Representative traces showing the larger spontaneous firing rate after acute XE991 treatment (red trace) compared to control (Black trace). (B) Acute XE991 exposure significantly increased the ongoing firing rate by 1.7-fold (two-tailed paired t test: **P = 0.0012, n = 11, t = 4.495, df = 10). (C) With longer exposures to XE991, the spontaneous firing rate progressively decreased and returned to values close to those of untreated neurons [one-way ANOVA: F (4, 207) = 3.749, **P=0.0057, and Dunnett’s multiple comparisons test: significantly different for untreated (n = 84) vs. 1 h (n = 21) and untreated vs. 4 h (n = 39) but not significant for untreated vs. 24 h (n = 37) and untreated vs. 48 h (n = 26)]. (D) Analysis of the mean firing rate (MFR) at the level of network across 8 MEA experiments (238 single units) over the course of two days of recording in the presence of 10 µM XE991. Each recording consisted of 3 hrs of baseline and 48h following XE991 administration. (E) Statistical comparison of the time points shown (the same data as in D). Significant increase in MFR following acute and 2h of XE991 (219.9 ± 33.1% and 172.3 ± 17.8%, respectively) and MFR renormalization following 1d and 2d (140.0 ± 13.5 % and 103.5 ± 13.7%, respectively).
way ANOVA, Friedman test with Dunn’s multiple comparisons test. **P<0.0021, ***P<0.0002, ns-
one significant). (F) Mean firing rate of 48 hr control MEA recordings (average of 6 experiments (440 single units). Error bars represent SEM.

Figure 4: Chronic M-channel inhibition triggers a slow adaptation of E/I balance caused by changes in sEPSCs charge transfer. (A) Representative traces showing larger sEPSCs following acute XE991 exposure (red) compared to control (black). (B) Acute XE991 exposure significantly increased the sEPSCs charge transfer (n = 17, control: 25.1 ± 4.3 pA*s, acute XE991: 43.5 ± 9.5 pA*s, two-tailed paired t test: *P = 0.043, t=2.198, df=16). After four hours of XE991 treatment, the sEPSCs charge transfer was still larger than the control and it returned to values similar to baseline after extended treatments of 24 (n = 45) and 48 hours (n = 34) [one-way ANOVA: *P = 0.03, F (3, 193) = 3.044; post-hoc Tukey's Multiple Comparison Test: significantly different for untreated (n = 68) vs. 4 h (n = 50)]. (C) Representative traces of sIPSCs before (black) and following acute XE991 exposure (red). (D) Acute (two-tailed paired t-test, P = 0.0763, t = 1.895, df = 16) and prolonged XE991 exposures (one-way ANOVA: P = 0.5651, F = 0.6803, df = 3) did not significantly change the sIPSCs charge transfer. (E) E/I ratio was calculated by dividing sEPSCs by sIPSCs charge transfers of the same neurons. Acute XE991 significantly increased the E/I ratio (control: 0.35± 0.06, acute XE991: 0.77 ± 0.15, two-tailed paired t test: *P = 0.0214, t=2.549, df=16). E/I ratio following prolonged XE991 treatments reflected the changes observed on the sEPSCs charge transfer. The E/I ratio increased following four hours of XE991 exposure, while it returned progressively to baseline value after 24 and 48 hours of XE991 treatment (one-way ANOVA: P=0.1835, F (3, 193) = 1.631; n = 45-68).

Figure 5: Chronic M-channel blockage promotes slow post-synaptic modulations. (A-D) Bar charts and cumulative probability plots are shown for the same data in each panel. (A, B) the distribution of mEPSCs inter-event intervals was not significantly different throughout acute (n = 7, two-tailed paired t test: P = 0.3903, t=0.9257, df=6) and extended XE991 exposures (one-way...
ANOVA: \( P = 0.5963, F (3, 219) = 0.6301, n = 49-68 \). (C, D) the distribution of mEPSCs amplitudes was similar during acute M-channel blockade (two-tailed paired t test: \( P = 0.1419, t=1.691, df=6 \)) but was significantly reduced during exposure to XE991 for 48 hours (\( n = 49-68 \); one-way ANOVA: \( *P = 0.0117, F (3, 219) = 3.752 \); post-hoc Tukey's Multiple Comparison Test: significantly different untreated vs. 48 h). (E) Representative traces showing the decrease in mESPCs amplitude following 48 hours of XE991 treatment.

