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Changes in Synaptic Transmission, Calcium Current, and Neurite Growth by Perfluorinated Compounds Are Dependent on the Chain Length and Functional Group

CHUNYANG LIAO,[†,‡] THANH WANG,[†] LIN CUI,[†] QUNFANG ZHOU,[†] SHUMIN DUAN,[§] AND GUIBIN JIANG*[,†]

State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China, Yantai Institute of Coastal Zone Research for Sustainable Development, Chinese Academy of Sciences, Yantai, Shandong 264003, China, Institute of Neuroscience and Key Laboratory of Neurobiology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

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Scientific and public concerns on perfluorinated compounds (PFCs) are increasingly growing because of their environmental persistency, bioaccumulation, and extensive distribution throughout the world. Little is known about the effects of PFCs on neural function and the underlying mechanisms. Recent evidence suggests that the toxicological effects of PFCs are closely correlated with their carbon chain lengths. In this present work, the actions of PFCs with varying chain length on cultured rat hippocampal neurons and possible action patterns were examined. Increases in the frequencies of spontaneous miniature postsynaptic current (mPSC) were commonly found in cultured neurons when perfused with PFCs. The increase of mPSC frequency was in proportion to the carbon chain length, and the potency of perfluorinated carboxylates was less pronounced than that of perfluorinated sulfonates. A comparable but less perceptible trend was also found for the amplitudes of voltage-dependent calcium current (I_{Ca}). No regular change in pattern was observed for the effects of PFCs on activation and inactivation kinetics of I_{Ca}. Furthermore, prolonged treatment of PFCs inhibited the neurite growth of neurons to various degrees. Comparisons between nonfluorinated and perfluorinated analogues demonstrated that the fluorination in alkyl chain exerts stronger actions on neurons as compared to the surfactant activity. This study shows that PFCs exhibit adverse effects on cultured neurons to various extents, which is dependent on the carbon chain length and functional group attached to the fully fluorinated alkyl chain.

Introduction

Perfluorinated compounds (PFCs), a class of fully fluorinated man-made hydrocarbons, are becoming widespread environmental pollutants as indicated by studies on animal species and humans (*1*−*3*). PFCs are compounds in which all carbon−hydrogen (C−H) bonds in the carbon backbone are replaced by carbon−fluorine (C−F) bonds and have been produced in large quantities and extensively used in a variety of industrial and consumer products for over 50 years (*4, 5*). Because of their exceptionally stable molecular structures, PFCs are resistant to hydrolysis, photolysis, biodegradation, and metabolism and thus become persistent and bioaccumulative when released into the environment.

Although PFCs have been present in the environment and biota for many decades, their environmental and biological effects remain largely unknown and their exposure routes are difficult to assess. Various volatile PFCs have been shown to degrade or metabolize to more persistent nonvolatile PFCs such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) (*6, 7*). The PFC levels in humans and wildlife are steadily increasing (*8, 9*). There is evidence that the bioaccumulation levels of PFCs and their actions on organisms are closely correlated with the carbon chain lengths (*10*−*12*). Post et al. (*13*) revealed that the negatively charged amphiphile dodecylsulfate increased the amplitude and leftward shifted the current−voltage (*I*−*V*) of the calcium currents (I_{Ca}) of isolated rabbit myocytes through either insertion into the bilayer of cell membrane or alteration of the lipid environment of cells. The amphiphilic nature of PFCs implies that they may exert their effects on cells in a similar manner like dodecylsulfate.

Neuronal intracellular free calcium concentration, $[Ca^{2+}]_i$, plays a critical role in multiple cellular processes and can generally be modulated by Ca^{2+} influx through voltage-gated channels during membrane depolarization (*14*). $[Ca^{2+}]_i$ may be disturbed by a wide range of neurotoxicants (*15*), and the resulting perturbation of calcium homeostasis in neurons interferes with various physiological processes, such as synaptic transmission and neurite outgrowth (*16*). Recent studies demonstrated that PFOS may accumulate in the brain and cause neurotoxicity (*17*) and that PFOA may lead to endocrine dysfunction (*5*), suggesting that PFC administration is likely to induce neurotoxic impairment. However, the specific process and potential mechanism for PFCs with varying chain length on neurons remain largely unclear. The aim of this work is to examine the effects of the specific structural properties such as chain length, functional group, and fluorination of PFCs on cultured hippocampal neurons of rat, including synaptic transmission, calcium current, and neurite growth, with emphasis on the potential correlation between the varying chain lengths and the extents of the adverse effects of PFCs.

