Altered surface trafficking of presynaptic cannabinoid type 1 receptor in and out synaptic terminals parallels receptor desensitization

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Presynaptic cannabinoid type 1 receptors (CB1Rs) are major mediators of retrograde synaptic plasticity at both excitatory and inhibitory synapses and participate in a plethora of physiological functions. Whether presynaptic receptors, such as CB1R, display functionally relevant movements at the surface of neuronal membranes is not known. We analyzed the lateral mobility of native CB1Rs in cortical neurons by using single-quatum dot imaging. We found that CB1Rs are highly mobile and rapidly diffuse in and out of presynapses. Agonist-induced desensitization correlated with a reduction in the fraction of surface CB1Rs and a drastic decrease in the membrane dynamic of the CB1Rs that remained at the presynaptic surface. Desensitization specifically excluded CB1Rs from synapses and increased the fraction of immobile receptors in the extrasynaptic compartment. The results suggest that decrease of mobility may be one of the core mechanisms underlying the desensitization of CB1R, the most abundant G protein-coupled receptor in the brain.

Cannabinoid type 1 receptors (CB1Rs) and endocannabinoids (eCBs), as well as their synthesizing and degrading enzymes, constitute the eCB system (1, 2). In the CNS, the eCB system mediates retrograde signaling: postsynaptically produced eCBs cross the synapses and activate presynaptic CB1Rs (3). In addition to excytocysis/endocytosis, neurotransmitter receptor movement into and out of synapses may be one of the core mechanisms for rapidly changing the number of functional postsynaptic receptors (4, 5). Curiously, the lateral movements of presynaptic receptors, such as CB1Rs, have never been studied.

Determining whether CB1Rs diffuse in presynaptic membranes, similarly to postsynaptic ionotropic receptors (5), is necessary to understand the molecular logic of the eCB system (1). Because CB1Rs must be within reaching distance of eCBs to fulfill their presynaptic functions (3), surface diffusion potentially represents an efficient and rapid means of modulating retrograde signaling. CB1Rs are classical G protein-coupled receptors (GPCRs), and prolonged agonist treatment triggers both internalization via the clathrin-coated pit pathway (ref. 6, but see ref. 7) and uncoupling from their effector G proteins. A single in vivo exposure to Δ⁹-tetrahydrocannabinol abolishes eCB-mediated retrograde signaling and reduces the CB1R maximal efficacy without modifying total binding or coupling (8). Thus, another mechanism must exist to functionally desensitize CB1Rs in response to agonist exposure. A tantalizing hypothesis is that CB1Rs are moved away from their presynaptic site of actions following agonist exposure.

Here, we analyzed the surface mobility of native CB1Rs in cortical neurons in vitro by using single-quatum dot imaging (9). Our data support the idea that the dynamic exclusion of CB1Rs from a particular presynaptic subdomain might play a prominent role in the desensitization of the most abundant GPCRs in the brain.

Results

CB1Rs Are Highly Mobile on the Surface of Cortical Neurons. Live staining of surface CB1Rs revealed that in cortical primary neurons (9–10 days in vitro) the vast majority of surface CB1R immunolabeling colocalizes with the axonal marker Tau1 (Fig. 1A). There was nearly no colocalization between the somatodendritic marker microtubule-associated protein 2 (MAP2) and CB1Rs, showing that surface CB1Rs are predominantly axonal in mouse cortical neurons in vitro. We colabeled for CB1R and glutamic acid decarboxylase 65 (GAD65), a marker of GABAergic neurons, and found that only 13% ± 1% (n = 603) of all neurons were GAD65-positive. Moreover, 51% ± 11% (n = 89) of CB1Rs were detected on GAD65-positive neurites [supporting information (SI) Fig. S1], suggesting that both glutamatergic and GABAergic cortical neurons express CB1Rs in vitro. Further analysis revealed that 66.1% ± 5.7% (n = 69) of GAD65-positive neurons expressed CB1R immunolabeling and that 9.8% ± 1.6% (n = 346) of non-GAD65-positive neurons expressed CB1R immunolabeling.

