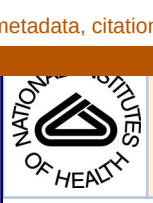

Published in final edited form as:

Neuroscience. 2013 September 17; 0: 562–570. doi:10.1016/j.neuroscience.2013.06.039.

Nerve growth factor/p75 neurotrophin receptor–mediated sensitization of rat sensory neurons depends on membrane cholesterol

Y.H. Zhang, **R. Khanna**, and **G.D. Nicol***

Department of Pharmacology and Toxicology, School of Medicine, Indiana University, Indianapolis, IN, USA 46202

Abstract

Nerve growth factor (NGF) is an important mediator in the initiation of the inflammatory response and NGF via activation of the p75 neurotrophin receptor ($p75^{NTR}$) and downstream sphingomyelin signaling leads to significant enhancement of the excitability of small diameter sensory neurons. Because of the interaction between sphingomyelin and cholesterol in creating membrane liquid-ordered domains known as membrane or lipid rafts, we examined whether neuronal NGF-induced sensitization via $p75^{NTR}$ was dependent on the integrity of membrane rafts. Here, we demonstrate that the capacity of NGF to enhance the excitability of sensory neurons may result from the interaction of $p75^{NTR}$ with its downstream signaling partner(s) in membrane rafts. Two agents known to disrupt membrane rafts, edelfosine and methyl-β-cyclodextrin (MβCD), block the increase in excitability produced by NGF. In contrast, treatment with MβCD containing saturated amounts of cholesterol does not alter the capacity of NGF to augment excitability. In addition, adding back MβCD with cholesterol restored the NGF-induced sensitization in previously cholesterol-depleted neurons, suggesting that cholesterol and the structural integrity of rafts are key in promoting NGF-mediated sensitization. Using established protocols to isolate detergent-resistant membranes, both $p75^{NTR}$ and the neuronal membrane raft marker, flotillin, localize to raft fractions. These results suggest that downstream signaling partners interacting with $p75^{NTR}$ in sensory neurons are associated with membrane raft signaling platforms.

Keywords

NGF; excitability; sensitization; membrane raft; dorsal root ganglia

*Corresponding Author: Grant Nicol, Ph.D., Department of Pharmacology and Toxicology, 635 Barnhill Drive, Indiana University School of Medicine, Indianapolis, IN USA 46202, Office: (317) 274-1570, FAX: (317) 274-7714, gnicol@iupui.edu.

The authors have no conflicts of interest to declare.

The fluid mosaic model of the plasma membrane (Singer and Nicholson 1972) proposed that proteins freely diffused in the lipid bilayer. However, membrane proteins may interact within microdomains wherein lateral mobility is restricted by packing of specific membrane lipids. These specialized complexes, referred to as membrane or lipid rafts, are domains of cholesterol and sphingolipids with long, mostly saturated acyl chains, which are packed tightly together to form a liquid-ordered phase that is scattered among a liquid-disordered matrix composed of unsaturated glycerolipids (Brown and London 1998, 2000; Simons and Toomre 2000; Hancock 2006; Shaw 2006; Jacobson et al., 2007; Simons and Gerl 2010). Membrane proteins associate differently between the liquid-ordered and –disordered domains allowing for dynamic inclusion/exclusion of proteins into or from liquid-ordered domains. When the membrane is solubilized with a detergent, the liquid-ordered structures give rise to detergent-resistant membrane domains that are enhanced in cholesterol, sphingolipids, and integral membrane proteins that can be enriched using density gradient centrifugation (Shogomori and Brown 2003). A variety of proteins are known to be associated with membrane rafts, and for many their activity depends on this association. Preferential inclusion or exclusion of proteins in these domains promotes interactions which can regulate intracellular signaling events spatially via localized compartments which can then modulate their temporal interaction. Thus, membrane rafts are important in protein sorting and serve as platforms that coordinate signal transduction with intracellular targets (Golub et al., 2004; Kiyokawa et al., 2005; Harding and Hancock 2008; Lingwood and Simons 2010).

