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The Antidepressant Fluoxetine Mobilizes Vesicles to the Recycling Pool of Rat Hippocampal Synapses During High Activity

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Abstract Effects of the antidepressant fluoxetine in therapeutic concentration on stimulation-dependent synaptic vesicle recycling were examined in cultured rat hippocampal neurons using fluorescence microscopy. Short-term administration of fluoxetine neither inhibited exocytosis nor endocytosis of RRP vesicular membranes. On the contrary, acute application of the drug markedly increased the size of the recycling pool of hippocampal synapses. This increase in recycling pool size was corroborated using the styryl dye FM 1-43, antibody staining with αSyt1-CypHer™5E and overexpression of synaptopHluorin, and was accompanied by an increase in the frequency of miniature postsynaptic currents. Analysis of axonal transport and fluorescence recovery after photobleaching excluded vesicles originating from the synapse-spanning superpool as a source, indicating that these new release-competent vesicles derived from the resting pool. Super resolution microscopy and ultrastructural analysis by electron microscopy revealed that short-term incubation with fluoxetine had no influence on the number of active synapses and synaptic morphology compared to controls. These observations support the idea that therapeutic concentrations of fluoxetine enhance the recycling vesicle pool size and thus the recovery of neurotransmission from exhausting stimuli. The change in the recycling pool size is consistent with the plasticity hypothesis of the pathogenesis of major depressive disorder as stabilization of the vesicle recycling might be responsible for neural outgrowth and plasticity.

Keywords Antidepressant · Fluoxetine · Fluorescence microscopy · Synaptic vesicles · Vesicle pools · Hippocampal neurons

Jasmin Jung and Kristina Loy, as well as Oliver Welzel and Teja W. Groemer contributed equally to this work.

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Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed class of antidepressants in the USA [1]. By preventing the uptake of exocytosed serotonin into the presynaptic terminal, SSRIs increase the reduced monoamine levels in the brain of depressed patients [2, 3]. Recently, the anesthetic ketamine was reported to lead to rapid antidepressant effects in patients who are nonresponders for typical antidepressants. The authors of this study could show that ketamine induced synaptogenesis and an amelioration of synaptic function [4], and thus pointing out the importance of synaptic transmission in major depressive disorder (MDD), resulting in a "synaptogenic hypothesis" of depression [4].

However, on the first look, the acute effects of SSRIs and ketamine do not seem to fit the clinical observation that

patients show an alleviation of their symptoms only after weeks. On the one hand, this delay could be explained by the "pharmacokinetic hypothesis" [5] which proposes a slow accumulation of antidepressants in acidic compartments of brain neurons [6, 7]. According to this hypothesis, therapeutic effects first set in when stable plateau concentrations are reached. On the other hand, the long-term changes could result from persisting acute effects of the drugs that ultimately facilitate the rewiring of neuronal networks. Changes in nerve activity are known to play a role at the formation and modification of synaptic connections [8–10] and at inducing presynaptic alterations [11], e.g., structural changes of the active zone [12] or adjustment of the different vesicle pools [10]. Moreover, it is known that classical antidepressant drugs are able to influence neurotransmission, e.g., by inhibiting exocytosis [13] or strengthening synaptic transmission during exhaustive stimuli [14]. Therefore, antidepressant drugs might be able to create new neuronal connections or strengthen those already existing [14, 15].

Although several studies found that both inhibition and potentiation of synaptic vesicle exocytosis could be evoked by psychotropic drugs [16, 17], the structural basis of these effects has remained elusive so far. In this study, we tested the acute influence of fluoxetine in its clinically relevant dose on synaptic vesicle recycling and plasticity and show that this SSRI mobilizes the resting pool of synaptic vesicles for secretion.

Materials and Methods

Cell Culture and Transfection

Hippocampal neuronal cultures were prepared from 1- to 4-day-old Wistar rats (Charles River, USA) as described [18]. Newborn rats were sacrificed by decapitation in accordance with the guidelines of the State of Bavaria. Hippocampi were removed from the brain and transferred into ice-cold Hank's salt solution, and the dentate gyrus was cut away. After digestion with trypsin (5 mg/ml), cells were triturated mechanically and plated in MEM medium, supplemented with 10 % fetal calf serum and 2 % B27 Supplement (all from Invitrogen, Karlsruhe, Germany). If required, neurons were transfected with synapto-pHluorin [19] or synaptophysin-monomeric red fluorescent protein (mRFP) [20] under control of a synapsin promoter on DIV3 with a modified calcium phosphate method as described [18, 21]. Experiments were performed between 25 and 30 days in vitro. In the latter, "short-term incubation" refers to a time interval of 10 to 20 min. For this incubation, fluoxetine (Sigma-Aldrich, Taufkirchen, Germany) was diluted to the respective concentration in extracellular medium (contents described below).

Imaging

Experiments were conducted at room temperature on a Nikon TI-Eclipse inverted microscope equipped with a ×60, 1.2 NA water immersion objective and Perfect Focus System™. Fluorescent dyes were excited by a Nikon Intensilight C-HGFI through excitation filters centered at 482 and 650 nm using dichroic longpass mirrors (cutoff wavelength 500 and 660 nm). The emitted light passed emission band-pass filters ranging from 500 to 550 nm and 650 to 700 nm (Semrock, Rochester), and was projected onto a cooled EM-CCD camera (iXon^{EM} DU-885 or iXon^{EM} DU-897, Andor, Belfast).

Cover slips were placed into a perfusion chamber (volume=500 μl) filled with extracellular medium containing (in mM): 144 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 2.5 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.5. For spontaneous labeling experiments, 1 μM TTX (Sigma-Aldrich, Taufkirchen, Germany) was added to the medium containing 0 mM Ca^{2+}. Synaptic boutons were stimulated by electric field stimulation (platinum electrodes, 10 mm spacing, 1 ms pulses of 50 mA and alternating polarity); 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris Bioscience) and 50 μM D-amino-5-phosphonovaleric acid (D,L-AP5, Tocris Bioscience) were added to prevent recurrent activity.

Recycled synaptic vesicles were labeled with 2.5 μM FM 1-43 (Invitrogen, Karlsruhe, Germany) as described in the respective experiments. Alternatively recycled synaptic vesicles were labeled using fluorescently tagged (CypHer™5E and Alexa Fluor® 488) antibodies directed to the vesicle protein synaptotagmin1 (αSyt1-CypHer5E and αSyt1-Alexa488; 1:100; Synaptic Systems, Goettingen). In some experiments, synapto-pHluorin-transfected neurons were additionally labeled using a fluorescently tagged antibody directed to the green fluorescent protein (GFP) labeled with CypHer™5E (αGFP-CypHer5E; 1:50; Synaptic Systems, Goettingen). To measure the calcium influx upon electrical stimulation, we incubated the cells with 2.5 μM of the calcium indicator fluo-4, AM (Invitrogen, Karlsruhe, Germany), for 30 min at 37 °C. An automated perfusion system (flow rate 0.2 ml/min, Fast-Step Valve Control Perfusion System VC-77SP8, Warner Instruments, Hamden, CT, USA) was used, e.g., to perfuse the neurons with the respective concentrations of the antidepressant fluoxetine. If required, image stacks were converted into tagged image file format.