To specifically track surface CB1Rs in real time, polyclonal antibodies directed against an extracellular N-terminus domain of CB1R were coupled to anti-rabbit F(ab')2 conjugated with Quantum Dots 655 nm (Qdots). Synapses were visualized with a fluorescent marker of active mitochondria, MitoTracker-Green, which colocalizes with presynaptic synaptotagmin clusters (10, 11). A real-time video of CB1R–Qdots recorded in 9- to 10-days-in-vitro cortical neurons (32°C) is available as Movie S1. Instantaneous diffusion coefficient (D), percentage of mobile receptors, synaptic dwell time, percentage of synaptic receptors, and mean square displacement (MSD) were all calculated from reconstructed CB1R–Qdot trajectories.

In control conditions, surface CB1Rs displayed a high level of lateral mobility: ≈83% were mobile (i.e., D > 0.005 μm²/s) (Fig. 1C). Within synaptic and extrasynaptic compartments CB1Rs alternated between periods of diffusive movement (Fig. 1B).

How long do CB1Rs spend within the synapse? The synaptic dwell time of CB1Rs was 6 times shorter than that of postsynaptic

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Desensitization Dramatically Reduces the Fraction of Mobile CB1Rs. In rodents, subchronic activation of CB1Rs strongly desensitizes CB1Rs (15, 16). In animal and cellular models, CB1R desensitization is achieved easily by prolonged treatment with a CB agonist (17–19). Cortical neurons were treated with the synthetic cannabinoid agonist (R)-(−)-(2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl)-1-naphthalenylmethane (WIN; 400 nM), the selective CB1 antagonist 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide (AM281; 400 nM), or vehicle (0.1% DMSO) for 20 h. In agreement with previous reports (20, 21), 20 h of WIN treatment strongly reduced surface CB1Rs. There were 61.5 ± 12.2 (n = 369) and only 19.1 ± 2.2 (n = 145) CB1R–Qdots particles per observation field after 20 h of treatment with vehicle and WIN, respectively (t test, P < 0.01). Chronic WIN treatment dramatically lowered the diffusion coefficients of surface CB1Rs (control: median = 0.135 µm²/s, IQR = 0.055–0.232 µm²/s, n = 369; AM281: median = 0.157 µm²/s, IQR = 0.081–0.261 µm²/s, n = 808; and WIN: median = 0.074 µm²/s, IQR = 0.0001–0.183 µm²/s, n = 145; P < 0.001; Fig. 2A). The apparent reduction in the diffusion coefficients could be due to a reduction of the diffusion of mobile CB1Rs or an increase in the fraction of immobile CB1Rs. Our data support the second possibility. The diffusion coefficients of all mobile receptors were not significantly different across the different groups (control: median = 0.157 µm²/s, IQR = 0.090–0.251 µm²/s, n = 320; AM281: median = 0.168 µm²/s, IQR = 0.101–0.267 µm²/s, n = 741; and WIN: median = 0.152 µm²/s, IQR = 0.090–0.242 µm²/s, n = 91; P > 0.05). In contrast, prolonged treatment with the cannabinoid agonist led to a large increase in the fraction of immobile CB1Rs. Chronic incubation with AM281, a described antagonist with inverse agonist properties, had no effect on the fraction of immobile CB1Rs (Fig. 2B). After 20 h of treatment, the percentage of mobile CB1Rs was 83.0% ± 4.6% (n = 369), 90.3% ± 1.9% (n = 808), and 57.6% ± 8.6% (n = 145) in control, AM281-treated, and WIN-treated neurons, respectively. Thus, the remarkable shift in the distribution of diffusion coefficients toward lower values observed after prolonged agonist exposure is due to an increase in the fraction of immobile receptors.