Sphingomyelins and ceramides are key membrane lipids that give rise to liquid-ordered membrane domains (Brown and London 2000). These rafts form scaffolding platforms where signaling molecules can be in close association and thereby facilitate transduction. Activation of the p75 neurotrophin receptor ($p75^{NTR}$) leads to stimulation of the sphingomyelin signaling cascade with a consequent liberation of ceramide (Dobrowsky et al., 1994). Previous studies indicated that $p75^{NTR}$ can associate with membrane rafts (Wu et al., 1997; Bilderback et al., 1997). In addition, our previous work demonstrated that NGF via activation of $p75^{NTR}$ and a neutral sphingomyelinase, significantly enhanced the excitability of small diameter sensory neurons through the modulation of a tetrodotoxin-resistant sodium current and an outward potassium current(s) (Zhang et al., 2002; 2008; 2012; Zhang and Nicol 2004). In recent studies examining the downstream signaling pathways activated by $p75^{NTR}$ (Zhang et al., 2012), use of the inhibitor, edelfosine, hinted that this pathway may involve membrane rafts. Taken together, these results raise the question as to whether the enhanced excitability mediated by NGF and activation of $p75^{NTR}$ depends on the integrity of membrane rafts in the neuronal membrane.

Experimental Procedures

Isolation and maintenance of adult rat sensory neurons

Sensory neurons were isolated from young adult male Sprague-Dawley rats (100–150 g) using procedures developed by Lindsay (1998) with slight modifications (Chi and Nicol 2007). Briefly, the young rats were killed by placing them in a chamber filled with CO_2. The dorsal root ganglia (DRGs) were collected in a culture dish filled with sterilized Puck's

solution. The ganglia were transferred to a conical tube with F-12 media containing papain (10 U/ml) and incubated for 15 min at 37° C, followed by incubation with 1 mg/ml collagenase IA and 2.5 mg/ml dispase for 25 min at 37° C. The suspension was centrifuged for 60 s (1000 × g) after which the enzyme-containing supernatant was removed. The pellet was resuspended in F-12 media supplemented with nerve growth factor (30 ng/ml) (Harlan Bioproducts, Indianapolis, IN) and 10% heat-inactivated horse serum (Gibco #26050-088). Cells were mechanically dissociated with fire-polished pipettes. Isolated cells were plated onto plastic coverslips previously coated with poly-D-lysine (100 μg/ml) and laminin (5 μg/ml). Isolated cells were maintained in culture at 37°C and 3% CO_2 for 18–24 hrs before electrophysiological recordings were obtained. All procedures were approved by the Animal Use and Care Committee of Indiana University School of Medicine.

Electrophysiology

Recordings were made using the whole-cell patch-clamp technique (Zhang et al., 2012; Hamill et al., 1981). Briefly, a cover slip with sensory neurons was placed in a recording chamber where neurons were bathed in normal Ringers solution of the following composition (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, pH at 7.4 with NaOH. Recording pipettes were pulled from capillary glass tubing (Model G85165T-4, Warner Instruments, Hamden, CT) and typically had resistances of 2–5 MΩ when filled with the following solution (in mM): 140 KCl, 5 $MgCl_2$, 4 ATP, 0.3 GTP, 2.5 $CaCl_2$, 5 EGTA (calculated free Ca^{2+} concentration of ~100 nM), and 10 HEPES, at pH 7.3 with KOH. Whole-cell voltages were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The whole-cell recording configuration was established in normal Ringers solution. The data were acquired and analyzed using pCLAMP 6.04 or pCLAMP 9.0 (Molecular Devices). All drugs were applied by external superfusion of the recording chamber using a VC-8 bath perfusion system (Warner Instruments, Hamden, CT). To assess excitability in the current-clamp experiments, neurons were held at their resting potentials (range between −45 to −65 mV) and ramps of depolarizing currents (1 s in duration) were used to evoke 2–4 action potentials (APs) under control conditions. The same ramp was then used throughout the recording period for each individual neuron. The current clamp traces were filtered at 5 kHz and sampled at 1 kHz. At the end of each recording, the neuron was exposed to 400 nM capsaicin for approximately 30 s. This neurotoxin was used to distinguish capsaicin-sensitive sensory neurons as these neurons are believed to transmit nociceptive information (Holzer 1991). However, the correlation between a neuron being a nociceptor and having capsaicin sensitivity is not absolute. Some nociceptive neurons are insensitive to capsaicin and some capsaicin-sensitive neurons are not nociceptors (see Petruska et al., 2000). Therefore this agent was used to define a population of small-diameter sensory neurons that could serve a nociceptive function. The results reported in the electrophysiological recordings were obtained from only capsaicin-sensitive neurons. All experiments were performed at room temperature (~23° C).