Experimental Section

The abbreviation and molecular structures of nine perfluorinated and one nonfluorinated chemicals tested are listed in Table S1 in the Supporting Information. The chemical administrations (50, 100 μM) were in reference to previous works (*12, 18*). Primary hippocampal neuron cultures were prepared from Sprague−Dawley rats as described previously (*19*). Electrophysiological recordings were performed in a whole cell patch-clamp configuration (*20*). Neurons were transfected with green fluorescent protein (GFP) plasmid and were subjected to examine inhibition of PFCs to neurite outgrowth with an Olympus confocal laser-scanning mi-

* Corresponding author fax: 86-10-62849179; e-mail: gbjiang@rcees.ac.cn.

[†] Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences.

[‡] Yantai Institute of Coastal Zone Research for Sustainable Development, Chinese Academy of Sciences.

[§] Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

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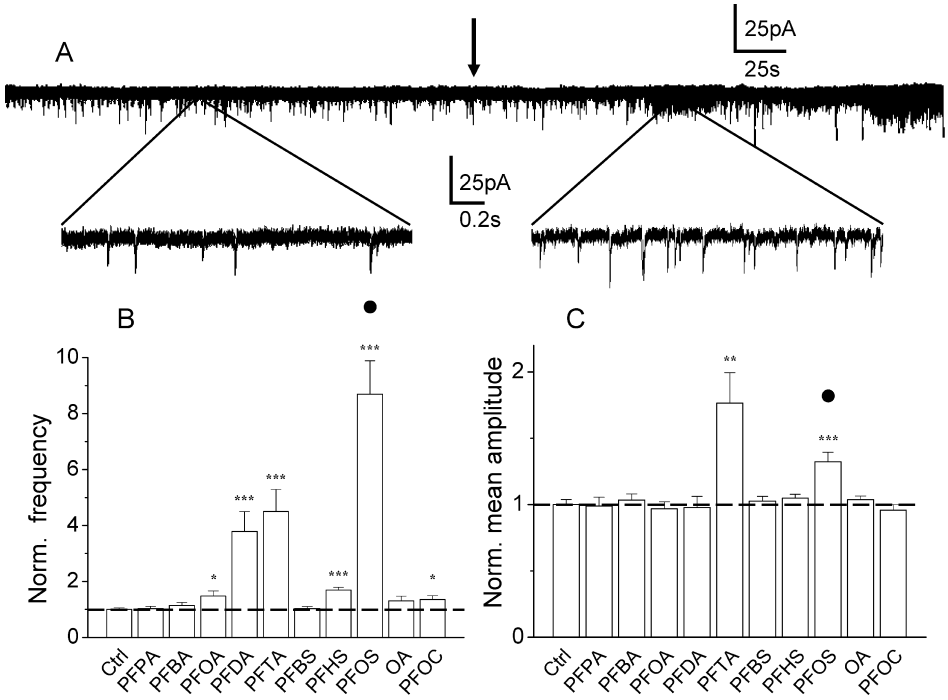

FIGURE 1. PFC-induced changes of mPSC in cultured hippocampal neurons are related to their chain length and functional group. (A) Typical trace of mPSC recorded before and after PFDA perfusion (indicated by arrow). Inset shows an expanded segment in which the mPSC frequency was increased by PFDA application. Scales: 25 pA, 25 s and 25 pA, 0.2 s for inset. (B) Normalized mPSC frequencies from ≥9 cells obtained from at least three separate cultures showing that PFCs increased the frequencies with increasing chain length. (C) Summarized results suggested that PFCs with longer alkyl chain including PFTA and PFOS can magnify the mean amplitude of mPSC. Data were compared to the control by paired *t*-test in both B and C: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Drug doses were all at 100 μM. Error bars represent SEM in this and subsequent figures. In B and C, the black circles indicate that the data regarding PFOS were cited from our previous paper (*30*) and here were incorporated into both histograms for discernible toxicity comparisons among selected PFCs.

croscope. Data are expressed as means ± SEM (standard error of the mean). Statistical differences were determined by paired *t*-test unless otherwise mentioned (significance level, 0.05). More experimental details can be found in the Supporting Information.

