Acute Enhancement of Synaptic Transmission and Chronic Inhibition of Synaptogenesis Induced by Perfluorooctane Sulfonate through Mediation of Voltage-Dependent Calcium Channel

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Perfluorooctane sulfonate (PFOS) is a persistent and bioaccumulative pollutant ubiquitous in wildlife and humans. Although the distribution and fate of PFOS have been widely studied, its potential neurotoxicity remains largely unknown. In the present study, the acute and chronic effects of PFOS on the development and synaptic transmission of hippocampal neurons was examined. Perfusion with PFOS markedly increased the frequency of miniature postsynaptic currents (mPSCs) and slightly elevated the amplitude of mPSCs in cultured hippocampal neurons. Perfusion with PFOS also increased the amplitude of field excitatory postsynaptic potentials (fEPSPs) recorded in the CA1 region of hippocampal slices. Both of these effects were largely blocked by the L-type Ca^{2+} channel antagonist nifedipine. Further studies showed that PFOS enhanced inward Ca^{2+} currents and increased intracellular Ca^{2+} in cultured neurons; these effects were also substantially inhibited by nifedipine. Moreover, prolonged treatment with PFOS moderately inhibited neurite growth and dramatically suppressed synaptogenesis in cultured neurons in a nifedipine-sensitive manner. Thus, through enhancement of Ca^{2+} channels, PFOS may exhibit both acute excitotoxic effects on synaptic function and chronically inhibit synaptogenesis in the brain.

Introduction

Perfluorooctane sulfonate (PFOS) belongs to a class of man-made, fully fluorinated organic chemicals, which are exceptionally stable to metabolic and environmental degradation (1–3). Because of its special properties, especially the oil- and water-resistance, PFOS and its derivatives have been widely used in a variety of products, such as surfactants, emulsifiers, nonstick pans, carpets, furniture, household

cleaners, shampoos, shoes/clothing, and packaged food containers (3). Release of PFOS and its derivatives into the environment occur from the product manufacturing processes, supply chains, and product use and disposal. Previous studies have shown that PFOS is a persistent pollutant, which is ubiquitous in wildlife (2, 3) and humans (4).

Because of its unique properties, PFOS does not accumulate in fat, like many other persistent pollutants, but may accumulate in proteins. PFOS and its derivatives are not easily eliminated (the half-life of PFOS in humans falls in the range of several years) and can build up to high levels, posing a great threat to human health (5). Evidence showed that PFOS accumulates up to 1–10 mg/kg (approximately 2–20 μM) in some animal tissues (1) and 1.82 μM in serum of occupational workers (4). The potential toxicity of PFOS, such as hepatotoxicity, interference with mitochondrial bioenergetics, impeding intercellular communication through gap junctions, endocrine dysfunction, effects on development and reproduction, and carcinogenicity, has been documented (3, 6, 7). A previous study revealed that PFOS accumulates in rat brain after subchronic exposure (6). However, the consequences of acute and chronic PFOS exposure on neural function and the underlying mechanisms have not been explored.

Calcium homeostasis is critical for various physiological and pathological processes in neurons (8, 9). Superfluous intracellular Ca^{2+} , either *via* influx from the extracellular space or released from internal stores, disturbs cellular calcium homeostasis and in turn influences development and function of neurons (9). Because of its huge concentration gradient across the plasma membrane (1.5 mM outside and 0.1 μM inside), Ca^{2+} can be driven into the cell through Ca^{2+} -permeable influx channels (10). Ca^{2+} influx is mainly mediated through three pathways: voltage-dependent calcium channels (VDCCs), transient receptor potential (TRP)-related calcium channels, and store-operated calcium channels (SOCCs) (11). In vertebrates, VDCCs are classified into L-, N-, P/Q-, R-, and T-types (12). Activation of VDCCs is critical for neurotransmitter release, neuronal excitability, and postsynaptic Ca^{2+} signaling (8, 13). In this work, using multiple approaches, including whole cell patch-clamp recording, field potential recording, calcium imaging, and immunocytochemical assay, we found that PFOS had both acute and chronic effects on development and function of hippocampal neuronal synapses, probably *via* enhancement of Ca^{2+} influx through VDCCs.