Detergent-free Membrane Raft Isolation

Membrane rafts were prepared as described previously (Macdonald and Pike 2005). Briefly, cells were washed, scraped into base buffer (20 mM Tris-HCL pH 7.8, 250 mM sucrose, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and pelleted by centrifugation at 250xg for 5 min. The cells were

resuspended in 1 ml base buffers supplemented with protease inhibitors (PMSF, benzamidine-HCl, calpain I, calpain II, leupeptin, pepstatin A, aprotinin) and homogenized using 20 strokes of a 2 ml dounce homogenizer. The lysed cells were then centrifuged at 1,000xg for 10 min. The supernatant was collected and transferred to a 5 ml ultracentrifuge tube (Sorvall) and mixed with an equal volume of 50% Optiprep® density gradient medium (Sigma-Aldrich, St. Louis, MO) diluted in base buffer. A 20-0% Optiprep® gradient was gently layered on the lysate using a Gilson Minipuls 3 gradient maker. The membrane rafts were separated by a 52,000xg 90 min centrifugation at 4°C in a Sorvall Discovery M150 SE ultracentrifuge using a Sorvall S52ST-0065 swinging bucket rotor. Following the spin, 12 separate 412 μl fractions were collected beginning at the top of the gradient and descending downward through the gradient.

Cholesterol Assay

The cholesterol content of fractions was assessed using the fluorometric Amplex Red® reagent cholesterol assay kit (Invitrogen) as per the manufacturer's instructions. Briefly, a 50 μl aliquot of each fraction was combined with 50 μl working reagent as described in the kit into separate wells of a 96 well plate. Following a 30 min incubation at 37°C in the dark, the fluorescence of each well was measured using a 1 second exposure Victor V3 Plate fluorescent plate reader using detection parameters at an excitation of 485 nm and emission of 535 nm. The cholesterol assay was calibrated by using cholesterol standards ranging from 0 to 20 μM (0, 0.5, 1, 2.5, 5, 10, and 20). The relation between the fluorescence reading and cholesterol concentration was fitted by a linear regression line having an R^2 value of 0.996 (Origin 8.6).

Western Blot Analysis

This was performed as described previously (Khanna et al., 2001). Briefly, aliquots of fractions from membrane raft isolations were separated on 4–12% sulfate-polyacrylamide gel (SDS-PAGE) and electrophoretically transferred to a PVDF membrane. The membranes were blocked 1 hr to overnight at 4°C in 5% dry milk diluted in 0.5% bovine serum albumin. After incubation of membranes with polyclonal antibodies against flotillin-1 (1:1000 dilution, Cell Signaling Technology, Danvers, MA) or $p75^{NTR}$ (1:400 dilution, Pierce Biotechnology, Rockford, IL), the blots were washed five times for five minutes in Tris-buffered saline with Tween-20 (TBST). The membranes were then incubated with secondary HRP goat anti-rabbit or anti-mouse antibodies diluted in blocking buffer at a dilution of 1:8000–1:10,000 for 1 hr. The blots were washed extensively in TBST and probed with Enhanced Chemiluminescence Western blotting substrate (Thermo Scientific) before exposure to photographic film. Blots were exposed for a range of durations to ensure the generation of a print in which the film was not saturated. Films were then scanned, digitized, and quantified using Un-Scan-It gel V6.1 scanning software (Silk Scientific Inc., Orem, UT), limiting the analysis to the linear range.

MβCD-cholesterol solution

A stock solution of 10 mM MβCD (Sigma-Aldrich) was made in normal Ringers solution. The protocol previously described in Christian et al., (1997) and Levitan et al., (2000) was used to prepare the MβCD-saturated cholesterol solution. Briefly, a stock solution of

cholesterol (5 mg/ml) was prepared in a choloform:methanol (1:1, vol:vol) solution in a glass vessel. The solvent was evaporated using a gentle stream of N_2 gas, leaving a dried film on the bottom. The MβCD-Ringers solution at 1 mM MβCD was added and sonicated; this solution was then slowly shaken overnight at 37° C. This solution of 1 mM MβCD-saturated cholesterol Ringers was then used to treat isolated sensory neurons.