Results

Miniature Postsynaptic Current. Spontaneous miniature postsynaptic currents (mPSCs) were recorded in gap-free mode from hippocampal neurons at 8−15 days in vitro (DIV). Figure 1A shows an example recording of mPSC in the absence (control) or presence (drug perfusion) of 100 μM perfluorododecanoic acid (PFDA), which directly suggests that the mPSC frequency in cultured neurons was increased by PFDA application. The increase in frequency was in proportion to the carbon chain length of PFCs (all at 100 μM). Among perfluorinated carboxylates, both perfluoropropionic acid (PFPA, three carbons in its alkyl chain, C-3) and perfluorobutyric acid (PFBA, C-4) did not show any significant effects on the frequency, while PFOA (C-8), PFDA (C-12), and perfluorotetradecanoic acid (PFTA, C-14) increased the frequency by 1.48, 3.79, and 4.49 fold, respectively, as compared to the control (Figure 1B, $P < 0.05$). A similar alteration pattern of the mPSC frequency was found for perfluorinated sulfonates including perfluorobutane sulfonic acid (PFBS, C-4), perfluorohexanesulfonic acid potassium salt (PFHS, C-6), and PFOS (C-8). Comparatively, the increase of mPSC frequencies by PFCs can be considered to be lower for those containing a carboxylic group than those containing a sulfonic group. Among the PFCs containing the carboxylic group, PFTA magnified the mPSC amplitudes (Figure 1C, $P < 0.01$) while the rest did not affect the amplitudes. Similarly, only PFOS showed a clear impact on the mPSC amplitudes among the PFCs containing the sulfonic group (Figure 1C, $P < 0.001$). Cumulative probability diagrams of mPSC amplitudes for PFCs suggested that only PFTA exhibited a rightward shift of the curve (Figure S1 in the Supporting Information, $P < 0.001$, two-way ANOVA). Other PFCs, regardless with carboxylic group or sulfonic group, had no apparent effects on the curves (Figure S1 in the Supporting Information, $P > 0.05$, two-way ANOVA). In general, PFC-induced alterations in synaptic transmission were relevant to their chain length and functional group.

Voltage Dependence Nature of Calcium Channel. The inward calcium currents (I_{Ca}) were recorded in the absence or presence of PFCs (all at 100 μM) with a ramp depolarization pulse (Figure S2A in the Supporting Information). The voltage values were recorded and plotted versus the corresponding I_{Ca} every 5 mV, and the resulting current−voltage ($I-V$) relationship curve of PFOA is shown in Figure 2A as an example. The peak values of I_{Ca} at 0 mV were normalized to the control to compare effects of the selected PFCs on calcium channel (Figure 2C). Of the carboxylic PFCs, PFPA and PFBA did not magnify I_{Ca}, while PFOA and PFDA increased I_{Ca} by 27% and 25%, respectively, over the control. Surprisingly, PFTA, containing the longest chain length in all PFCs investigated, had no significant effect on the I_{Ca}. All three sulfonic PFCs steadily increased I_{Ca} with apparent correlation with their chain lengths; PFOS exerted more effects than PFBS and PFHS (Figure 2C, $P < 0.05$). The magnification of I_{Ca} by PFCs containing the carboxylic group was relatively lower than that containing the sulfonic group (Figure 2C).

The effect of PFCs (all at 100 μM) on the kinetics of I_{Ca} was further investigated by examining the steady-state activation and inactivation of calcium channel $I-V$ curves. The typical activation $I-V$ curve, such as that for PFOA, exhibits that the voltage showing the maximum current amplitude was increased and shifted toward negative potentials (Figure 2A).