**Figure 6: Lack of homeostatic plasticity at the level of intrinsic excitability in GABAergic inhibitory neurons following chronic XE991 treatment.** (A) red fluorescence identification of GABAergic inhibitory neurons in primary hippocampal cultures (14 DIV) by infection with the recombinant AAV virus derived from the AAV-viral vector driving the expression of the fluorescent protein mCherry under the control of the specific GABAergic mDlx enhancer. (B) Representative traces of solitary spike discharge evoked by 2 ms step injection of depolarizing currents with increments of 25 pA in the presence of synaptic blockers: in control conditions (black trace) and upon acute XE991 exposure (red trace), as well as following 4 hours exposure to XE991 (blue trace). (C) Acute XE991 exposure significantly decreased the threshold current in GABAergic neurons (\( n = 27 \), control: 480 ± 20 pA, acute XE991: 369 ± 17 pA; one-way ANOVA, Tukey's multiple comparisons test: \( P=0.0013, F (2, 69) = 7.324 \)). Following four hours incubation with XE991, the threshold current was not significantly different from that of the acute treatment (\( n = 18 \), 4 h XE991: 431 ± 31 pA; one-way ANOVA: \( P = 0.1441 \)). (D) Representative spike discharge in GABAergic neurons evoked by 400-ms step injection of 250 pA depolarizing current in the presence of synaptic blockers in control conditions (black trace) and upon acute XE991 exposure (red trace), as well as following 4 hours exposure to XE991 (blue trace). (E) F-I curves in GABAergic neurons. Acute XE991 (10 µM) exposure significantly increased the frequency of evoked spike discharge (\( n = 26 \); Bonferroni multiple comparison test, control vs acute: 250 pA, \( **P = 0.0036, t=3.209, df=25 \); 300 pA, \( **P = 0.0042, \).
However, following four hours incubation with XE991, the F-I curve still showed increased frequency of spike discharge and was not significantly different from acute XE991 treatment (n = 20-26, two-tailed unpaired t test, where multiple comparisons were adjusted with multiple t-test bonferroni correction; acute vs. 4h: 150 pA, P = 0.6136, t=0.5088, df=41; 200 pA, P = 0.6117, t=0.5112 df=44; 250 pA, P = 0.6625, t=0.4394, df=44; 300 pA, P = 0.9546, t=0.05730, df=44; 350 pA, P = 0.9136, t=0.1092, df=42; 400 pA, P = 0.3126, t=1.025, df=34; 450 pA, P = 0.5447, t=0.6174 df=18). (F) Representative spike discharge in pyramidal-like excitatory neurons evoked by 400-ms step injection of 150 pA depolarizing current in the presence of synaptic blockers in control conditions (black trace) and upon acute XE991 exposure (red trace), as well as following 4 hours exposure to XE991 (blue trace). (G) F-I curves in pyramidal-like excitatory neurons. Acute XE991 exposure significantly increased the frequency of evoked spike discharge but sustained block of M-channels (4h XE991) led to rapid adaptive changes that brought the values back to those of untreated neurons (n = 47-68; Bonferonni multiple comparison test, control vs. acute: 50 pA, *P = 0.0137 , t=2.985, df=10; 100 pA, *P=0.0166, t=2.863, df=10; 150 pA, **P = 0.0074, t=3.348, df=10; control vs 4h XE991: 50 pA, P = 0.3988, t=0.8470, df=113; 100 pA, P = 0.9400, t=0.07548, df=113; 150 pA, P = 0.6537, t=0.4498, df=112; 200 pA P = 0.5964, t=0.5311, df=113; 250 pA, P = 0.5722, t=0.5665, df=113). (H) Representative AIS FGF14 (green) and vGAT (red) co-immunostaining labels in GABAergic neurons. (I) Following 4h XE991 treatment no changes were observed in the distance between the soma and the proximal boundary of the FGF14 immunoreactive signal (n = 90-93; unpaired two-tailed t test: P = 0.7632, t=0.3017, df=181). (J) Following 4h XE991 treatment no changes were observed in the total length of the FGF14 immunoreactive segment (n = 90-93; unpaired two-tailed t test: P = 0.2049, t=1.272, df=181).

Figure 7: Lack of homeostatic plasticity at the level of spontaneous MFR in GABAergic inhibitory neurons following chronic XE991 treatment. (A) Representative traces showing the larger
spontaneous firing rate after acute XE991 treatment (red trace) compared to control (black trace). (B) Representative traces showing the higher spontaneous firing rate after 48 h XE991 treatment (blue trace) compared to untreated neurons (black trace). (C) A significant increase in the ongoing MFR rate was found following acute M-channel inhibition, which was still observed following 48 h of XE991 chronic treatment (n = 24-27; control: 1.86 ± 0.35 Hz, acute XE991: 3.72 ± 0.41 Hz and 48 h XE991: 3.68 ± 0.50 Hz; one-way ANOVA and Tukey's multiple comparisons test: P=0.0048, F (2, 72) = 5.754; control vs acute: P= 0.0122; control vs 48 h XE991: P= 0.0122).