Data analysis

Data are presented as the means ± s.e.m. Statistical differences between the control recordings and those obtained under various treatment conditions were determined by using either an ANOVA or repeated-measures (RM) ANOVA whenever appropriate. When a significant difference was obtained, post hoc analyses were performed using a Holm-Sidak all pairwise test. If the data set failed the normality test, a Kruskal-Wallis analysis of variance on ranks was performed using a Dunn's all pairwise test. Values of $P<0.05$ were judged to be statistically significant.

Chemicals

Edelfosine was purchased from EMD Bioscience (San Diego, CA). Tissue culture supplies were purchased from Fisher (Pittsburgh, PA). All other chemicals were obtained from Sigma-Aldrich Chemical Corp. (St. Louis, MO). For the electrophysiology experiments, capsaicin and edelfosine were dissolved in 1-methyl-2-pyrrolidinone (MPL, HPLC grade). These stock solutions were then diluted with Ringers solution to yield the appropriate concentration. The vehicle, MPL, was typically used at 1000–5000 fold dilutions; we demonstrated previously that the vehicle 1-methyl-2-pyrrolidinone has no effect on AP firing or the activation of either the tetrodotoxin-resistant sodium current or the voltage-dependent potassium current (Zhang et al., 2002).

Results

Increased excitability produced by NGF is blocked by edelfosine

As part of our studies investigating the downstream signaling pathways activated by NGF via $p75^{NTR}$, we found that the phospholipase C inhibitors U73122 and neomycin were without effect (Zhang et al., 2012). In a separate series of studies to corroborate these previous observations obtained with U73122 and neomycin, we used another putative phospholipase C inhibitor, edelfosine (ET-18-OCH3). Surprisingly, pretreatment of sensory neurons with edelfosine blocked the increase in excitability produced by NGF. As shown in Fig. 1, treatment with 100 ng/ml NGF produced an increase in the number of action potentials (APs) evoked by a ramp of depolarizing current. Under control conditions the ramp evoked 3 APs (panel A) whereas after a 6 min exposure to NGF the number of APs increased to 9 (panel B). Panel C summarizes the sensitization produced by NGF wherein after a 6 min exposure to NGF, the number of evoked APs was significantly increased to 7.8 ± 1.2 compared to a control value of 2.8 ± 0.2 APs ($P<0.001$, RM ANOVA, n=5); the AP values recorded at 6, 10, and 20 min were not different. Associated with the increased AP firing, NGF produced a significant time-dependent depolarization of the neuronal resting membrane potential wherein the control value of −55.1 ± 0.2 mV increased to −49.8 ±1.0, −47.6 ± 1.2, −46.4 ± 1.2, and −44.1 ± 0.7 after exposure to NGF for 2, 6, 10, and 20 min,

respectively (P<0.001 for all values compared to control, RM ANOVA, n=5, data not shown). In contrast, pretreatment with 10 μM edelfosine for 30 min completely suppressed the sensitization of AP firing produced by NGF (panel C). The average AP value obtained for NGF in the presence of edelfosine was not different from the control values (3.3 ± 0.4 APs for control vs. 3.7 ± 0.5 APs after 20 min NGF, P=0.28 RM ANOVA, n=7). In addition, after edelfosine treatment, NGF failed to depolarize the resting membrane potential (−52.1 ± 1.7 mV for control vs. −49.7 ± 2.1 mV after 20 min NGF, P=0.06 RM ANOVA, n=7, data not shown). These results raise the question as to why edelfosine blocked the actions of NGF while U73122 or neomycin was ineffective. In addition to acting as a phospholipase C inhibitor, edelfosine has other actions. In its capacity as an anti-cancer chemotherapeutic agent, edelfosine interacts with membrane rafts causing their disruption. In fact, edelfosine has a high affinity for cholesterol and prior disruption of membrane rafts by methyl-β-cyclodextrin (MβCD), a cholesterol chelating agent prevents the capacity of edelfosine to induce apoptosis (van der Luit et al., 2002; Zaremberg et al., 2005; Ausili et al., 2008; Mollinedo et al., 2010). These results suggest that the enhanced excitability produced by NGF may involve membrane rafts.