Data Analysis

All image analysis was performed using custom-written routines in MATLAB (The MathWorks, Natick, MA). Recorded image stacks were used to automatically define peak regions of interest (ROI) of synaptic bouton size [22], where a stimulation-evoked fluorescence decrease or increase occurred in difference images. The average of each ROI was

calculated for each image to obtain fluorescence intensity profiles for each bouton. The absolute fluorescence change ΔF was calculated as the difference of the mean of at least three values before the onset of the respective stimulation and after the end of the stimulus. Kymographs were generated using the dropdown menu "kymograph" in MetaMorph® 7.5.1 (Molecular Devices, Sunnyvale, CA). Only straight axonal segments were selected for analysis to avoid velocity artifacts originating from form factors such as strong axon covings. Kymographs were analyzed using the Hough transform as described [20]. Graphs were generated using MATLAB (The MathWorks Inc., Natick), Microsoft® Excel 2010 (Microsoft® Corporation, Redmond), or GraphPad Prism® 5.04 (GraphPad Software, San Diego, CA). Error bars and specified values are all standard deviation if not indicated otherwise. Depending on the sample size and on the respective distribution, statistical comparisons were made with two-sample *t* tests, one-sample *t* tests, one-way ANOVAs, Wilcoxon rank sum test, or a Kruskal–Wallis test, respectively. Distributions were compared using a two-sample Kolmogorov–Smirnov test. In all figures, one asterisk indicates $p < 0.05$, two asterisks indicate $p < 0.01$, and three $p < 0.001$.

Electrophysiology

Whole-cell recordings of cultured hippocampal neurons (DIV25 to DIV40) were performed in voltage-clamp mode at room temperature. The miniature postsynaptic currents (mPSC) were recorded at a membrane holding potential of −70 mV. The recordings were filtered at 2 kHz (eight-pole Bessel filter) and sampled at 10 kHz using a Multiclamp 700B amplifier in conjunction with a Digidata 1322A interface and pClamp10 software (all from Molecular Devices/MDS Analytical Technologies, Sunnyvale, CA). Patch pipettes from borosilicate glass (Harvard Apparatus, Edenbridge, UK) were pulled and firepolished using a DMZ-Universal Puller (Zeitz, Munich, Germany), to yield a tip resistance of 2.7–4.8 MΩ. Cell capacitance ranged between 20 and 40 pF and access resistance between 6.5 and 15 MΩ in whole-cell configuration. Series resistance compensation was 50 %. The recordings started 4 min after breaking through the cell membrane. After 5 min of recording, to ensure a stable mPSC frequency, fluoxetine (1 μM solved in extracellular medium) or control solution was applied using a rapid, gravity-driven Y-tube system and mPSCs were recorded for the following 15 min. The intracellular solution contained (mM): 136 potassium gluconate, 4 MgATP, 0.3 Na_2GTP, 1 EGTA, 18 HEPES, 0.6 $MgCl_2$ (pH 7.3; 310 mOsm). The extracellular solution contained (mM): 145 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 HEPES, 10 TEA (pH 7.4; 330 mOsm). The recordings were analyzed offline using custom-written routines in MATLAB (The MathWorks Inc., Natick) based on a scaled template (threshold criterion=4) event detection routine [23].

Fluorescence Spectrophotometry

All fluorometric measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies Deutschland GmbH, Boeblingen). All solutions were measured in quartz cuvettes (sample volume=400 μl) at an excitation wavelength of 473 or 640 nm with excitation and emission slits set at 5 nm. FM®1-43, αSyt1-CypHer5E and αSyt1-Alexa488 were solved in their corresponding concentrations in extracellular medium with pH 7.4 or pH 5.3. For the determination of FM dye emission spectra, samples, including calibration samples, were mixed with 20 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [24, 25] which is a zwitterionic detergent used to increase the absolute fluorescence of FM dyes (Fluka™, Buchs) [25].

Western Blotting

Hippocampal neurons were grown in six-well plates for 20 to 25 days. After an incubation of 20 min in extracellular medium (see above) containing receptor blockers with or without 1 μM fluoxetine, the cells were lysed using 100 μl RIPA buffer (45 mM Tris/HCl, 150 mM NaCl, 1 % NP-40, 0.5 % sodium desoxycholate, 0.5 % SDS) containing protease inhibitor (cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Mannheim, Germany). Protein lysates were denatured and total protein concentration was quantified by BCA assay, then 10 μg of total protein per well was loaded on SDS-PAGE gels (12 % Mini-PROTEAN® TGX™ Precast Gel, Bio-Rad, Munich, Germany). In a semi-dry blot, proteins were electrophoretically transferred to a nitrocellulose membrane. The latter was blocked in 2.5 % milk in PBST buffer (phosphate-buffered saline, PBS (Biochrom, Berlin, Germany) with 1 % Tween 20) for 1 h at room temperature. Afterwards, the membranes were washed three times with PBST (gently shaking), then the primary antibody, diluted in 0.5 % milk in PBST buffer, was applied for 2 h at room temperature. After another PBST washing step, the membrane was incubated with the secondary antibody (HRP-labeled, diluted 1:20,000 in 0.5 % milk in PBST buffer) for 2 h at room temperature, again washed in PBST buffer, and treated with ECL Select solution (Bio-Rad, Munich, Germany). The luminescence signal was detected using the Fluor-S® MAX MultiImaging System (Bio-Rad, Munich, Germany). For comparing expression levels of different synaptic proteins in drug-treated and control lysates, the antibodies against the following proteins were used in a dilution of 1:5,000: CDK5 (mouse mAb, Life Technologies, Darmstadt, Germany), synaptotagmin1 (1:1,000, mouse mAb, Synaptic Systems, Goettingen, Germany), and dynamin (mouse mAb,

Becton Dickinson, Heidelberg, Germany). As a loading control, an antibody against GAPDH was used (1:200,000, mouse mAb, Billerica, USA). As a standard, MagicMark™ XP Western Protein Standard (20–220, Life Technologies, Darmstadt, Germany) was used. Images were analyzed using the ImageJ gel analysis tool. For quantification, resulting intensities were normalized to GAPDH loading control. Obtained values were normalized to respective control.

Electron Microscopy

For electron microscopy, cell cultures were grown on Thermanox coverslips (Plano) and incubated in extracellular medium with or without 1 μM fluoxetine for 20 min. Cells were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, postfixed in 2 % buffered osmium tetroxide, dehydrated in graded alcohol concentrations, and embedded in epoxy resin according to standard protocols. Ultrathin tangential sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (EM 906E; Carl Zeiss, Oberkochen, Germany)

Super Resolution Microscopy

The direct stochastical optical reconstruction microscopy (dSTORM) technique was used to perform super resolution microscopy experiments [26–28]. Experiments were conducted at room temperature on a custom-built setup based on a Nikon TI-Eclipse inverted microscope equipped with a ×100, 1.49 NA oil immersion objective. Fluorescent dyes were excited by a solid-state 488 nm laser (Sapphire 488, Coherent, Dieburg, Germany) using dichroic longpass mirrors (cutoff wavelength 500 nm). The emitted light passed an emission band-pass filters ranging from 500 to 550 nm (Semrock, Rochester, NY, USA) and was projected onto a water-cooled EM-CCD camera (iXon^{EM} DU-897, Andor, Belfast, UK). Images were acquired using Andor™ Solis 4.20 (Andor, Belfast, UK)

Cell cultures were grown on chambered coverglass (Lab-Tek, borosilicate coverglass, eight-well, Thermo Fisher Scientific Inc., Waltham, MA, USA). For super resolution experiments, cells were fixed with Roti®-Histofix 4 % (Carl Roth, Karlsruhe, Germany), washed with PBS (Invitrogen, Karlsruhe, Germany), and chambers were filled with PBS at pH 8.2 containing 100 mM mercaptoethylamine-hydrochloride (Sigma-Aldrich, Taufkirchen, Germany) to ensure optimal photoswitching [26, 28]. Super resolution images were reconstructed using rapidSTORM 3.2 software [29–31].

The number of neighbors was determined by counting automatically detected spots [22] in a circular region within an area of approximately 50 μm^2 [18]. The peak detection diameter was set to 600 nm as ultrastructural analysis revealed a synapse diameter around this value [32] excluding detection of multiple synapses within this distance.