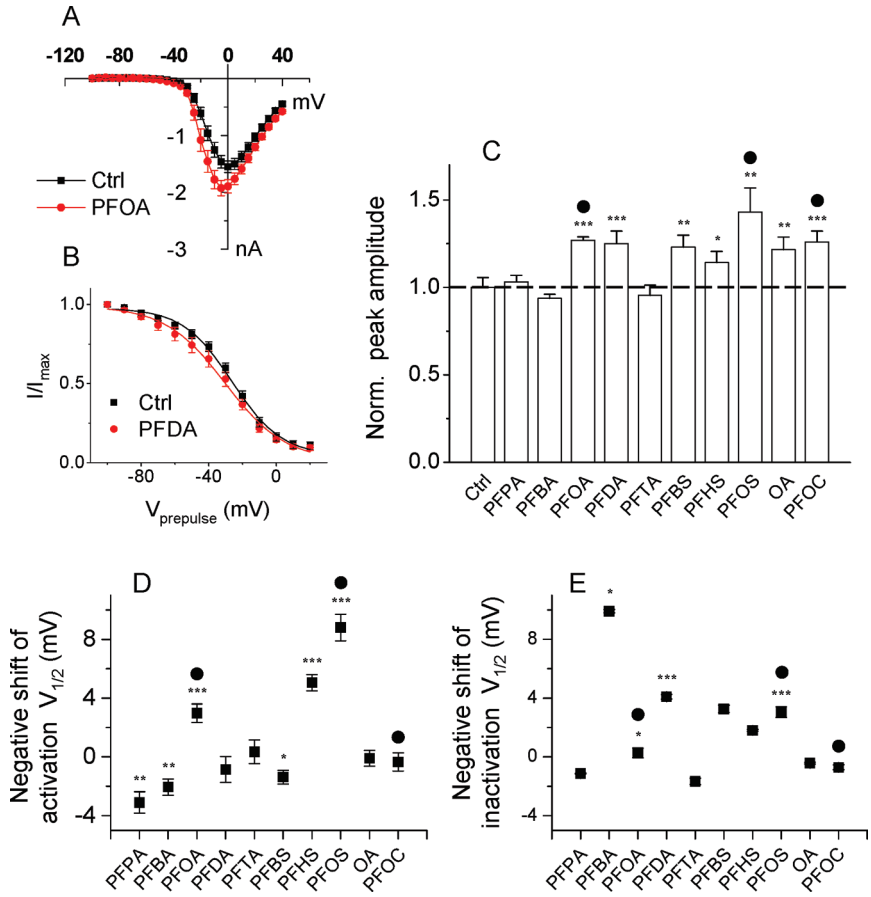

FIGURE 2. Involvement of PFCs and OA in calcium channels of cultured hippocampal neurons. (A) The *I*−*V* curve of I_{Ca} was evidently leftward shifted by PFOA. (B) Normalized peak amplitudes of the I_{Ca} (I/I_{max}) were plotted versus the inactivating prepulse for application of PFDA, and the smooth curve was fitted using the Boltzmann equation. (C) Average peak amplitudes of I_{Ca} at 0 mV, by normalization to the corresponding control, showing that I_{Ca} was basically elevated by PFCs with the increase of chain length. (D) Summary of activation $V_{1/2}$, obtained via normalization by reference to the maximum amplitude of *I*−*V* curves (see text), indicated that the effect on activation kinetics of I_{Ca} differs with PFCs and OA. In C and D, $n = 9-12$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control, paired *t*-test. (E) Summary data of inactivation $V_{1/2}$ showed that no apparent differences were observed in comparison of the effects of perfluorinated sulfonates to inactivation kinetics of I_{Ca} with those of perflurinated carboxylates. $n = 9-14$, *$P < 0.05$, ***$P < 0.001$ vs control, two-way ANOVA. Drug doses were all at 100 μM. In E, error bars are embedded in the symbols because of their small ranges; in E−G, the black circles indicate that the data regarding PFOS, PFOA, and PFOC were cited from our previous paper (*30*).