Experimental Section

Heptadecafluorooctane sulfonic acid potassium salt [$\text{CF}_3(\text{CF}_2)_7\text{SO}_3\text{K}$, MW 538.22, 98%], pentadecafluorooctanoic acid [$\text{CF}_3(\text{CF}_2)_6\text{COOH}$, MW 414.07, 96%], and 1H-heptadecafluorooctane [$\text{CF}_3(\text{CF}_2)_7\text{H}$, MW 420.07, 99%] were used as the standards for PFOS, PFOA, and PFOC, respectively. The doses (1, 10, and 100 μM) of PFCs were administrated according to previous literature (7, 14). Primary hippocampal neuron cultures and acute hippocampal slices from Sprague–Dawley rats were prepared as described previously (15, 16). Voltage-clamp recordings were performed in a whole cell patch-clamp configuration, and field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA1 region of hippocampal slices (17, 18). Calcium imaging was performed in an Olympus confocal laser-scanning microscope. Cultured neurons transfected with green fluorescent protein (GFP) plasmid and immunostained with PSD95 marker were respectively subjected to examination of PFOS effect on

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FIGURE 1. PFOS-induced increase in mPSC frequency in cultured rat hippocampal neurons. (A) Typical mPSC recording from a neuron at 8 DIV. Black bar indicates perfusion with PFOS. Insets are expanded segments showing mPSC frequency was increased by PFOS application. (B) Normalized mPSC frequency from >10 neurons from at least 3 separate cultures showing a dose-dependent increase in the frequency. $***P < 0.001$ vs control, paired t -test. This increase was significantly decreased by coapplication of PFOS and nifedipine ($***P < 0.001$, one-way ANOVA). (C) Summary data showing mean mPSC amplitude per cell in each condition. The numbers on the X-axis indicate the dose of PFOS used (Nife = nifedipine, 20 μ M); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs control, one-way ANOVA. (D) Average frequency versus mPSC amplitude showing that amplitude distribution was not shifted by PFOS (100 μ M). The black and red histograms indicate the control and PFOS groups, respectively. Error bars represent SEM in these and subsequent histograms.

neurite outgrowth and synaptogenesis under the Olympus confocal microscope. Results represent mean $\pm$ SEM. Statistical differences were determined by ANOVA or paired t -test where appropriate (significance level, 0.05). Detailed information regarding to experiments can be found in the Supporting Information.

Results

Potentiation of Synaptic Transmission in Hippocampal Neurons by PFOS. Spontaneous mPSCs were recorded in hippocampal neurons at 7–12 DIV. The mPSC frequency dose-dependently increased by 2.29 and 8.69 fold compared to control following application of 10 and 100 μ M PFOS, respectively (Figures 1A and 1B, $P < 0.001$, one-way ANOVA). The normalized mean mPSC amplitudes per cell were slightly increased by 100 μ M PFOS application but not elevated by 1 or 10 μ M PFOS (Figure 1C). Average mPSC frequency plotted against amplitude showed that Gaussian distributions of amplitude were not shifted by PFOS although the mPSC frequency was markedly enhanced (Figure 1D and Figures

S2A–C, left panels). The corresponding cumulative probability diagrams also showed that the distribution difference between control and PFOS was not significant (Figures S2A–C, right panels, $P > 0.05$, two-way ANOVA).

We used nifedipine, a specific L-type calcium channel blocker, to determine which subtype of calcium channel is involved in the effects of PFOS on synaptic transmission. Nifedipine (20 μ M) alone did not affect mPSCs but significantly depressed the increase in their frequency in response to 100 μ M PFOS by 51% (Figure 1B; increase in frequency: 8.69 fold versus 4.29 fold for PFOS only and PFOS plus nifedipine, respectively; $P < 0.001$, one-way ANOVA), suggesting that this neuromodulatory effect of PFOS may be mainly mediated by L-type VDCC. Application PFOS also induced a small increase in the amplitude of mPSC, but this increase was not significant inhibited by nifedipine (Figure 1C; increase in amplitude: 1.32 fold versus 1.26 fold for PFOS alone and PFOS with nifedipine, respectively; $P > 0.05$, one-way ANOVA).

FIGURE 2. Synaptic potentiation induced by PFOS in CA1 neurons of hippocampal slices. (A) Upper panels: fEPSP recordings before (black lines) and after (red lines) perfusion with PFOS (400 μ M, left), nifedipine (Nife, 40 μ M, middle), and PFOS plus nifedipine (right). Lower panel: the enhancement of fEPSP amplitude by PFOS was almost completely blocked by nifedipine. Black bar indicates perfusion with drugs. (B) Summary data (average fEPSP values from 10 to 30 min in (A)) illustrating that augmentation in fEPSP amplitude by PFOS was markedly reduced by nifedipine. Data normalized to average fEPSP amplitude in the control period (~10–15 min) before drug application for each recording. $n = 7-9$, * $P < 0.05$ vs control, t -test.