FRAP

For fluorescence recovery after photobleaching (FRAP) experiments, hippocampal neurons transfected with either synaptophysin-mRFP or synapto-pHluorin were incubated in extracellular medium (see above) with or without 1 μM fluoxetine for 15 min at 37 °C. After acquiring one image using a laser scanning microscope (LSM 780; Zeiss, Jena, Germany; 20× objective, ZEN® software; open pinhole), three square regions (20.75×20.75 μm) per coverslip were bleached with a 561-nm laser or a 488-nm laser, respectively. These regions were monitored for another 20 min (acquisition interval 2 min). Twelve minutes after the end of the bleaching period, the cells were electrically stimulated with 1,200 pulses at 40 Hz. For the detection of active synapses, synaptophysin-mRFP-transfected neurons were co-stained with 2.5 μM FM 1-43 in 40 mM KCl for 5 min at 37 °C.

Results

Fluoxetine Alleviates Synaptic Fatigue in Repeatedly Stimulated Neurons

In order to obtain a dose–response relationship for the antidepressant fluoxetine, we performed experiments using neurons transfected with synapto-pHluorin (spH), an optical probe consisting of a pH-sensitive GFP fused to the intravesicular domain of synaptobrevin2 [33, 34]. Our experiments consisted of ten consecutive electrical stimuli of 50 pulses at 10 Hz each 90 s apart [16]. During minutes 4–12 of the experiment, the cells were perfused with the respective concentration of fluoxetine or vehicle (Fig. 1a). This enabled us to reveal short-term effects on synaptic vesicle exocytosis. We observed a reversible, dose-dependent reduction of the evoked fluorescence increase upon stimulation depending on the fluoxetine concentration. Due to the perfusion system, the first stimulus after fluoxetine application still evoked a fluorescence increase whereas the following stimuli triggered responses that declined in a dose-dependent fashion (Fig. 1b). We determined the half maximal inhibitory concentration (IC_{50}) to be 4.31 μM (95 % confidence interval: 3.38, 5.23 μM). As 1 μM is in the range of clinical use [35], we focused on this concentration and found it not to inhibit stimulation-dependent exocytosis of synaptic vesicles (Fig. 1c). Higher concentrations of fluoxetine hindered exocytosis, which is in accordance with a previous study [14]. With 1 μM fluoxetine having no immediate inhibitory effect on synaptic vesicle exocytosis, we decided to use this concentration in further experiments.

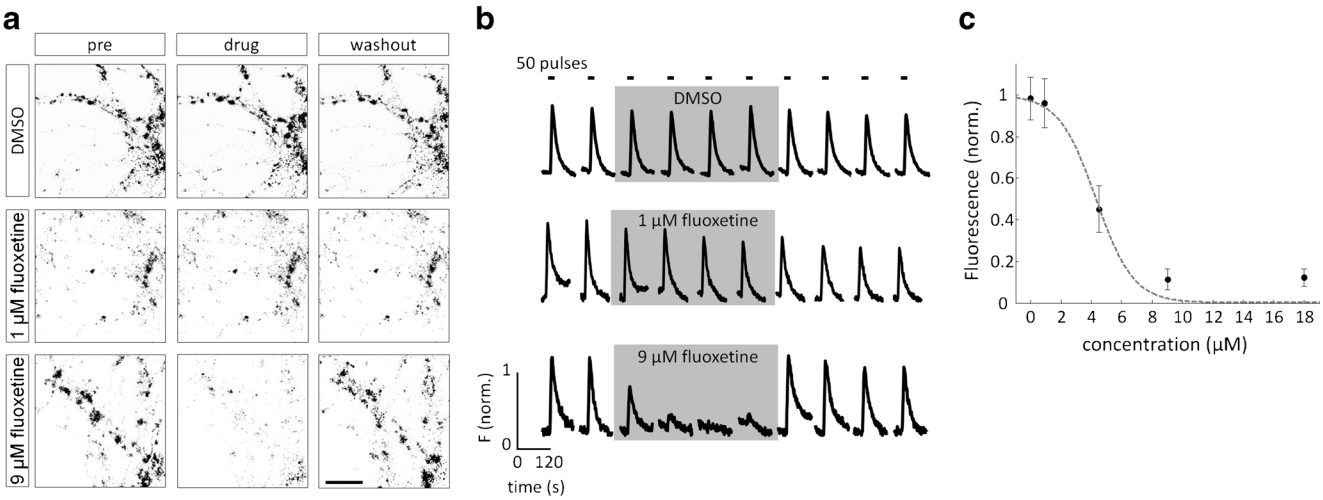

Fig. 1 Fluoxetine in low micromolar concentrations has no influence on the amount of vesicle exocytosis. **a** Exemplary fluorescence difference images of fluoxetine- and DMSO-treated spH-transfected hippocampal neurons before, during, and after drug application. *Scale bar*, 20 µm. **b** Protocol for generating the dose–response curve. Neurons transfected with spH were stimulated with ten trains of 50 pulses at 10 Hz with inter-stimulation intervals of 85 s. Cells were sequentially perfused with extracellular medium, followed by a fluoxetine solution with different concentrations (0.9, 4.5, 9, 18 µM) or a DMSO control solution and extracellular medium again. Exemplary normalized fluorescence profiles for 9 µM solutions and corresponding DMSO control are shown. **c** Dose–response curve for fluoxetine (0.9 µM, $N=6$; 4.5 µM, $N=5$; 9 µM, $N=3$; 18 µM, $N=4$; DMSO, $N=5$)

Nevertheless, 1 µM fluoxetine might have an influence on synaptic vesicle recycling, if stronger stimuli are applied, i.e., stimuli that are known to recycle the entire recycling pool. Therefore, we examined the ability of fluoxetine to ameliorate the exhaustive effects multiple rounds of stimuli have on synapses [14, 36]. SpH-transfected neurons were stimulated with 600 pulses at 30 Hz for six times, while the inter-stimuli intervals were halved every consecutive stimulus (Fig. 2a). With growing stimulus number, the amplitudes of spH fluorescence decreased (Fig. 2b, c). Short-term incubation with 1 µM fluoxetine attenuated this reduction significantly (Fig. 2c). Since it is known that calcium triggers synaptic vesicle exocytosis, and fluoxetine has been described to influence calcium-permeable ion channels [37] or calcium signaling [38], we next tested the effect of 1 µM fluoxetine on action potential-triggered calcium influx using the same stimulus protocol as before. As a calcium sensor, we used fluo-4, while synaptic boutons were detected with an antibody against synaptotagmin1 coupled to a pH-sensitive Cy5-variant fluorescent at an acidic pH and therefore in synaptic vesicles (αSyt1-CypHer™5E). In these synaptic regions, fluo-4 fluorescence changes were quantified (Fig. 2d). No influence of fluoxetine on presynaptic calcium influx upon repetitive stimulation could be observed (Fig. 2e, f). Changes in the levels of the calcium sensor protein synaptotagmin1 [39–43] or the vesicle-associated membrane protein synaptobrevin2 were excluded using a protein biochemical analysis (Fig. S1).

In order to clarify if the observed amelioration was due to an increase in the number of active vesicles after fluoxetine incubation, we measured the spH fluorescence increase following full de-acidification of the recycling pool with the v-ATPase blocker folimycin (80 nM) using the same stimulus protocol as above (Fig. S2). The results showed that the same number of synaptic vesicles was mobilized during the first 600 pulses both in the case of fluoxetine or vehicle (Fig. S2b). As expected, in control experiments, additional stimulations led only to a small increase in fluorescence ([44, 45]; Fig. S2b). The antidepressant fluoxetine caused a significantly higher fluorescence increase when compared to control, indicating that more synaptic vesicles are mobilized (Fig. S2b, c). In contrast to these results, no obvious reduction in fluorescence changes, and thus synaptic fatigue, could be observed if the inter-stimuli intervals remained constant (percent of first stimulation amplitude, indices correspond to stimulation number: $\Delta F_2=111.12\pm10.35$, $\Delta F_3=112.56\pm11.94$, $\Delta F_4=105.93\pm9.59$, $\Delta F_5=106.94\pm12.23$, $\Delta F_6=102.55\pm10.69$; Kruskal–Wallis test: $\chi^2_{5,30}=1.97$, $p=0.85$; $n=1{,}992$, $N=6$), which is in accordance with recently published data [46, 47]. To further confirm that this 1 µM fluoxetine effect only arises after applying 600 pulses or more, synaptic vesicles were loaded with FM 1-43 using a small stimulus known to release only docked vesicles [48] and subsequently de-stained after washout (fluorescence change: controls, 1,671.88±313.93 a.u.; fluoxetine, 2,151.69±405.73 a.u.; Wilcoxon rank sum test, $p=0.63$; control, $n=801$, $N=3$; fluoxetine, $n=1{,}048$, $N=4$). As expected, no differences between treated and untreated cells were observed.