Increased excitability produced by NGF is blocked by MβCD

Because of the cholesterol dependency for structural stability of membrane rafts, compounds that extract cholesterol from the membrane, such as MβCD (Brown and London 2000; Zidovetzki and Levitan 2007), have been widely used to disrupt these domains and thereby establish functional roles of membrane rafts. To test the possibility that edelfosine acts by disrupting the organization of membrane rafts, isolated sensory neurons were pretreated with 10 mM MβCD for 30 min and then exposed to NGF. As shown in Fig. 2A, MβCD completely blocked the ability of NGF to increase the number of APs. In addition, treatment with MβCD prevented the NGF-induced depolarization of the resting membrane potential (control −54.1 ± 1.4 mV vs. NGF 20 min −53.2 ± 1.5 mV, P=0.32 RM ANOVA, n=6, data not shown). It is possible that MβCD disrupts NGF-mediated signaling pathways independently of its capacity to chelate cholesterol. To test this idea, sensory neurons were treated with either 1 mM MβCD Ringers solution alone or 1 mM MβCD Ringers solution with saturating levels of cholesterol for 60 min. Similar to the 30 min treatment, a 60 min treatment with 1 mM MβCD blocked the enhanced excitability (both increased AP firing and membrane depolarization) produced by NGF (see Fig. 2B). In contrast, a 60 min pretreatment with 1 mM MβCD and saturating concentrations of cholesterol did not affect the ability of NGF to augment AP firing wherein a significant increase in AP firing was observed after only a 2 min exposure to NGF. Under the MβCD + saturated cholesterol condition, NGF significantly depolarized the membrane potential from a control value of −54.8 ±1.9 mV to −47.2 ± 2.1 mV after 10 min NGF (P<0.001 RM ANOVA, n=8). To further demonstrate that membrane cholesterol was a key component to the sensitization produced by NGF, sensory neurons were pretreated with 10 mM MβCD for 30 min prior to recording. After attaining the whole-cell configuration, neurons were exposed to a Ringers solution containing 10 mM MβCD and 100 ng/ml NGF. Over a 10 min exposure to MβCD and NGF, eight sensory neurons did not exhibit an increase in the number of evoked APs (see Fig. 2C). These neurons were then treated with Ringers solution containing 10 mM MβCD + 100 ng/ml NGF + saturating cholesterol wherein after only 6 min, there was a

significant increase in the number of APs that was maintained for the 20 min recording period. These results indicate that returning cholesterol to the neuronal membrane is a critical factor in the capacity of NGF to enhance the excitability of these neurons. We believe that the suppression produced by MβCD does not result from non-specific membrane effects such as changes in membrane fluidity since in the absence of NGF there were no changes in the resting membrane potential. In addition, previous studies demonstrated that 1 mM MβCD for 10 min significantly depleted cholesterol without compromising membrane stability (Zidovetzki and Levitan 2007) and 10 mM MβCD for 30 min did not affect the membrane resistance of isolated trigeminal neurons (Gnanasekaran et al., 2011). Taken together, these results suggest that the enhanced excitability produced by NGF may involve the interaction of $p75^{NTR}$ and/or its downstream signaling partners in a membrane raft domain.

$p75^{NTR}$ is found in the membrane raft fractions

Additional studies were performed to determine whether $p75^{NTR}$ was detected in density fractions obtained from isolated sensory neurons. The representative Western blot shown in Fig. 3 demonstrates that $p75^{NTR}$ was found in both the raft (fractions 6–8) and non-raft (fractions 9–12) membrane density fractions. In addition, fractions 6–8 contained the established membrane raft marker, flotillin (Bickel et al., 1997; Lang et al., 1998). To corroborate the idea that elevated levels of cholesterol were associated with the raft-containing density fractions 6–8, the cholesterol content of the 12 density fractions was determined. As shown in Fig. 4A, there were two peaks of cholesterol content, one associated with fractions 5–8 and the other with fractions 9–12. Treatment with 10 mM MβCD for 30 min at 37°C reduced the concentration of cholesterol detected in fractions 5–11 obtained from isolated sensory neurons (Fig. 4A). The ability of MβCD to deplete sensory neurons of their cholesterol was concentration-dependent with 10 mM significantly reducing the content of fractions 6–8 by about 35% (see Fig. 4B). Thus, these results indicate that MβCD effectively lowers cholesterol in sensory neurons.