Moderate Desensitization Is Sufficient to Reduce CB1R Surface Mobility. We tested the effects of short agonist treatments on CB1R lateral mobility. Cortical neurons were incubated for 30 min or 2 h with the agonist WIN, the antagonist AM281, or vehicle. Thirty minutes of treatment had no consequence on CB1R mobility (Fig. S2). In contrast, a 2-h treatment with the cannabinoid agonist diminished CB1R mobility: the diffusion coefficients of all CB1Rs were lowered (control: median = 0.151 µm²/s, IQR = 0.062–0.259 µm²/s, n = 434; AM281: median = 0.164 µm²/s, IQR = 0.086–0.255 µm²/s, n = 785; and WIN: median = 0.124 µm²/s, IQR = 0.008–0.227 µm²/s, n = 194; P < 0.001; Fig. 2C). We compared the diffusion coefficients of surface mobile receptors in vehicle versus treated cultures and found the different groups to be identical (control: median = 0.175 µm²/s, IQR = 0.088–0.272 µm²/s, n = 396; AM281: median = 0.175 µm²/s, IQR = 0.099–0.263 µm²/s, n = 739; and PAR were similar (14). Further analysis revealed that the distribution of the diffusion coefficients of mobile extrasynaptic and synaptic CB1Rs were identical (833 extrasynaptic CB1Rs: median = 0.297 µm²/s, IQR = 0.154–0.465 µm²/s; 25 synaptic CB1Rs: median = 0.225 µm²/s, IQR = 0.083–0.506 µm²/s; P > 0.05). Within the extrasynaptic subdomain, the MSD of extrasynaptic CB1Rs as a function of time was linear, indicating free diffusive movement (Fig. 1E). In contrast, the MSD values of CB1Rs within the spatially restricted synaptic subdomain reflected spatially confined movement (Fig. 1E).

GluR2-containing AMPA receptors (AMPArs; 0.2 ± 0.05 s, n = 24, and 1.3 ± 0.4 s, n = 20, respectively; see Fig. 4B) (12, 13), likely due to differences in the environment of presynaptic CB1Rs and postsynaptic GluR2-containing AMPARs.

A comparison of the distribution of diffusion coefficients of the global population of CB1Rs (i.e., mobile and immobile) revealed no difference between the mobility of synaptic and extrasynaptic CB1Rs [extrasynaptic: median = 0.213 µm²/s, interquartile range (IQR) 25–75% = 0.019–0.405 µm²/s, n = 1,072; synaptic: median = 0.182 µm²/s, IQR = 0.02–0.42 µm²/s, n = 30; P > 0.05; Fig. 1D]. Compared with other neurotransmitter receptors, the diffusion coefficients of CB1R and AM-
CB1R Desensitization Does Not Alter the Mobility of AMPARs. AMPARs underlie most of the excitatory currents at glutamatergic excitatory synapses, and we have studied extensively their movements on neuronal membranes (4, 5, 22, 23). We tested the impact of subchronic treatment with either the CB agonist or the CB1R antagonist on the mobility of GluR2-containing AMPARs. Such treatments had no effect on the mobility of GluR2-containing AMPARs (Fig. 3 C and D), strongly suggesting that CB1R desensitization does not result in a general alteration of neurotransmitter receptor surface movements.

**Desensitization Excludes CB1R from Synapses.** During desensitization, the fraction of immobile receptors increased significantly. The synaptic terminal is composed of several subcompartments with different functional specialization (24). Are the CB1Rs within these compartments affected equally by agonist treatment? We compared the effects of CB1R desensitization on the mobility of CB1Rs in the extrasynaptic and synaptic compartments. Both 2 h and 20 h of agonist-induced desensitization reduced the fraction of synaptic CB1Rs (Fig. 4 A). Agonist treatment reduced by as much as 60% the fraction of synaptic CB1Rs. In vehicle-treated neurons, 3% of CB1Rs (±0.6%, n = 434) were in synapses. Less than 1% of CB1Rs (0.7% ± 0.2%, n = 194) remained within synapses after 2 h of agonist treatment. The CB1R antagonist AM281 had no effect on the number of synaptic AMPARs. Blue and red lines represent the extrasynaptic and the synaptic parts of the trajectories, respectively. (Scale bar, 0.5 µm.)
24; WIN: dwell time = 0.2 ± 0.03 s, n = 18, P > 0.05; Fig. 4B). Similarly, the exchange rate of CB1Rs between synaptic and extrasynaptic compartments was not affected. The percentages of mobile CB1Rs that alternated between synaptic and extrasynaptic membrane were 19% ± 2.9% (n = 1,016) in control conditions and 21.9% ± 5.3% after agonist exposure (n = 343; P > 0.05).