In order to exclude the possibility that the attenuation of synaptic fatigue was an artifact of the shortened inter-stimuli intervals and was due to accelerated endocytosis, we used

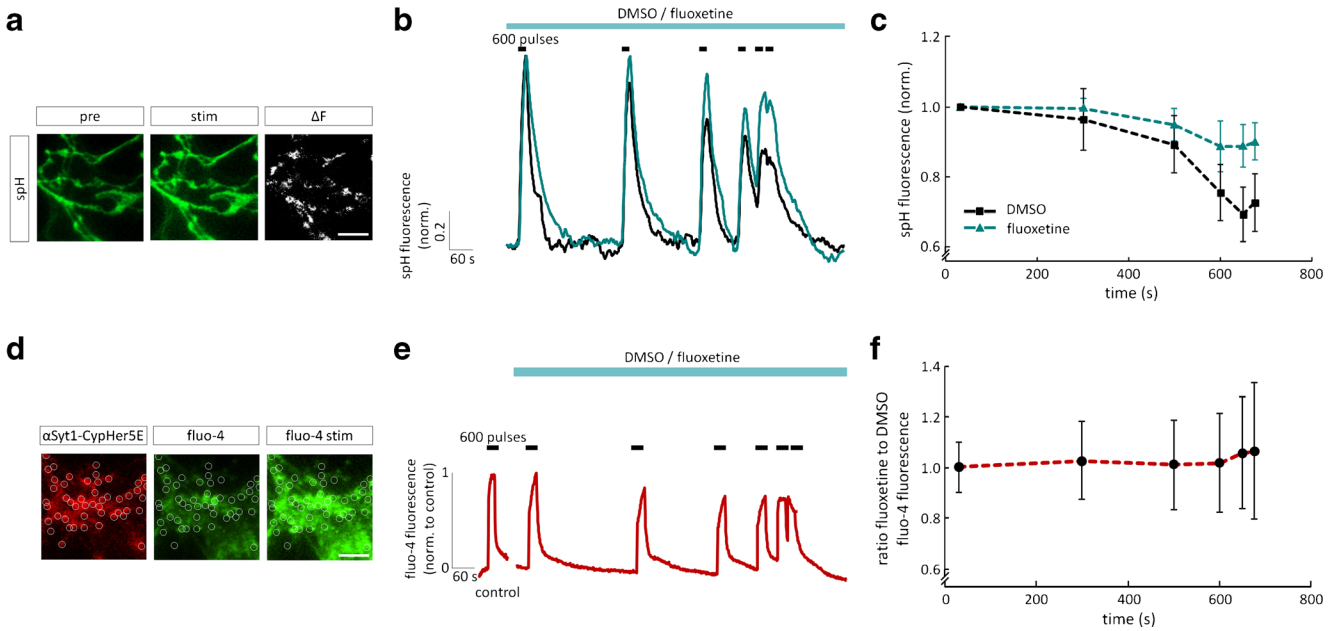

Fig. 2 Fluoxetine alleviates synaptic fatigue in repeatedly stimulated neurons in a calcium-independent fashion. **a** Exemplary images of spH-transfected neurons treated with 1 μM fluoxetine or DMSO, respectively, before (*pre*) and during (*stim*) stimulation and corresponding difference image (Δ*F*). *Scale bar*, 10 μm. Cells were repeatedly stimulated with 600 pulses at 30 Hz with decreasing inter-stimulation intervals after 30, 300, 500, 600, 650, and 675 s, respectively. **b** Mean fluorescence profile for fluoxetine-treated cells and control conditions. **c** Stimulation-induced fluorescence changes of neurons treated with 1 μM fluoxetine or vehicle (DMSO). Fluorescence changes were normalized to their first amplitude. Cells treated with fluoxetine exhibited a lesser fatigue compared to controls (Kruskal–Wallis test, control: $\chi^2_{5,18}=11.17$, $p=0.04$; fluoxetine: $\chi^2_{5,18}=6.02$, $p=0.31$; $N=4$ each). **d** Fluoxetine causes no changes in stimulation-dependent calcium influx. Exemplary images of cells incubated with fluo-4 and an anti-synaptotagmin1 antibody labeled with the pH-dependent fluorophore CypHer™5E before and during stimulation, respectively. *Circles* illustrate active synapses detected with αSyt1-CypHer™5E. *Scale bar*, 10 μm. **e** Mean fluorescence profile for control condition and corresponding control stimulation before start of the experiment to which all further fluorescence changes were normalized. Neurons treated with 1 μM fluoxetine or DMSO were stimulated using the same protocol as described in **a**. **f** Ratio of fluo-4 fluorescence changes of fluoxetine-treated cells to DMSO-treated cells. One micromolar fluoxetine did not change stimulation-dependent calcium influx (one-way ANOVA: $F_{5,30}=0.02$, $p=0.99$; $N=6$ each)

spH-transfected cells labeled with an αGFP-CypHer™5E antibody, which binds to the pHluorin epitope of transfected cells (Fig. S3a-d). Similarly to pHluorin, changes in the fluorescence intensity of CypHer™5E mirror full rounds of exo- and endocytosis, whereas CypHer™5E has inverse fluorescence properties, and thus is quenched at exocytosis and becomes fluorescent upon reacidification. Comparison of the kinetics of the fluorescence profiles revealed no differences between fluoxetine-treated synapses and control (fluorescence changes due to stimulation, two-sample *t* test, spH: $p=0.58$, CypHer™5E: $p=0.91$; $N=4$ each). In order to avoid possible overexpression artifacts resulting from the spH overexpression, terminals were labeled with an antibody against endogenous synaptotagmin1 tagged with CypHer™5E (αSyt1-CypHer™5E) at 37 °C for 60 min to visualize synaptic vesicles. Neurons were stimulated once with 600 pulses at 30 Hz, and after a short-term incubation with either vehicle or 1 μM fluoxetine, stimulated a second time (Fig. S3e; Kruskal–Wallis test: $\chi^2_{3,16}=0.55$, $p=0.91$; $N=5$ each). A protein known to be responsible for the pinch-off of synaptic vesicles from the plasma membrane, and thus for proper vesicle endocytosis, is dynamin1 [49, 50]. By determining its protein levels in both fluoxetine-treated and untreated cells (Fig. S1), we wanted to prove that the endocytosis apparatus was not altered in drug-treated cells. Again, no difference in dynamin1 levels between controls and fluoxetine-treated neurons could be detected (Fig. S1).