Discussion

In this report, we demonstrate that NGF enhanced the capacity of small diameter sensory neurons to fire APs and that this may result from the interaction of $p75^{NTR}$ with its downstream signaling partner(s) in membrane liquid-ordered domains known as membrane rafts. Two established agents known to modify membrane cholesterol levels and therefore disrupt membrane rafts, edelfosine and MβCD, blocked the increased excitability produced by NGF. When sensory neurons were treated with MβCD containing saturated amounts of cholesterol, the capacity of NGF to augment AP firing was quickly restored to levels that we previously observed under normal recording conditions (Zhang et al., 2002, 2006; 2012), suggesting that the loss of cholesterol from the neuronal membrane and consequently disruption of raft structural integrity could be key in promoting the sensitization produced by NGF. The relatively rapid recovery for the NGF-induced enhancement of AP firing with the re-addition of MβCD + cholsterol is consistent with previous reports. In A431 cells, a human epithelial carcinoma cell line, cholesterol depletion with 5 mM MβCD completely suppressed the bradykinin-induced turnover of phosphatidylinositol within 10 min;

application of MβCD with cholesterol fully restored this bradykinin turnover within 20 min of application (Pike and Miller 1998). The binding of stromal cell-derived factor 1α (a ligand for CXCR4) was restored within 10 min after adding back βCD + cholesterol to T cells that had been previously treated with βCD to deplete cholesterol (Nguyen and Taub 2002). Similarly, treatment with 10 mM MβCD significantly decreased the amplitude of the excitatory post-synaptic potential recorded from crayfish muscle, which was completely recovered after only 10 min of adding back 10 mM MβCD + cholesterol (Zamir and Charlton 2006). In addition, using established protocols to isolate raft and non-raft membranes, $p75^{NTR}$ as well as the neuronal marker for membrane rafts, flotillin, were localized to these raft fractions. Therefore, our results clearly demonstrate that the NGF-mediated enhancement of sensory neuron excitability requires the presence of membrane cholesterol. Although these observations are not definitive for a causal role of membrane raft domains, they do suggest that rafts may play an important role in the interaction of $p75^{NTR}$ with its downstream effector molecules.

In support of the idea regarding the raft dependence of $p75^{NTR}$ signaling, previous studies have found that $p75^{NTR}$ can be localized to membrane raft fractions. Early studies using synaptic plasma membranes isolated from rat forebrain, NIH-3T3, or PC12 cells demonstrated that the highest levels of $p75^{NTR}$ were detected in the low-density fractions that were associated with the membrane raft resident proteins caveolin (3T3 cells) or caveolin-like membrane fractions (PC12 cells) although lower levels of $p75^{NTR}$ were also observed in the higher-density or detergent soluble fractions (Wu et al., 1997; Bilderback et al., 1997; Huang et al., 1999). These results are consistent with our observations wherein $p75^{NTR}$ was detected in both the low-density (fractions 6–8) and the higher density fractions (9–12). In PC12 cells, treatment with NGF (2 nM for 1 hr) did not alter the levels of $p75^{NTR}$ expressed in the raft fractions (Huang et al., 1999), however in mouse cerebellar granule neurons, which express $p75^{NTR}$ and not TrkA, exposure to 200 ng/ml NGF for 10 min increased the levels of $p75^{NTR}$ by about two fold that were found in the insoluble raft fractions (Higuchi et al., 2003), suggesting that the ability of NGF to translocate $p75^{NTR}$ into or out of rafts may depend on the model system.