We found that CB1R desensitization was characterized by a selective decrease in the diffusion coefficients of extrasynaptic CB1Rs (Fig. 4C). In control conditions, the diffusion coefficients of all CB1Rs in the extrasynaptic (median = 0.213 μm²/s, IQR = 0.019–0.405 μm²/s, n = 1,072) and the synaptic (median = 0.182 μm²/s, IQR = 0.02–0.42 μm²/s, n = 30) compartments were not significantly different (P > 0.05). When CB1Rs had been desensitized, there was a selective decrease in the diffusion coefficients of extrasynaptic CB1Rs (median = 0.023 μm²/s, IQR = 0.0002–0.318 μm²/s, n = 585). In contrast, desensitization did not modify the diffusion coefficients of synaptic CB1Rs (median = 0.17 μm²/s, IQR = 0.075–0.455 μm²/s, n = 22; P < 0.001). The reduction in the diffusion coefficients of all CB1Rs could originate from a reduction of the diffusion of mobile CB1Rs or an increase in the number of immobile CB1Rs.

In both conditions, the CB1Rs that remained mobile diffused with similar characteristics (control extrasynaptic: median = 0.297 μm²/s, IQR = 0.154–0.465 μm²/s, n = 832; control synaptic: median = 0.225 μm²/s, IQR = 0.083–0.506 μm²/s, n = 25; WIN extrasynaptic: median = 0.292 μm²/s, IQR = 0.12–0.478 μm²/s, n = 314; and WIN synaptic: median = 0.212 μm²/s, IQR = 0.11–0.455 μm²/s, n = 20; P > 0.05). Thus, an increase in the fraction of immobile extrasynaptic receptors is at the origin of the global reduction in the diffusion of CB1Rs that characterizes agonist-induced desensitization.

Our data are consistent with a model where agonist-induced desensitization affects the mobility of CB1Rs by diminishing the number of surface CB1Rs, reducing the fraction of synaptic CB1Rs, and augmenting the fraction of immobile CB1Rs in the extrasynaptic compartment (Fig. 4D).

**Discussion**

The significant therapeutic potential of drugs acting on the eCB system and the fact that CB1R is the principal molecular target of the most widely used illegal drug (marijuana) have generated great interest in the elucidation of the eCB system (1, 3, 25).

Here, single-quantum dot imaging was combined with pharmacological approaches to identify how agonist desensitization has an impact on the surface movements of native CB1Rs and controls their behavior in cortical synapses in vitro. We present evidence that a presynaptic receptor, CB1R, displays significant movements within axon terminals. Together, the results are consistent with the broad notion that receptor mobility plays a major role in regulating the functions of presynaptic receptors. Our data indicate that agonist desensitization has an impact on the mobility of axonal CB1Rs and results in their synaptic exclusion.