Fluoxetine Recruits Synaptic Vesicles from the Reserve Pool

Obviously, upon fluoxetine treatment, more synaptic vesicles were recruited to the recycling pool. To find out where these additional vesicles originated from, neurons whose extracellular medium contained folimycin were stimulated with 600 pulses at 30 Hz, a stimulus known to turn over the whole recycling pool [45, 51]. After this first control stimulus and a following short-term incubation with 1 μM fluoxetine, neurons were stimulated a second time (Fig. S4). As expected, due to the used stimulation paradigm, the fluorescence level increased only a little further under control conditions (see above, and [44, 45]). This is because all recycling pool vesicles had had access to folimycin and thus were already fluorescent before the second round of exocytosis. However, fluoxetine-treated neurons exhibited a larger increase in

fluorescence during the second stimulation indicating new vesicles had joined the recycling pool (Fig. S4b, c). To exclude possible interactions of fluoxetine with folimycin or artifacts due to the transfection with spH, recycling synaptic vesicles were labeled using αGFP-CypHer™5E (Fig. S4d) or the styryl dye FM 1-43 (Fig. S4f) and stimulated using either 40 mM KCl at 37 °C for 10 min or 1,200 pulses at 40 Hz. Again 1 μM fluoxetine raised the number of recycling vesicles (Fig. S4e, g). In order to exclude the possibility that the increase in CypHer™5E or FM 1-43 fluorescence was an artifact derived from a possible interaction between fluoxetine and these dyes, spectrophotometric measurements were performed. Apparently, fluoxetine did not augment the fluorescence intensity of the fluorophores itself. (Fig. S5; CypHer™5E: controls, 57.34±1.43 a.u.; fluoxetine, 51.98±0.67 a.u.; Wilcoxon rank sum test: $p=0.1$; FM 1-43: 90.55±8.71 a.u.; fluoxetine, 79.53±7.15 a.u.; Wilcoxon rank sum test: $p=0.2$, $N=3$ each). To clarify these effects by using a method independent of possible overexpression artifacts, we adopted an experimental design that had been published previously to quantify the increase of the recycling pool [52]. Terminals were maximally stained with 2.5 μM FM 1-43 using 600 pulses at 10 Hz (Fig. 3a–d). As reported, a second round of stimulation led to a small increase in fluorescence, when dye was present [52]. However, when cells were incubated with 1 μM fluoxetine prior to the second staining, the observed fluorescence increase was much higher (Fig. 3b, c). These newly stained vesicles are release-competent because, after washing external dye away, the terminals could be de-stained (Fig. 3c). We calculated the average increase in recycling pool size to be 1.61-fold higher compared to controls (Fig. 3d; ratio first load to second load: controls, 1.03±0.02; fluoxetine, 1.66±0.49; Wilcoxon rank sum test: $p=0.03$, $N=5$ each). To directly quantify the fraction of the recycling pool to the total pool, we used the genetically encoded reporter spH (Fig. 3e–g). Experiments were conducted in the presence of the v-ATPase inhibitor folimycin to obtain a cumulative amount of exocytosis [52]. Neurons were stimulated with 1,200 pulses at 40 Hz, a stimulus known to release the complete recycling pool [45, 46, 51]. At the end of the experiment, cells were perfused with NH_4Cl to de-quench vesicles in the acidic state [34] and thus obtain a measure for the total vesicle pool. This allowed us to calculate the recycling pool size. In untreated neurons, the recycling pool made up for 36.73±2.50 % of the total pool, which is in accordance with previous studies [53, 54]. In the case of short-term application of 1 μM fluoxetine, the recycling fraction increased to 46.70±6.23 % of the total pool at the expense of the resting pool (control: $n=1{,}963$, $N=8$; fluoxetine: $n=1{,}405$, $N=8$) (Fig. 3e, g). From these data, we conclude that short-term application of fluoxetine is able to increase the size of the recycling vesicle pool. In additional experiments equal to Fig. 3a–d, we measured 50 μM roscovitine, a CDK5 inhibitor [52, 55], as an internal control and detected that 1 μM fluoxetine exhibited a similar effect like roscovitine (normalized fluorescence: controls, 1.00±0.18; roscovitine, 1.31±0.17; fluoxetine, 1.36±0.04; effect size Cohen's $d_{\text{fluoxetine,roscovitine}}=0.33$; two-sample t test: $p_{\text{fluoxetine,roscovitine}}=0.66$; one-way ANOVA: $F_{2,9}=6.79$; $p_{\text{control,fluoxetine,roscovitine}}=0.02$; $n>800$ each, $N=4$ each).

Next, we wanted to exclude that the additionally recruited vesicles originated from a source other than the resting pool. Since synaptic vesicles are shared between adjacent synapses [56–58], it is possible that fluoxetine might increase this sharing of release-competent vesicles moving between neighboring boutons. To examine this, we first analyzed the axonal transport properties of the synaptic vesicle protein synaptophysin, i.e., velocity, pausing time, and percent moving particles (Fig. 4a). We could not detect any differences between the fluoxetine-treated and the control group (Fig. 4b; percent moving particles: controls, 53.87±11.11 %; fluoxetine, 42.48±5.76 %; two-sample t test: $p=0.43$; $N=5$ each). Additionally, we sought to rule out the possibility that fluoxetine altered sharing or capturing of synaptic vesicles by adapting a previously published FRAP experiment [59]. Therefore, we transfected hippocampal neurons with synaptophysin-mRFP (syp-mRFP) or spH, respectively. In order to make sure that we monitored active synapses in the syp-mRFP-transfected neurons, we co-stained these cells with FM 1-43 using a 40 mM KCl solution. Most syp-mRFP-positive regions on neuronal processes had also taken up FM dye; thus, we can conclude that the majority of red fluorescent spots represent synapses (Fig. 4c). After incubation with either 1 μM fluoxetine or control solution, square regions were bleached and the time courses of the fluorescence recovery were investigated over 20 min (Fig. 4d). In order to analyze the impact of strong stimulation on synaptic capture, the neurons were stimulated with 1,200 pulses at 40 Hz 12 min after the end of the bleaching period (t_1+12 min) (Fig. 4e). Importantly, since synaptophysin is located almost exclusively intracellularly, here the only source for an increase in fluorescence is synaptic capture, whereas spH has a surface expression of up to 30 % [19]. Therefore, in this case, fluorescence recovery is also caused by fluorescent molecules that diffuse freely in the cell membrane. The comparison of the time courses of fluorescence intensity in the observed regions revealed no differences between fluoxetine-treated and control group (Kolmogorov–Smirnov test, syp-mRFP: $p=0.9913$; spH: $p=0.7864$). As a measure for the extent of synaptic capture, we used the difference in fluorescence intensity between t_1 and the end of the experiment (t_1+20 min), i.e., fluorescence recovery (Fig. 4f). This analysis showed that short-term incubation with fluoxetine did not alter the synaptic capture properties of hippocampal neurons (syp-mRFP, control: 0.10±0.0096, $n=30$, $N=10$; fluoxetine: 0.09±0.0049, $n=27$, $N=9$; two-sample t test: $p=0.5961$; spH,