We next examined whether PFOS also affects synaptic transmission in brain slices. PFOS (400 μ M) significantly increased the fEPSP amplitudes. While nifedipine (40 μ M) alone did not affect basal synaptic transmission (Figure 2A), it largely blocked PFOS-induced augmentation of fEPSP (Figure 2B). This finding indicated that PFOS regulated synaptic transmission in brain slices mainly mediated by the L-type calcium channel, which was consistent with the results from cultured neurons.

Effect of PFOS on Calcium Channel. To further confirm involvement of VDCC in the PFOS-induced responses, we directly examined the effect of PFOS on the properties of depolarization-evoked calcium current in cultured neurons. Inward calcium currents (I_{Ca}) were recorded during a depolarizing pulse (0 mV, 150 ms) with a prepulse of -90 mV for 300 ms. The peak amplitude of I_{Ca} was dose-dependently increased by PFOS and the maximum enhancement of 53% occurred at 100 μ M (Figure 3B, $P < 0.01$, paired t -test).

Considering that PFOS is negatively charged when dissolved in water, we next determined the current-voltage (I - V) curves of calcium channels in the presence of PFOS (14). The recordings were made during a 500 ms ramp pulse from -100 to $+40$ mV with a 300 ms prepulse of -100 mV to avoid inactivation of calcium channels. To provide a simplified figure, we took a voltage value every 5 mV to plot against the corresponding current value and obtained the I - V relationships. 100 μ M PFOS not only markedly enhanced I_{Ca} but also caused a clear left-shift of the curve (Figure 3D, $P < 0.001$, two-way ANOVA). VDCCs are involved in various neuronal functions, among which the L-type has been widely studied (19). To further characterize the PFOS effect on calcium channel subtypes, nifedipine was added to the bath solutions during current recordings. Total I_{Ca} was reduced to 75% by 20 μ M nifedipine, and additional application of PFOS (100 μ M) did not elevate the current, indicating that the PFOS-induced enhancement of the Ca^{2+} current is mainly mediated by L-type VDCC (Figure 3E).

The effect of PFOS on the voltage-dependence of the calcium channel was evaluated as the half activation voltage ($V_{1/2}$) and measurement of steady-state inactivation of calcium currents. PFOS shifted the voltage, moving the maximum current amplitude toward a more negative potential (Figure 3D). A concentration of 100 μ M PFOS induced a significantly leftward shift (8.8 mV) of the $V_{1/2}$ activation, an effect that was significantly inhibited in the presence of nifedipine (decreased to 4.53 mV, $P < 0.001$, paired t -test;

Figure S4A). The effect of PFOS (100 μ M) on the voltage-dependence of steady-state inactivation was assessed with a double-pulse regime (see details in the legend of Figure S4). The relationship between prepulses and the amplitude of calcium current, normalized to the maximum amplitude, was fitted by a single Boltzmann function: $I/I_{max} = 1/(1 + \exp((V - V_{1/2})/S))$, where $V_{1/2}$ and S indicate half-inactivation voltage and the slope factor. Statistical analysis suggested that the voltage-dependence of inactivation was significantly leftward shifted although the shift was comparatively small (3 mV, $P < 0.001$, two-way ANOVA; Figure S4D).

PFOA and PFOC are structurally similar to PFOS, and all three are eight-carbon perfluorinated chemicals (PFCs). When dissolved in water, both PFOS and PFOA are in ionic form with one negative charge, while PFOC is in molecular form. In this study, PFOA was utilized for toxicity comparison with PFOS while PFOC was used to reveal what role the negative charge played in affecting I_{Ca} . A dose-dependent increase of I_{Ca} evoked by a depolarizing pulse was recorded in neurons with PFOA application (Figure 3B, $P < 0.05$, paired t -test), although the enhancement was slightly less than that of PFOS. A similar pattern of increased I_{Ca} was found with PFOC (Figure 3B). Then the I - V curves were measured using the ramp protocol outlined above in the presence of PFOA and PFOC (both 100 μ M), and the two compounds elevated the peak amplitudes but not as markedly as PFOS (Figure 3E). PFOA negatively shifted $V_{1/2}$ activation by 2.97 mV while PFOC did not (Figure S4A). $V_{1/2}$ measurements suggested that neither compound negatively shifted the steady-state inactivation curve of I_{Ca} (Figure S4D). These results indicated that all three perfluorinated compounds increased the calcium current to different degrees, and both PFOS and PFOA influenced the voltage-dependence, while PFOC did not.