**Mobility of Presynaptic CB1Rs.** Receptor trafficking regulates synaptic transmission (4, 26). Trafficking of AMPARs in and out of the synapses is one of the core mechanisms underlying the expression of long-term plasticity (26). The discovery that surface neurotransmitter receptors are mobile and rapidly exchange in and out of the postsynaptic specialization provides synapses with an additional means to quickly control their gain (4, 5, 23). Constitutive endocytosis of CB1R in somatodendritic membranes participates in their polarized distribution (21, 27–30). In accord with their inhibitory functions, the majority of surface CB1Rs are located in the axonal compartment and the postsynaptic terminal (31, 32). Until the present study, the mobility of presynaptic receptors has never been evaluated. Surface CB1Rs are remarkably dynamic: ~80% of all CB1R were mobile, a value substantially higher than that reported for postsynaptic ionotropic receptors (12, 33). Similarly to other postsynaptic receptors (5), CB1Rs alternate between periods of...
diffusive movement within the synaptic and extrasynaptic compartments. The short synaptic dwell time of CB1Rs compared with postsynaptic AMPARs (12, 13) may be due to intrinsic receptor properties, differences in anchoring, and interacting partner proteins, and more generally to the marked structural differences in presynaptic and postsynaptic specializations. In accord with the concept that receptor dynamics reflect the features of their immediate surroundings, extrasynaptic (i.e., axonal) CB1R behaviors indicated free diffusive movement, whereas CB1Rs displayed more confined movement within the spatially restricted synaptic domain.

Desensitization Has an Impact on CB1R Mobility. During chronic consumption of cannabis derivatives, such as marijuana, CB1Rs show a marked tolerance (17–19). Desensitized CB1Rs are internalized via the clathrin-coated pit pathway. The dynamics of the CB1Rs that remain on the neuronal surface after desensitization have never been explored. We found that a reduction in the mobility of CB1Rs reflects agonist-induced desensitization. This effect was due to a massive increase in the fraction of immobile CB1Rs and not a global reduction in the diffusion of mobile CB1Rs, showing that agonist-induced desensitization controls discrete features of CB1R dynamics.

Desensitization Excludes Surface CB1Rs from the Synaptic Terminal. The eCB system is a retrograde signaling system where eCBs produced in the postsynapse cross the synaptic cleft, bind to presynaptic CB1Rs, and inhibit transmitter release (3). A unique in vivo exposure to Δ9-tetrahydrocannabinol causes a reversible loss of CB1R function that is not due to CB1R internalization or uncoupling (8, 16). The present data identify a mechanism allowing synapses to efficiently reduce CB1R response after agonist activation. Desensitization was accompanied by a marked decrease in the fraction of immobile receptors and a selective decrease in the diffusion coefficients of extrasynaptic CB1Rs. The data show that desensitized CB1Rs are gradually slowed down and immobilized in the extrasynaptic zone, resulting in a progressive loss of synaptic CB1Rs. The functional characteristics of immobilized CB1Rs are unknown, but an exciting hypothesis is that they are incompetent in signaling. In all cases, it is likely that receptors outside the synapse receive fewer signaling eCBs than their synaptic counterparts.

Conclusions

Our data shed light on the dynamic behavior of native presynaptic CB1Rs and suggest that subtle regulations of CB1R movements participate in the complex molecular cascade underlying agonist-induced desensitization. The presently revealed mechanism has significant implications for the study of mechanisms underlying tolerance of other presynaptic GPCRs.

Materials and Methods

Cortical Cell Culture. Cultures of cortical neurons were prepared from Swiss mice (Janvier). On the day of birth the pups were killed by cervical dislocation, and dissected cortices were dissociated with the use of papain and were finally triturated mechanically. Cells were plated at a density of 360,000 cells per ml and grown on poly-L-lysine-coated coverslips in MEM supplemented with Serum Supreme (BioWhittaker). Polyclonal CB1R N-terminus-specific antibodies were visualized with Alexa 488-conjugated anti-rabbit IgG (Invitrogen). The monoclonal anti-MAP2 (1:500), anti-Tau1 (1:500), and anti-GAD65 (1:500) antibodies were acquired with an epifluorescence microscope (Leica DM microscope; oil immersion 100×, N.A. 1.35 objective). Single-particle tracking was performed at 32°C with an inverted microscope (Olympus IX71) equipped with an oil immersion 100×, N.A. 1.35 objective. QDots and MitoTracker were illuminated with a mercury lamp (Olympus) and detected by using appropriate excitation (HQ560/55, HQ480/40) and emission (D655/20, HQ535/50) filters (Chroma Technology). A total of 1,500 consecutive frames were acquired at 20 Hz with an EM-CCD camera QuantEM (20 Hz) or a CCD camera CoolSNAPq (2.5 Hz; Figs. 2 and 4A) (Photometrics).