To address the negative shifts, we quantified the half-activation voltage ($V_{1/2}$), which is the voltage value showing half-maximum amplitude of I_{Ca}, for the different groups (Figure 2D). For the carboxylic PFCs, PFPA and PFBA shifted the *I*−*V* curves of I_{Ca} toward positive potentials while PFOA drove the curve toward negative potential (Figure 2D and 2A, $P < 0.01$). No significant shifts in *I*−*V* curves were observed by perfusions of PFDA and PFTA. For the sulfonic PFCs, PFBS slightly rightward shifted the *I*−*V* curve but PFHS and PFOS effectively leftward shifted the *I*−*V* curves (Figure 2D, $P < 0.05$). A typical trace of inactivation of *I*−*V* curve showed that PFDA leftward shifted the inactivation *I*−*V* curves (Figure 2B). For the carboxylic PFCs, PFPA and PFTA did not show any significant effects on the inactivation *I*−*V* curves of I_{Ca}, whereas PFBA, PFOA, and PFDA leftward shifted the curves (Figure 2E, $P < 0.05$). For the sulfonic PFCs, both PFBS and PFHS shifted the inactivation *I*−*V* curves slightly toward the left, while PFOS induced a marked leftward shift of the curves (Figure 2E, $P < 0.001$). Comparatively, PFCs containing the sulfonic group showed stronger effects on the activation *I*−*V* curves of I_{Ca} than those containing the carboxylic group, but no differences were observed in effects between the two classes for the inactivation curves of I_{Ca}.

Neurite Outgrowth Observation. We next explored the chronic effects of PFCs (all at 50 μM) on neuronal development functions such as neurite growth and bifurcation. Typical images showed that the effects of PFCs on neurite outgrowth of neurons appeared to vary with the chain length and the functional group attached to the fully fluorinated chain (Figure 3A). The incidences of development inhibition by PFCs in cultured neurons were studied in detail. For the carboxylic PFCs, PFPA and PFBA had no significant effect on the lengths of the longest neurites, but PFOA, PFDA, and PFTA suppressed the lengths about 20% shorter compared to the control (Figure 3B, $P < 0.05$, one-way ANOVA). For the sulfonic PFCs, PFOS reduced the lengths by 25% below that of the control whereas PFBS and PFHS had no evident influences (Figure 3B, $P < 0.001$, one-way ANOVA). Of the carboxylic PFCs, PFPA and PFOA basically did not affect the sum lengths of neurites per neuron while PFBA, PFDA, and PFTA decreased the sum lengths up to about 19% (Figure 3C, $P < 0.05$, one-way ANOVA). Of the sulfonic PFCs, PFBS and PFOS reduced the sum lengths by 25% and 31%, respectively, below that of the control (Figure 3C, $P < 0.01$, one-way ANOVA), whereas PFHS slightly suppressed the sum lengths by about 10% although this change was not significant. The change pattern of the number of bifurcated neurites was similar to that of the sum lengths of neurites per neuron