Increased Intracellular Calcium by PFOS. To directly observe effects of PFOS on $[Ca^{2+}]_i$, cultured neurons were loaded with Ca^{2+} dye fluo-4 AM and fluorescence signal was imaged. We found that the basal level of fluorescence intensity, which represents basal $[Ca^{2+}]_i$ produced by spontaneous neuronal activities that may induce membrane depolarization and thus activation of VDCC, was significantly increased shortly (within 10–20 s) after application of PFOS (Figures S5 and 4A, $P < 0.001$, one-way ANOVA). To assess the mechanical effects of perfusion on fluorescence intensity, PFOS-free saline was also perfused into the recording bath and no change in the fluorescence intensity was found (Figure

FIGURE 3. Involvement of PFOS in calcium channel changes in cultured rat hippocampal neurons. (A) I_{Ca} recordings obtained during a depolarizing pulse (0 mV, 150 ms) with a prepulse of -90 mV for 300 ms (upper panel) showing that PFOS markedly increased I_{Ca} (lower panel). (B) Summary data of I_{Ca} elicited by the depolarizing pulse. Data normalized to mean peak amplitude of I_{Ca} before drug application (control) in each neuron. Peak I_{Ca} was dose-dependent. $n = 7-9$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control, paired t -test. (C) I_{Ca} induced by a 500 ms ramp pulse from -100 to +40 mV with a 300 ms prepulse of -100 mV in absence and presence of PFOS. Inset shows the protocol. (D) The $I-V$ curve was significantly left-shifted by 100 μ M PFOS. Black squares and red circles show control and drug application, respectively. (E) Average peak amplitude of I_{Ca} obtained with the ramp pulse protocol. Both PFOA and PFOC elevated I_{Ca} but not as much as PFOS (all at 100 μ M). I_{Ca} inhibition by nifedipine was not increased by additional application of PFOS (Nite = nifedipine, 20 μ M; $P > 0.05$, one-way ANOVA). $n = 9-14$, ** $P < 0.01$, *** $P < 0.001$ vs control, paired t -test.

4A). The fluorescence enhancement became evident at 10 μ M PFOS and showed a clear dose-response relationship (Figure 4A). Perfusion with Ca^{2+} -free extracellular solution (ECS) blocked $[Ca^{2+}]_i$ elevation induced by PFOS at doses less than 100 μ M, indicating that PFOS-induced $[Ca^{2+}]_i$ elevation resulted from Ca^{2+} influx. A small increase in $[Ca^{2+}]_i$ was observed when PFOS was applied at 300 μ M in Ca^{2+} -free ECS (Figure 4B), which may be caused by PFOS-induced nonspecific toxic effect or PFOS-induced Ca^{2+} release from intracellular stores.

To verify whether VDCCs are indeed involved in the PFOS-induced increase in $[Ca^{2+}]_i$, high- K^+ saline (90 mM) was used to evoke depolarization, which in turn activates VDCCs. We found that perfusion of 90 mM KCl markedly increased the fluorescence in neurons and additional application of PFOS (100 μ M) further enhanced this increase. The PFOS-induced increase in fluorescence intensity was inhibited by application of 20 μ M nifedipine (Figures 4C and 4D), confirming involvement of L-type calcium channels in the PFOS-induced increase in $[Ca^{2+}]_i$.