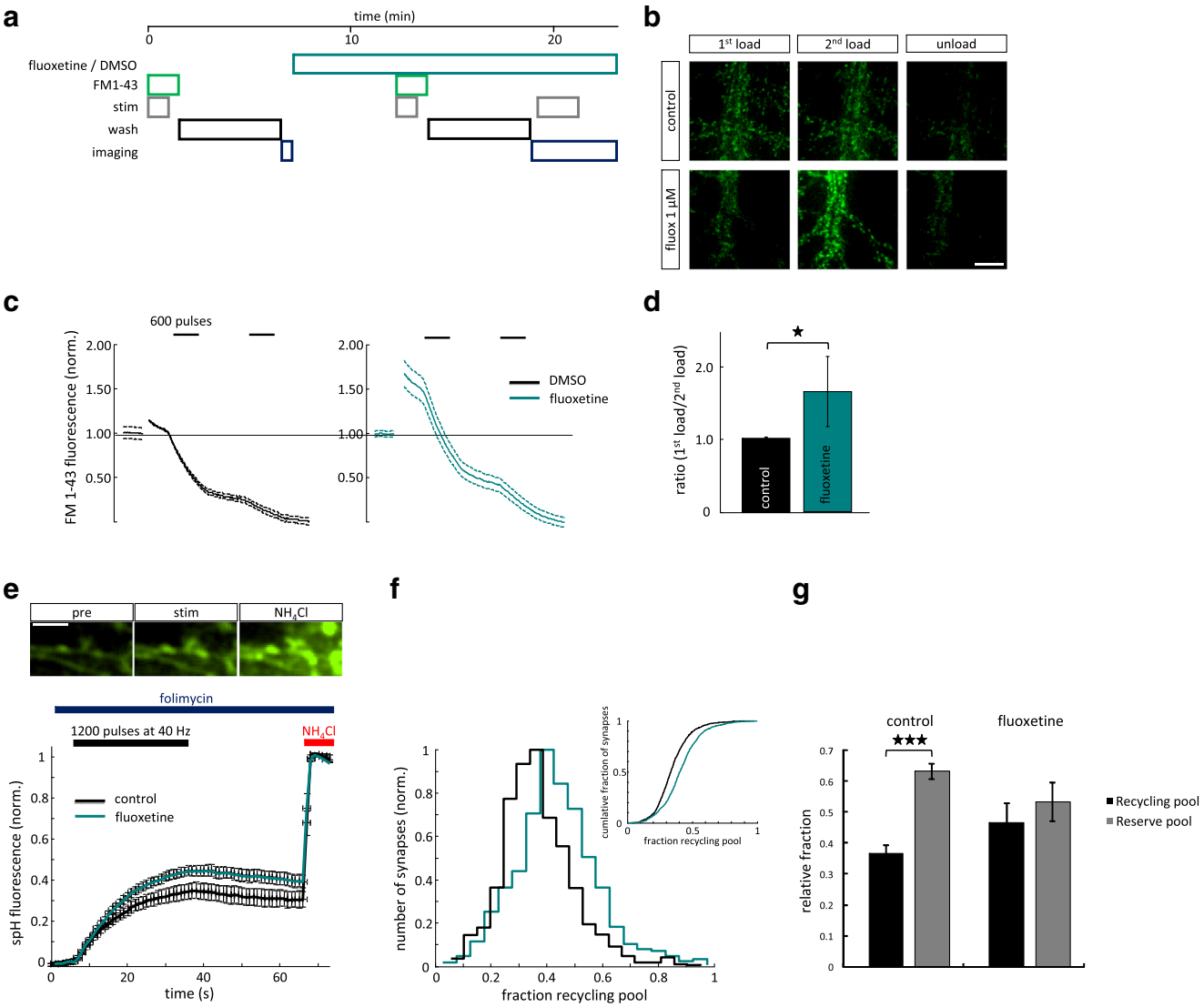

Fig. 3 Fluoxetine increases the recycling pool at the expense of the resting pool in hippocampal nerve terminals. **a** Experimental setting according to [25]: Recycling synaptic vesicles of hippocampal neurons were loaded with FM 1-43 two times with a stimulus of 10 Hz for 60 s and external dye was extensively washed away. After a first round of loading, the fluorescence signal was measured. After a short-term incubation with either DMSO or 1 μM fluoxetine, the same cells were loaded with FM 1-43 in the second round and the fluorescence was then determined by unloading all synaptic vesicles with two stimuli at 10 Hz for 60 s. **b** Representative images for the two rounds of stimulation. *Scale bar*, 10 μm. **c** Average fluorescence profiles normalized to the respective first round of loading ($N=5$). *Error bars* indicate standard error of the mean. Corresponding bar diagram depicts mean of all experiments and standard deviation (**d**) of fluorescence increase (Wilcoxon rank sum test: $p=0.03$). **e** Hippocampal neurons transfected with spH were stimulated with 1,200 pulses at 40 Hz under the v-ATPase inhibitor folimycin. At the end of the experiments, cells were perfused with NH_4Cl to obtain a measure for the total pool size or the relative resting pool size, respectively. Average fluorescence profiles of neurons after short-term incubation with 1 μM fluoxetine or vehicle 10 min before and during the experiment ($N=7$ each). Profiles were normalized to fluorescence values obtained during NH_4Cl treatment. *Scale bar*, 5 μm. *Error bars* indicate standard error of the mean. **f** Treatment with 1 μM fluoxetine increased the fraction of the recycling pool size 1.19-fold. *Inset*, empirical cumulative distribution function. **g** Relative fraction of recycling and reserve pool for control and fluoxetine conditions (control: two-sample t test, $p<0.001$; fluoxetine: two-sample t test, $p=0.49$)

control: 0.47±0.03, $n=15$, $N=5$; fluoxetine: 0.44±0.01, $n=18$, $N=6$; two-sample t test: $p=0.2967$).

Furthermore, we investigated the influence of short-term application of fluoxetine on either synaptic pattern or morphology. Synaptic pattern can be analyzed by counting the number of neighbors in a predefined circular area [18]. Due to the diffraction limit of the conventional fluorescence microscopy, nearby synapses might appear as one single synapse and thus the number of synapses might be underestimated. Therefore, we made use of the dSTORM super resolution technique [28] with a localization precision of 29.34±4.99 nm in our imaging setup [60, 61]. Recycling synaptic vesicles were