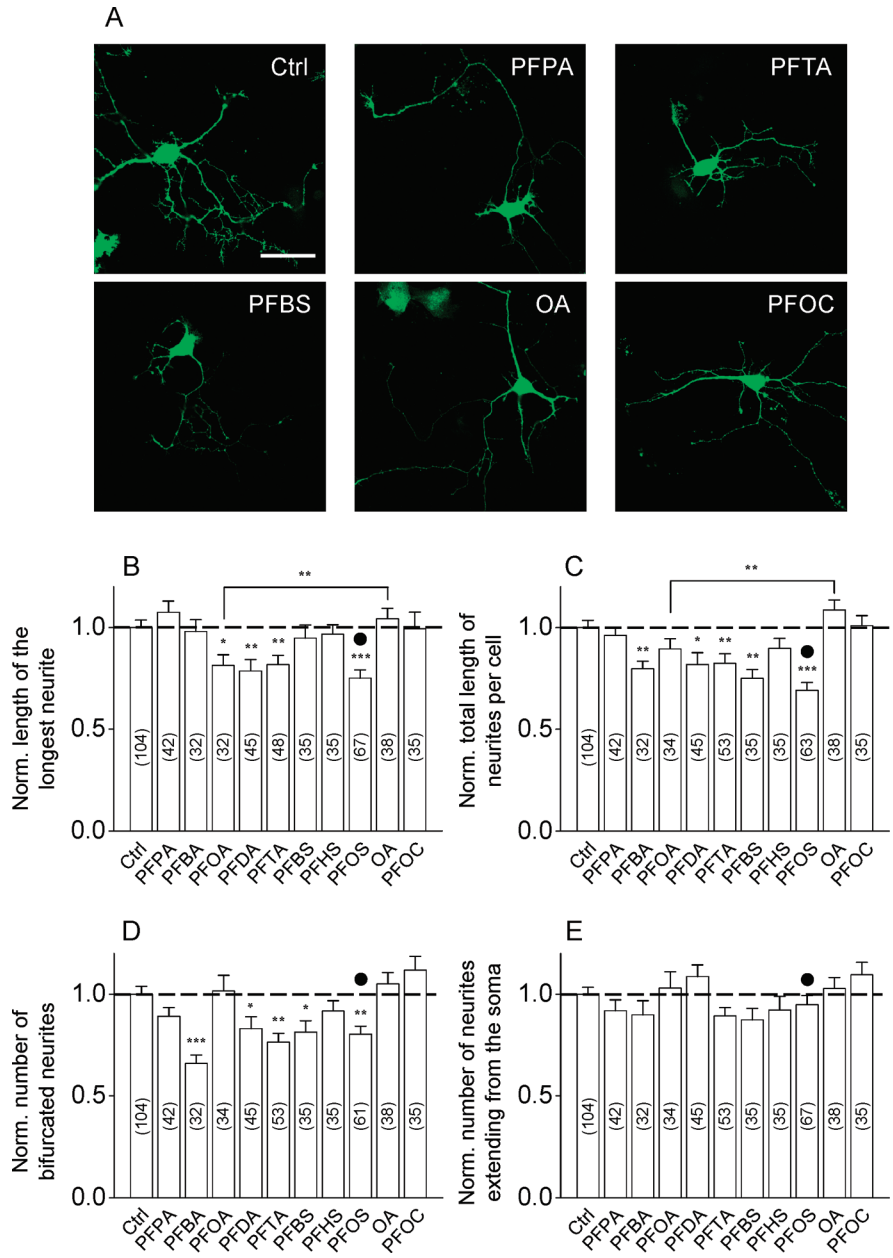

FIGURE 3. Effects of PFC and OA exposures on neurite growth in cultured hippocampal neurons. (A) Representative images showing morphologies of GFP-transfected neurons at 4 DIV chronically treated with various PFCs and OA (as indicated in each image, all at 50 μM). Scale bar, 50 μm. (B) Quantitative measurements indicated that PFCs with longer carbon chains suppressed the lengths of the longest neurites. (C, D) No regular trends were found on effects of PFCs and OA to the sum length of neurites per neuron (C) and the number of bifurcated neurites (D). (E) Neither PFCs at varying chain length nor OA affected the number of neurites extending from the soma. The numbers in parentheses indicate the number of cells tested. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$, as compared with control or compared between the two groups indicated, one-way ANOVA. In B–E, the black circles indicate that the data regarding PFOS were cited from our previous paper (*30*).

(Figure 3D). All PFCs, at 50 μM, had no apparent effects on the number of neurites extending from the soma (Figure 3E).

Effects of Fluorination and Negative Charge. A nonfluorinated hydrocarbon, octanoic acid (OA, C-8), was used as a comparison to study the effect of fluorination on the neurotoxicity. OA is a surfactant, as are all the PFCs tested in this work, and was used to examine whether the fluorination or surfactant action plays a more important role in interferences of PFCs on neurons. To study the role of the negative charge on neurotoxicity, the nonionic 1*H*-perfluorooctane (PFOC) was used as a comparison to PFOA and PFOS as all three compounds belong to the eight-carbon (C-8) perfluorinated chemicals.

The effects of OA, PFOA, PFOC, and PFOS (all at 100 μM) on the mPSC frequencies and mean amplitudes are compared in Figure 1B and 1C, respectively. OA slightly, but not significantly, increased the mPSC frequency by 30% over the control ($P > 0.05$), and the increase was less than that by PFOA (48% over the control, $P < 0.05$). PFOC significantly elevated the frequency by 36%, which was somewhat lower than that by PFOA and considerably smaller in increase than that by PFOS (8.69 fold compared with the control, $P < 0.001$). Also, of the four compounds, only PFOS exhibited significant effects on the mPSC amplitudes. Summarized data with respect to the normalized peak values of I_{Ca} at 0 mV demonstrated that the four compounds (at 100 μM) magnified the amplitudes of I_{Ca} in the order OA < PFOC < PFOA <