Chronic Effects of PFOS on Neurite Growth and Synaptogenesis. Pre-experiments showed no significant cytotoxicity was observed in cultured neurons treated with relatively high dose (50 μ M) of PFOS for 2 weeks (data not shown), which suggested the doses set in this work were reasonable for evaluation of the *in vitro* toxicity of PFOS to

neurons. Cultured neurons were transfected with GFP so that neuronal processes could be clearly discerned. Four parameters were measured: (1) the length of the longest neurite, irrespective of whether it was the axon or not, (2) the summed length of neurites per cell, (3) the number of neurite bifurcations, and (4) the number of neurites extending from the soma. Neurite length was markedly decreased by PFOS treatment (Figure 5A). Exposure to 10 μ M PFOS slightly decreased the length of the longest neurite by 8% (Figure 5B, $P > 0.05$, one-way ANOVA). Furthermore, 50 μ M PFOS significantly decreased the length by up to 25% ($P < 0.001$, one-way ANOVA). Coexposure to PFOS (50 μ M) and nifedipine (20 μ M) effectively reduced the decrease in length of the longest neurites as compared to PFOS alone (86% versus 75% of control), which revealed that the involvement of L-type VDCC is mainly response $((0.86-0.75)/(1-0.75) \times 100\% = 44\%)$ for this process and other subtypes of VDCCs are involved as well. This also confirmed the speculation that PFOS-induced elevation of $[Ca^{2+}]_i$ was mainly mediated by L-type VDCC. A similar pattern was found in the summed length of neurites per cell (Figure 5C). The number of neurite bifurcations decreased to 80% of control with PFOS alone (50 μ M) but not with PFOS plus nifedipine (Figure 5D). No significant differences were found in the number of neurites extending from the soma (Figure 5E). These results indicated

FIGURE 4. Time course of fluorescence changes by PFOS in cultured rat hippocampal neurons. (A and B) $[Ca^{2+}]_i$ was significantly enhanced by PFOS application (black bar) both in normal ECS (A) and Ca^{2+} -free ECS (B). The fluorescence enhancement showed a clear dose–response relationship ($P < 0.001$, one-way ANOVA). (C) Increase of fluorescence in the presence of 90 mM KCl (black bar) was further elevated by application of PFOS alone but was reduced by coapplication of PFOS and nifedipine (PFOS, 100 μ M; Nife = nifedipine, 20 μ M). (D) Summary results from experiments as shown in (C). The intensities of three scanned images at the peak of the fluorescence curves were averaged for each group (K^+ = KCl, 90 mM; $**P < 0.01$, $***P < 0.001$ compared to the “ K^+ ” group, one-way ANOVA). Note: in A and B numbers refer to dose (μ M) of PFOS, and in A, B, and C numbers in parentheses refer to the number of cells tested.

that a relatively high dose of PFOS caused developmental inhibition of neurite growth in cultured neurons.

To further investigate the developmental inhibition induced by PFOS, neurons were continuously treated with both PFOS and nifedipine, and then synapses were visualized using the marker PSD95 (20). In control and 10 μ M PFOS exposure, PSD95 clusters were clearly present on dendrites (Figure 5F). The clustering density decrease in dendrites was quite clear after 14 or 15 days of exposure to 50 μ M PFOS (Figure 5G, $P < 0.001$, one-way ANOVA). Coexposure to PFOS (50 μ M) and nifedipine (20 μ M) potently reduced the depression in cluster density as compared to PFOS exposure alone (75% versus 52% of control).

Discussion

PFOS Induced Ca^{2+} Influx through Action on Ca^{2+} Channels.

Recent studies have begun to elucidate the specific actions and potential mechanisms of PFOS on the cell membrane (21, 22). In view of its amphiphilic nature, PFOS may act primarily on the cell membrane by increasing the fluidity (7) and permeability (21). Such alterations are likely due to PFOS decreasing the cholesterol content of cell membrane (21). There is evidence that alterations in membrane properties have a considerable impact on its various functions and substructures (23). In the present study we found that PFOS evidently augmented I_{Ca} (Figure 3E) and significantly shifted the I – V curves of I_{Ca} in the negative direction (Figures 3D and S4A), consistent with previous reports (14, 24). Together with calcium imaging experiments (Figures 4C and 4D), our findings indicated that the PFOS-induced increase in I_{Ca} was

FIGURE 5. PFOS exposure inhibited neuron growth. (A) Representative images showing that neurite growth in 4 DIV neurons transfected with GFP was inhibited by PFOS exposure. Scale bar, 20 μ m. (B) Coexposure to PFOS (50 μ M) and nifedipine (20 μ M) effectively reduced the decrease in length of the longest neurites compared with exposure to PFOS alone. $*P < 0.05$ between exposure to nifedipine alone and coexposure of PFOS and nifedipine. (C) Similar changes were found in the summed length of neurites per cell. (D) The number of neurite bifurcations was decreased to 80% of control by PFOS alone but not by PFOS plus nifedipine. (E) No significant differences were found in the number of neurites extending from the soma. (F) Representative images showing that PSD95 cluster density in neurons labeled with Cy3 was decreased by PFOS exposure. Scale bars, 50 μ m; 10 μ m for insets. (G) Coexposure to PFOS (50 μ M) and nifedipine (20 μ M) potently reduced the depression in cluster density compared to exposure to PFOS only (75% versus 52% of control). Note, in B to E and G, the numbers on the X-axis indicate the dose of PFOS, Nife = nifedipine (20 μ M), and the numbers in parentheses indicate the number of cells tested. $**P < 0.01$, $***P < 0.001$ vs control, one-way ANOVA.