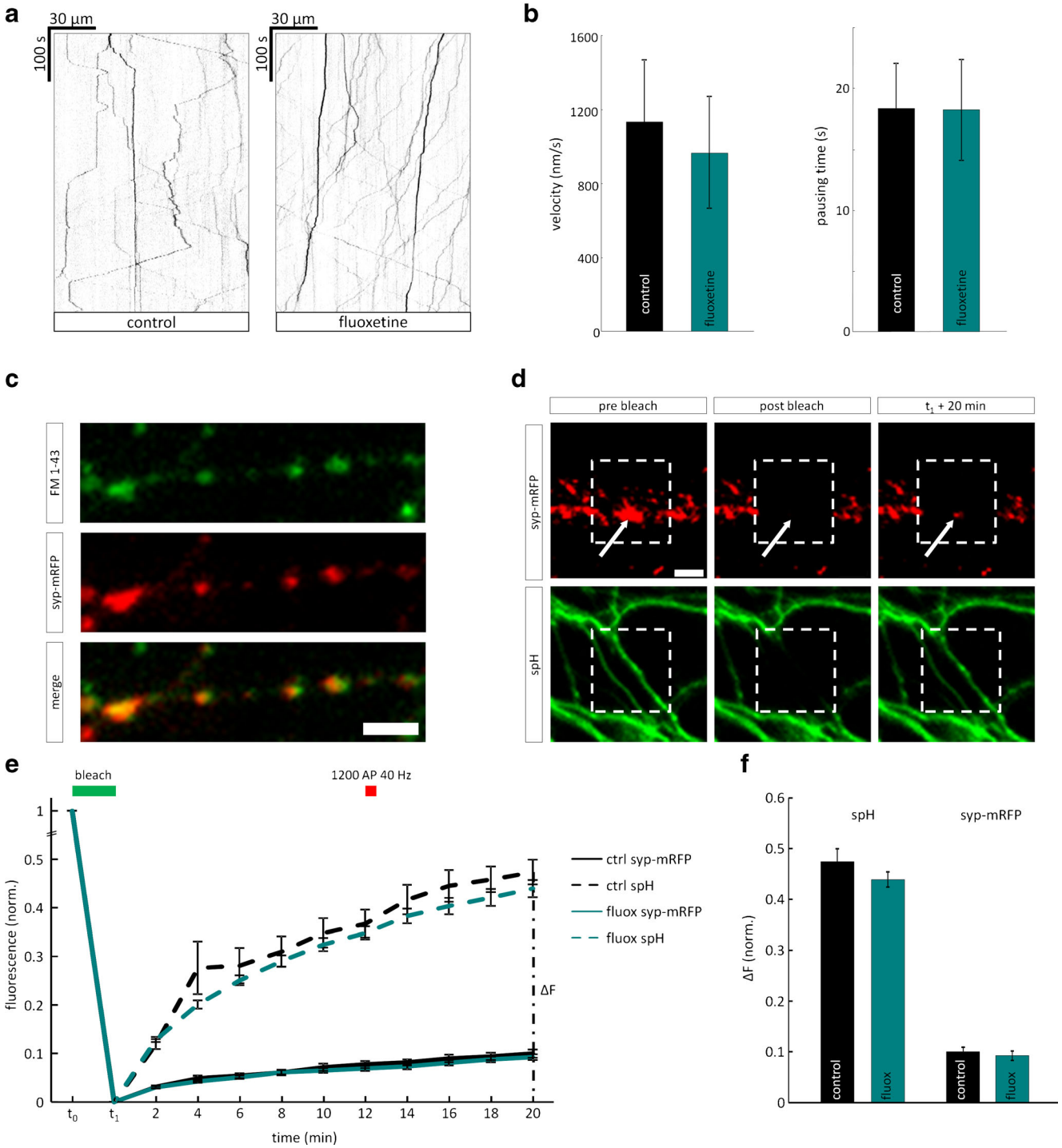

Fig. 4 Fluoxetine has no influence on axonal transport or synaptic capture. **a** Exemplary kymographs obtained from time-lapse movies (1 Hz) of hippocampal axons for control and 1 µM fluoxetine condition. Transport vesicles are visualized by synaptophysin labeled with mRFP (syp-mRFP). Kymograph images were analyzed using the Hough transform [29]. Quantification (**b**) of velocity and pausing time of transport vesicles (velocity, two-sample *t* test: $p=0.10$; pausing time, two-sample *t* test: $p=0.20$; $N=5$). **c** Exemplary images of a hippocampal axon stained with FM 1-43 (40 mM KCl, 5 min at 37 °C) and transfected with syp-mRFP, and resulting merge image. *Scale bar*, 5 µm. **d** Exemplary synaptic regions of a syp-mRFP-transfected and a spH-transfected neuron, respectively, both directly before and after bleaching, and 20 min after the end of the bleaching period. *Squares* indicate bleached regions. *Arrows* indicate a recovering synapse at the three time points. *Scale bar*, 10 µm. **e** Mean fluorescence profiles of fluoxetine-treated and control cells, normalized to start and end of bleaching. Twelve minutes after the end of the bleaching period, the cells were electrically stimulated with 1,200 pulses at 40 Hz. t_0, start bleaching. t_1, stop bleaching. *Error bars* indicate standard error of the mean. ΔF, difference of fluorescence intensity between time points t_1 and t_1+20 min. **f** Quantification of ΔF for fluoxetine-treated and control cells (two-sample *t* test: syp-mRFP, $p=0.5961$; spH, $p=0.2967$). *Error bars* represent standard error of the mean

labeled using an antibody against synaptotagmin1 coupled to Alexa488 (αSyt1-Alexa488), a fluorophore which was shown to be well-suited for dSTORM [26, 28]. Neurons were incubated in extracellular medium containing the labeled antibody for 60 min at 37 °C. Subsequent short-term incubation with 1 μM fluoxetine in 40 mM KCl solution had no impact on the number of detected synapses (Fig. S6). Although spectrophotometric measurements indicated that fluoxetine did not influence the emission spectra of the Alexa488 dye (Fig. S5c), the fluorescence intensity was slightly reduced in the fluoxetine-treated samples (controls, 232.53±2.56 a.u.; fluoxetine, 216.99±1.18 a.u.; Wilcoxon rank sum test: $p=0.1$, $N=3$ each). However, this reduction had no effect on the detection of single molecules and thus on our high resolution images. Another morphological analysis using electron microscopy (Fig. S6c) did not reveal any influence of short-term application of 1 μM fluoxetine (number of vesicles: controls, 81.05±1.57; fluoxetine, 87.60±1.34; two-sample t test: $p=0.5656$; 20 synapses each) (Fig. S6d), which gave additional evidence that this SSRI did not evoke ultrastructural changes in hippocampal neurons.

Fluoxetine Increases the Frequency of Miniature Postsynaptic Currents and the Amount of Spontaneously Recycling Vesicles

The above experiments showed that short-term application of 1 μM fluoxetine is able to increase the recycling pool of synaptic vesicles. As miniature activity was shown to correlate with the recycling pool size [62], an increase in miniature frequency evoked by fluoxetine was expected. Therefore, we analyzed the influence of 1 μM fluoxetine on mPSCs as it was previously shown that this antidepressant is able to increase their frequency [17]. After obtaining a stable mPSC frequency as an internal control, 1 μM fluoxetine or vehicle was applied using a fast perfusion system and mPSCs were recorded for the following 15 min. Application of the drug had no effect on the amplitude of mPSCs (Fig. 5c; two-sample t test: $p=0.16$), whereas their frequency was slightly, but significantly increased (Fig. 5b, d; ratio after/before, control=0.97±0.16, ratio after/before, fluoxetine=1.27±0.32; two-sample t test: $p=0.02$). To further consolidate these findings, we analyzed the amount of spontaneously recycling vesicles using fluorescence microscopy (Fig. 5e). For this purpose, neurons were incubated with FM 1-43 for 30 min at 37 °C in medium containing 1 μM TTX but no $Ca2^{+}$ to block voltage-gated sodium channels. After considerable washout, spontaneously stained synaptic vesicles were de-stained using 40 pulses at 20 Hz followed by a complete unloading of the dye (Fig. 5f). One micromolar fluoxetine increased the number of spontaneously recycling vesicles, thus confirming the results achieved using electrophysiology (Fig. 5g).

Discussion

It has been shown that antidepressant drugs and hereby especially SSRIs like fluoxetine do not only affect serotonin reuptake but also have a variety of other effects including activity-dependent inhibition of voltage-gated sodium channels [63] or inhibition of the acidic sphingomyelinase [64]. Furthermore, they can affect dopamine and norepinephrine transporter binding [65] and growth factor modulation [66]. In the present work, we studied acute effects of the SSRI fluoxetine on synaptic vesicle recycling. We were able to prove that this antidepressant inhibits synaptic transmission in a dose-dependent manner. Our work revealed that a clinically relevant dose of the drug ameliorated presynaptic depression, which was caused by exhaustive stimuli, by increasing the number of recycling vesicles.

The dose–response relationship demonstrated that concentrations above 8 μM inhibited exocytosis almost completely, which is in accordance with previous results [14]. This observation was expected as fluoxetine administration in this range of concentration was reported to block Na^{+}, Cl^{-}, and Ca^{2+} channels [67–71]. We showed that the clinically relevant dose had no influence on synaptic vesicle recycling when cells were stimulated with weak stimuli like 50 pulses at 10 Hz (Fig. 1) or pulses known to release the readily releasable pool of synaptic vesicles (see "Results"). However, we demonstrated that treatment with fluoxetine is able to increase the recycling pool size of synaptic vesicles upon the application of exhaustive stimuli, e.g., 600 or more pulses or incubation with a high KCl solution for at least 10 min.

By now, several mechanisms and substances which are capable of expanding the recycling pool or of recruiting vesicles from the resting pool have been identified: For example, phorbol esters were shown to potentiate spontaneous and evoked release [72]. These effects were partly mediated through L-type calcium channels, affected mainly the readily releasable pool [72] and were shown to be induced only in mature synapses [73]. Additionally, phorbol esters exhibited different effects on spontaneous and activity-dependent pools of vesicles indicating different priming mechanisms for spontaneous and evoked fusion [73]. In contrast to phorbol esters, fluoxetine had no effects on calcium influx (Fig. 2) and addressed the total recycling pool instead of the readily releasable pool. Both substances were able to increase the number of spontaneously fused vesicles, though.

NMDA receptor-dependent synaptic potentiation [10] could be excluded to be the cause for the observed fluoxetine effect since postsynaptic blockers (AP5 and CNQX) were present in all experiments, and all results were normalized to corresponding controls if the experimental design allowed such an analysis (e.g., Fig. 2).