PFOS (Figure 2C, $P < 0.01$). The half-activation and inactivation $V_{1/2}$ of $I-V$ curves of I_{Ca} for the tested compounds (at 100 μM) are summarized in Figure 2D and 2E, respectively. OA and PFOC had no obvious effects on the kinetics of I_{Ca}, whereas PFOA and PFOS shifted both the activation and the inactivation $I-V$ curves toward negative potential ($P < 0.01$). Meanwhile, the four features concerning neurite growth and bifurcation of neurons as stated above were determined for the four compounds (all at 50 μM). The lengths of the longest neurites and the sum length of neurites per neuron were not affected by OA and PFOC but were reduced by PFOA and PFOS (Figure 3B and 3C, $P < 0.01$). Comparison of the decrease in neurite length between OA and PFOA proved that PFOA caused a greater development inhibition indicating that the fluorination in carbon chain played a more important role in this process as compared to the surfactant action.

Discussion

PFC-Induced Changes in Cultured Neurons Were Dependent on Their Chain Length. Considerable evidence suggests that the effects of PFCs on organisms vary with their carbon chain lengths. A study by Upham et al. (*21*) demonstrated that inhibitions of perfluorinated fatty acids (PFFAs) with chain lengths of 7−10 carbons on gap junctional intercellular communication (GJIC) depended on the carbon number of their fluorinated backbone. Expanding Upham's studies, Hu et al. (*22*) indicated that the optimum chain length for inhibition of PFFAs on GJIC is 10 carbons while that for the perfluorinated sulfonic acids is 8 carbons. Further, shorter PFCs such as PFBS and PFHS, four-carbon (C-4) and six-carbon (C-6) chains, respectively, did not inhibit GJIC. By using PC12 cell lines as in vitro model, Slotkin et al. (*23*) evaluated the essential features of the developmental neurotoxicity such as inhibition of DNA synthesis, deficits in cell numbers and growth, oxidative stress, reduced cell viability, and so forth of four PFCs. They found that the rank order of adverse effects was perfluorooctane sulfonamide (PFOSA, C-8, a less polar precursor of PFOS) > PFOS > PFBS ≈ PFOA. Their results also suggested that all PFCs were different in their impact on neurodevelopment in PC12 cell lines. Our data, especially concerning the effects of selected PFCs (both perfluorinated carboxylates and sulfonates) on the mPSC frequencies and amplitudes of I_{Ca} in cultured neurons, are in agreement with these previously published results (Figures 1 and 2). In considering the effect on the amplitudes of I_{Ca}, optimum chain length for the two classes of fluorinated compounds was found to be both eight carbons (C-8), which confirms studies by other researchers (*18, 22*). Inhibition of neurite growth was common in cultured neurons treated with various PFCs irrespective of carboxylates or sulfonates (Figure 3). There seemed to be no apparent correlation between the inhibition rate and the chain length of PFCs while compounds with longer carbon chains generally exerted more forceful effects. Taken together, these findings exemplify that the interferences of PFCs on neurons vary with the carbon number of their fluorinated backbone.

Effects of Functional Group and Charge. Among the perfluorinated compounds, PFOS and PFOA are of special interest because they are the predominant PFCs found in tissues of wildlife as many commercially used perfluorinated products break down or metabolize to them. While the actions of PFCs on organisms are not fully understood, the results obtained from our experiments imply that perfluorinated sulfonates exert more potent actions on neurons compared with perfluorinated carboxylates. The difference is especially remarkable when comparing PFOS with PFOA (Figures 1−3). Previous study indicated that PFOS is likely to be incorporated into the lipid bilayer of cell membranes, which might attribute to the differences in neuronal features between the sulfonates and the carboxylates (*13*). Both PFOS and PFOA are anionic surfactants when dissolved in water, whereas PFOC remains neutral. Their effects on neurons were compared in Figures 1−3, where it can be seen that PFOC increased the frequency of mPSC and amplitude of I_{Ca}, but did not affect the activation/inactivation kinetics of I_{Ca}, and neurite outgrowth and bifurcation, which demonstrated that negative charge only played a minor role in these processes. PFOS and PFOA exerted more potent effects on cultured neurons than PFOC probably because of the attached sulfonic and carboxylic groups to the fluorinated backbone of PFOC, which might validate above speculation that potencies of PFCs are sensitive to the nature of their functional groups. The $I-V$ curves of I_{Ca} were leftward shifted by both PFOS and PFOA, but not by PFOC, further suggesting that the negative charge played a critical role in actions on voltage-dependent nature of I_{Ca}.