mainly mediated by L-type calcium channel. However, involvement of other subtypes of calcium channels cannot be excluded, since PFOS-induced $[Ca^{2+}]_i$ increase was not completely blocked by nifedipine. As for the specific mechanism of action, Harada and co-workers (14) deduced that PFOS may directly activate calcium currents at specific lipid

sites or have a direct action on the channel proteins themselves. Direct protein binding assays demonstrated binding between PFOS and proteins in serum (25). PFOS has a sulfonic group attached to the perfluorinated chain, and PFOA has a carboxylic group. PFOS is likely to become incorporated into the lipid bilayer of the cell membrane (24), which may explain the differential effects of the two compounds on the Ca^{2+} channels (Figure 3). In contrast to PFOS and PFOA, PFOC is an uncharged perfluorinated compound. PFOC caused less increase in Ca^{2+} current than that induced by PFOS or PFOA and did not induce apparent shift of the $I-V$ curves of the Ca^{2+} channels (Figure S4). The negative charged PFOS and PFOA may reduce the membrane potential by inserting into the lipid bilayer, resulting in a negative shift in the $I-V$ curve of I_{Ca} . Although not altering the membrane potential, PFOC may, like PFOS and PFOA, interfere with the membrane lipid environment surrounding the calcium channel protein and modulate the properties of the channel.

Acute Effects of PFOS on Synaptic Transmission. PFOS substantially induced Ca^{2+} entry into neurons through Ca^{2+} channels and thereby led to perturbations in $[\text{Ca}^{2+}]_i$. The perturbation of calcium homeostasis interferes with physiological processes in neurons, including synaptic activity (9). The mPSC frequency in cultured neurons was markedly increased by PFOS, indicating that neurons became excessively excitable after acute PFOS application (Figure 1B). The excitotoxicity contributes to neuronal degeneration in many acute CNS diseases (9). L-Type calcium channel antagonist nifedipine did not affect basal synaptic transmission but significantly depressed the increase in mPSC frequency following PFOS alone (Figure 1B). Since changes in the mPSC frequency mainly reflect presynaptic modulation of the synaptic transmission, our results suggest that PFOS may mainly influence presynaptic transmission. We found that the mPSC amplitude was slightly increased by application of high concentration of PFOS (Figure 1C), which may result from either pre- or postsynaptic modulation.

Our studies also showed that PFOS modulated synaptic transmission in hippocampal slices. PFOS significantly increased fEPSP amplitude and the facilitation lasted for at least 20 min (Figure 2A). Increasing evidence demonstrates a role for strong activation of L-type calcium channel in excitatory synaptic plasticity (26). Interestingly, PFOS-induced enhancement of fEPSP amplitude was largely prevented by joint application of nifedipine (Figures 2 and S3). Thus, the PFOS-induced potentiation of synaptic transmission in both cultured neurons and brain slices involves Ca^{2+} influx mainly through the L-type VDCC.

Chronic Effects of PFOS on Neurite Growth and Synaptogenesis. $[\text{Ca}^{2+}]_i$ modulation is important for neuronal functions because it influences a broad range of biological events, including differentiation, neurite outgrowth, and synaptogenesis (10, 27). Neuronal calcium regulation is especially robust because a massive calcium influx occurs through multiple subtypes of VDCCs during membrane depolarization (27, 28). The proper development of complex neural networks occurs only at an optimal range for $[\text{Ca}^{2+}]_i$ (29) in which neurons maintain a baseline $[\text{Ca}^{2+}]_i$ (30). Each form of neurite outgrowth and branching results from specific molecular mechanisms that remain still elusive. Evidences show that an abnormal and high $[\text{Ca}^{2+}]_i$ can inhibit neurite outgrowth (30–32), although other studies have found that elevating $[\text{Ca}^{2+}]_i$ in growth cones promotes neurite outgrowth (33–35). Similarly, enhanced synaptic activity may cause either down-regulation or up-regulation in synaptogenesis. These discrepancies might be explained by many factors, such as differences in resting $[\text{Ca}^{2+}]_i$ relative to the optimal 'set point' for different types of neurons, or the experimental conditions used (30). Our findings showed that the length of neurites and number of bifurcations in cultured neurons