A recent study detected CDK5 to be a major control point for neurotransmitter release [52]. Nevertheless, in our work,

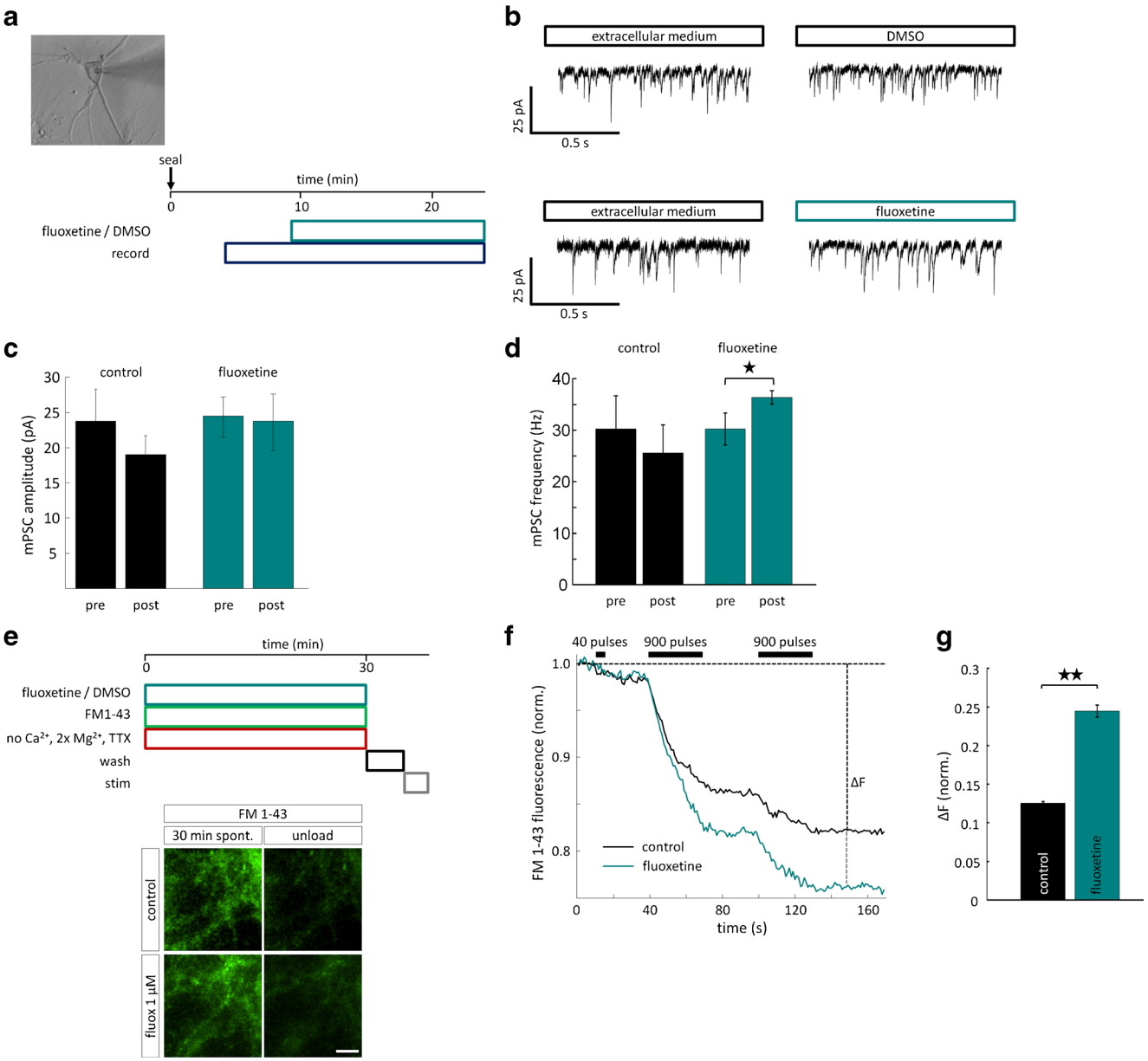

Fig. 5 Fluoxetine increases the amount of spontaneously recycling synaptic vesicles. **a** Electrophysiology measurement protocol. **b** Sample traces from whole-cell recordings of rat hippocampal neurons for the application of vehicle ($N=6$) or 1 μM fluoxetine ($N=7$). Average mPSC amplitudes (**c**) and frequencies (**d**) for the two recording conditions. *Error bars* indicate standard error of the mean (amplitude, paired-sample t test: $p=0.16$; frequency, paired-sample t test: $p=0.02$). **e** Fluorescence microscopy: unloading protocol and exemplary images of hippocampal neurons stained by spontaneous FM 1-43 uptake (30 min at 37 °C) under 1 μM fluoxetine or DMSO control and following de-staining. *Scale bar*, 10 μm. **f** Corresponding exemplary fluorescence intensity profiles normalized to the initial fluorescence for fluoxetine-treated neurons and controls. **g** Quantification of stimulation-induced fluorescence change (controls, $N=6$; fluoxetine, $N=7$; two-sample t test: $p=0.002$)

we could not prove that CDK5 was responsible for altered neuronal transmission as fluoxetine at a concentration of 1 μM did not affect the protein levels of CDK5 (Fig. S1).

Recently, it was shown that nitric oxide (NO) participates in presynaptic plasticity [74]. By inhibition of NO synthase, a tetanus-induced increase in the recycling pool fraction was blocked [10]. Fluoxetine is able to increase extracellular levels of NO in the anterior hypothalamus [75] and to prevent a reduction of NO synthesis when tested in the acute forced swim test [76]. Additionally, NO regulates brain-derived neurotrophic factor (BDNF) production via positive feedback and BDNF itself induces NO synthase [77, 78]. Several studies suggest a link between a lack in BDNF and MDD [79]. A possible influence of fluoxetine on NO may thus be in accordance with the clinical lag in onset of its antidepressant action, which is a main reason for non-compliance in SSRI

pharmacotherapy and linked to the delay in elevation of BDNF [80–82]. However, we do not have any data on that. Nevertheless, recent findings support the importance of BDNF in MDD, as the rapid antidepressant effect of the NMDA receptor antagonist ketamine is mediated by the inactivation of eukaryotic elongation factor 2 kinase and the resulting decrease in BDNF [83, 84].

The recently discovered synaptic vesicle super-pool [57, 58, 85] could be excluded as a possible source for the additional recycling vesicles recruited by fluoxetine. As our experiments indicated that there was neither a net change in axonal transport nor in the synaptic capture properties of the protein synaptophysin. Moreover, we could show that the membrane diffusion of spH was not altered by acute administration of fluoxetine (Fig. 4).

Our finding that the fluoxetine-induced increase of the recycling pool is associated with the enhanced frequency of electrophysiologically determined miniature events also contributes to the ongoing debate about the origin of synaptic vesicles fusing at rest [86–89]. If synaptic vesicles that release neurotransmitter both on stimulation and at rest belong to the same pool [88–90], an increase in the recycling vesicle pool could be directly linked to an increase in the mPSC frequency. We could show this to be one of the effects of clinically relevant doses of the antidepressant fluoxetine (Fig. 5).

As recently reported, antipsychotic drugs are able to inhibit neuronal transmission in a use-dependent manner since these drugs are enriched in synaptic vesicles at least 60-fold [16]. Fluoxetine, like many other antidepressants which are weak bases, accumulates in acidic cellular compartments, e.g., synaptic vesicles, too [91, 92]. Thus, it also might attenuate synaptic transmission. The effects of fluoxetine with regard to synaptic recycling, namely inhibiting exocytosis at higher concentrations and recruiting release-competent vesicles from the resting pool, antagonize, but during enhanced neuronal activity the increase of the recycling pool might dominate. However, the augmented activity at rest shown by raised mPSCs frequency and spontaneously recycling synaptic vesicle (Fig. 5) is per definition insensitive to evoked transmission and is thought to be involved in the regulatory role in neuronal outgrowth and plasticity [86] as well as MDD [93]. Our data therefore support the plasticity hypothesis of MDD since strengthened neurotransmitter release is associated with, among others, recovered hippocampus volume [94].

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Conflict of Interest The authors declare no conflict of interest.

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