Fluorination Exerted More Potent Actions on Neurons than Surface Activity. The eight-carbon-chained (C-8) OA is an anionic surfactant which can be converted to PFOA when all the hydrogen atoms in its aliphatic chain are substituted by fluorine. When comparing the effects of OA and PFOA on cultured neurons, it was found that OA showed an insignificant increase of the mPSC frequency but evidently elevated the amplitude of I_{Ca} (Figures 1B and 2C) indicating that the surface activities of OA (similar to the PFCs) can interfere with the physiological processes of neurons, which is in agreement with past studies on other negatively charged surfactants (*13, 24*). The same but more observable trends were observed on neurons with PFOA (Figures 1B and 2C). While OA did not affect the kinetics of I_{Ca} or the neurite outgrowth, PFOA at the same concentration leftward shifted the $I-V$ curves of I_{Ca} and inhibited the neuronal outgrowth (Figures 2 and 3). Our findings demonstrated that the fluorination in alkyl chains of PFCs likely plays a more crucial role in the processes stated above than the interfacial activity of these surfactants. This might be explained by the fact that the properties of the functional group for many PFCs are readily influenced by the fluorination of their alkyl chains. Similar results regarding effects of fluorination were found in recent studies. Upham et al. (*21*) reported that PFOA (C-8) and PFDA (C-12) dose dependently inhibited GJIC in rat liver epithelial cell line, while nonfluorinated fatty acids containing octanoic acid (OA), octanesulfonic acid (OSA), and decanoic acid (DA) did not. Boger et al. (*25*) revealed that the inhibition potency of GJIC by native fatty acids was weaker than that by PFFAs and that the optimum chain length for native fatty acids is 16−18 carbons compared to 8−9 optimum carbon chain length for PFFAs.

Implications and Relevance to Human Health. PFCs have been found to be ubiquitously present in the environment (*1, 5, 26*), and they are accumulating in the biota throughout the world (*27*). Potential routes of exposure to PFCs may be through air, water, food, and household dust (*28*). PFCs can readily be absorbed orally and can distribute primarily in the serum and liver, where it is difficult for the body to eliminate (*29*). Despite the hydrophilic nature of their sulfonyl or carboxyl head groups, PFOS and PFOA can cross the cell membrane and enter the developing brain possibly because of either their surfactant properties or the immaturity of the fetal/neonatal blood-brain barrier (*5, 26*). An in vitro study on cultured rat hippocampal neurons showed that through enhancement of Ca^{2+} channels, PFOS may exhibit both acute excitotoxic effects on synaptic function and may chronically inhibit synaptogenesis in the brain (*30*). Recent evidence indicated that PFCs show signs of a variety of toxicological effects to organisms, such as those mentioned previously, and the effects appear to differ with their carbon chain length (*5, 31*). In regard to human health concerns, there is some association between exposure to PFOS and PFOA and liver and bladder cancer. In addition, epidemiology shows that PFC exposures are related to endocrine disorders, disorders

of the liver and biliary tract, and reproductive disorders (*32*). Olsen et al. (*9*) found that the serum levels of PFOS and PFOA in occupationally exposed workers were up to 13 ppm (approximately 24 μM) and 114 ppm ($\sim$275 μM), respectively, which also demonstrates that the dose levels (50, 100 μM) of PFCs used in our experiments are within the range of the actual exposure levels. As PFCs are bioaccumulative and persistent, more attention should be raised regarding the environmental and health aspects of these chemicals.

Acknowledgments

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Supporting Information Available

Information showing experimental details including reagents, instrumentation, and morphological observation. This material is available free of charge via the Internet at http://pubs.acs.org.

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