were reduced with long-term exposure to PFOS (Figure 5). PFOS administration caused a transient but fairly large elevation of $[\text{Ca}^{2+}]_i$ (Figure 4), which may cause a cascade of transduction changes and eventually induce long lasting changes in the function and structure of neurons. Expression levels of PSD95 clustering in dendrites reflect synaptogenesis and synapse formation (20). We found that the dendritic PSD95 density was suppressed in cultured neurons exposed to PFOS for a long period (Figure 5G). These findings indicate that both acute and chronic exposure of PFOS influenced the normal development and functions of central synapses. The results that cotreatment with nifedipine largely prevented PFOS-induced inhibition on neurite outgrowth and synaptogenesis (Figure 5) indicate the involvement of L-type calcium channels, although other mechanisms may be also involved.

PFOS Toxicity and Relevance to Human Health. Widespread use of PFOS has caused a global pollution in various media (1–5). PFOS has been found at ppb level in various wildlife species and PFOS can accumulate up to 1–10 mg/kg (approximately 2–20 μM) in some animal tissues (1) and 1.82 μM in serum of occupational workers (4). PFOS is readily absorbed orally and distributes primarily in the serum and liver but poorly eliminated (5). PFOS presents moderate acute toxicity by the oral route with a rat LD_{50} of 251 mg/kg. Rodent studies show that a NOAEL of 1 mg/kg/day and a LOAEL of 5 mg/kg/day for developmental toxicity (36). Johansson et al. (37) reported that neonatal exposure (1.4 or 21 $\mu\text{mol/kg}$) of PFOS (~1.4 or 21 μM) caused irreversible neurobehavioral defects such as reduced and/or lack of habituation and hyperactivity in adult mice. Our data demonstrated PFOS (10 μM and above) induced a robust augmentation of mPSC frequency (Figure 1B), which may cause excessive excitability in neurons. This indicates that our results from *in vitro* exposure are fairly consistent with those of from *in vivo* exposure. Austin et al. (6) results showed that accumulation of PFOS in rat hippocampus ranged from 0.1–9 $\mu\text{g/kg}$ (~0.2–17 μM) after subchronic exposure (1, 10 mg/kg). For developmental studies, Lau et al. (38) reported a serum level of 100 $\mu\text{g/mL}$ in the newborn rats exposed to 5 mg/kg of PFOS prenatally, where 50% neonatal mortality was evident. Assuming PFOS in adult rat brain is about 5–6% of serum level, this would provide 5 $\mu\text{g/g}$ (~9 μM) of PFOS in the hippocampus, although PFOS is a charged molecule and therefore does not cross the blood–brain barrier readily. On the other hand, the blood–brain barrier may not be fully closed at birth, so one may argue for a higher concentration of PFOS accumulated in the brain, say a maximum of 30–40 μM . In our experiments, the effects of PFOS on mPSC and $[\text{Ca}^{2+}]_i$ were evident at 10 μM and above (Figures 1 and 4). Comparably the low doses of PFOS (1, 10 μM) used in our experiments are environmentally relevant and accordingly, the potential damage of PFOS to human health is suggested herein. Meanwhile, all responses tested were evident at 100 μM . Considering PFOS's bioaccumulation and persistence nature, the effects induced by 100 μM PFOS in the present study might be produced by chronic exposure of PFOS at relative low concentrations. Given that the tolerance of brain slice to contaminant is stronger than that of cultured neuron since brain slice is comparatively close to physiological status, a higher dose (400 μM) of PFOS is administrated to the brain slice, where the dose is still comparable to the LD_{50} of 251 mg/kg. The PFOS concentration in the general population in the U.S. is estimated to be about 30 ng/mL (~0.06 μM) (39). Although this level is slightly lower than the lowest dose in our experiment, the intracellular Ca^{2+} may still be interfered by PFOS accumulated in the body over a long time.

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Supporting Information Available

Details of experimental section including instrumentation, reagents, calcium imaging, and morphological observation. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

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