Single-Particle (Qdot) Tracking. Polyclonal CB1R N-terminus-specific antibodies were incubated with QDot 655 Fab (34); anti-rabbit (Thermo Fisher Scientific) in PBS for 2 h. The incubation was then blocked with casein for 30 min. CB1R antibodies coupled to QDots 655 (CB1R-QD655) were filtered through a column of Superdex 200 gel (Amersham Biosciences). The same protocol was applied for monoclonal anti-GluR2 antibodies (Chemicon) coupled with QDot 655 Fab(-), anti-mouse.

Neurons were incubated with CB1R-QD655 or GluR2-QD655 for 10 min at 37°C, washed, and incubated for 1 min with a synaptic marker MitoTracker (20 nM; Invitrogen). After the final wash, coverslips were mounted in a chamber with permanent perfusion of a recording medium (160 mM NaCl, 10 mM Hepes, 10 mM glucose, 2.4 mM KCl, and 1.5 mM CaCl2). The imaging of internalized CB1Rs, all of the movies were taken within 20 min. Control experiments performing acid stripping (pH 2.8, 4 min, 4°C) did not show internalized CB1R-QD655 after 20 min of incubation (data not shown) (34).

Trajectory Analysis. The CB1R-QD655 or GluR2-QD655 recording sessions were processed with MetaMorph software (Universal Imaging). As previously described (12), the spatial distribution of the signals of the CCD is fitted to a 2-dimensional Gaussian surface. The 2-dimensional trajectories of single molecules in the plane of focus were constructed by correlation analysis between consecutive images using a Vogel algorithm. Tracking of single QDots was performed with custom software written with Matlab (Mathworks). The MSD was calculated according to \( \langle r^2(t) \rangle = \sum_i (r_i(t) - r_i(0))^2 \) for reconnected trajectories. The diffusion coefficient (D) was calculated from the slope of the first 4 points of MSD curves versus time using \( \langle r^2(t) \rangle = 2Dt \) in 4D. To assign synaptic localization, trajectories were sorted into extrasynaptic and synaptic by using the mitochondria marker MitoTracker image (9–11): MitoTracker-positive pixels defined the synaptic zone (11). Synapse dwell time was calculated as a mean residency time of CB1R-QD655 within synapses. All of the trajectories of CB1R that did not leave the synapse during the imaging period were excluded.

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Supporting Information

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Fig. S1.  In cortical neuronal cultures, CB1Rs are expressed in both GABAergic and non-GABAergic neurons. (A) Illustrative image of the overlay of GAD65 immunoreactivity and bright-field image. (Scale bar, 10 μm.) (B) Quantification of percentage of GAD65-positive neurons calculated from 3 independent experiments (n = 603). (C) Illustrative image of overlay of GAD65 (red) and CB1R (green) immunoreactivity. (Scale bar, 6 μm.) (D) Quantification of percentage of CB1Rs detected on GAD65-positive neurites calculated from 4 independent experiments (n = 89).
Fig. S2. Short agonist treatment had no effect on CB1R surface mobility. Thirty-minute treatment with the cannabinoid agonist had no consequence on CB1R mobility (t test, \( P > 0.05 \)). Similarly, 30-min treatment with the antagonist AM281 had no effect (control \( n = 1,192 \), AM281 \( n = 1,116 \), and WIN \( n = 930 \) trajectories).
**Movie S1**. CB1R lateral mobility on the surface of cortical neurons. Sample video of lateral mobility of CB1R coupled with Quantum dot (CB1R-QD655). Synapses were visualized by an active mitochondrial marker (MitoTracker, green); CB1R-QD655 (red).