Little is known about the interaction of $p75^{NTR}$ and its downstream signaling partners that may occur in the raft domain. Membrane rafts are known to contain increased levels of sphingomyelin (Brown and Rose 1992; Brown and London 1998; Paratcha and Ibanez 2002). In rat T9 glioblastoma cells, NGF (100 ng/ml) exposure resulted in the hydrolysis of membrane sphingomyelin with the consequent generation of ceramide (Dobrowsky et al., 1994). Interestingly, treatment of PC12 cells with NGF reduced the levels of sphingomyelin only in the low-density raft fractions, suggesting that $p75^{NTR}$ was associated or interacted with the sphingomyelinase within a membrane raft domain (Bilderback et al., 1997). Similar changes in sphingomyelin and ceramide levels were reported in human fibroblasts after treatment with IL-1β (Liu and Anderson 1995). In addition, the activity of PI3K, a key molecule in many signaling cascades, appears to critically depend upon its localization to membrane rafts where its capacity to phosphorylate Akt requires membrane cholesterol (Zhuang et al., 2002; Peres et al., 2003; Arcaro et al., 2007). Consistent with these observations, we found that inhibition of neutral sphingomyelinase (Zhang et al., 2002) or PI3K blocked the capacity of NGF to enhance the excitability of small diameter sensory

neurons (Zhang et al., 2012). These results raise two important questions as to whether downstream signaling partners associated with $p75^{NTR}$ in sensory neurons are localized to raft signaling platforms and also whether NGF alters the distribution of $p75^{NTR}$ and/or downstream effectors into or out of raft platforms. These questions are clearly important areas for future investigations.

In addition, modulation of the activity of different ion channels has been associated with their localization to membrane raft domains, which could then potentially regulate neuronal excitability. The potassium channels Kv2.1 and Kv1.5 were detected in the low-density raft fractions when expressed in Ltk mouse cells (Martens et al., 2000; 2001; 2004). The activity of these potassium channels was linked to their association with membrane rafts where cholesterol depletion resulted in hyperpolarizing shifts in their activation curves (Kv1.5) and their steady-state inactivation relations (Kv2.1 and Kv1.5)(Martens et al., 2000; 2001). The rapidly inactivating potassium channels Kv1.4 and Kv4.2 have also been detected in the raft fractions isolated from rat hippocampal neurons (Wong and Schlichter 2004). The current conducted by the inward rectifying potassium channel Kir2 is regulated by the levels of membrane cholesterol such that MβCD treatment leads to enhanced current amplitudes (Romanenko et al., 2002, 2004). Similarly, Kv1.3 in both human and Jurkat T-lymphocytes is associated with membrane rafts wherein the levels of membrane cholesterol inversely regulate the current amplitudes (Hajdu et al., 2003; Bock et al., 2003). In addition, the formation of ceramide-enriched domains lead to inhibition of these currents (Bock et al., 2003). More recently, the tetrodotoxin-resistant sodium channel Nav1.8 was localized to both the insoluble and soluble fractions obtained from sensory neurons isolated from the dorsal root ganglia (Pristera et al., 2012). Although the amplitude of the TTX-resistant sodium current was not altered by 10 mM MβCD, the number of sensory neurons responsive (measured as an increase in Fluo-4 fluorescence) to mechanical stimulation of the membrane with a small glass probe (tip diameter 1 μm) was reduced by about 50% (Pristera et al., 2012). In support of the idea that $p75^{NTR}$ and its activation of downstream signaling plays a key role in enhancing the excitability of sensory neurons, we previously demonstrated that both NGF and brain derived neurotrophic factor augmented the tetrodotoxin-resistant sodium current and suppressed a voltage-dependent potassium current(s) (Zhang et al., 2002; 2008; 2012). Whether the activity of these ion channels is modulated directly within the raft domain is presently unknown.

In addition to voltage-dependent channels, ligand-gated channels such as P_2X_3 can be localized to flotillin-associated fractions obtained from rat cerebellar granule neurons and DRG sensory neurons using a detergent-free isolation procedure (Vacca et al., 2004). The currents conducted by P_2X_3 and P_2X_1 were both significantly reduced after treatment with 10 mM MβCD in isolated trigeminal neurons (Gnanasekaran et al., 2001) and smooth muscle cells (Vial and Evans 2005), respectively. Importantly, 10 mM MβCD did not alter the membrane resistance of trigeminal neurons (Gnanasekaran et al., 2001). In contrast, MβCD had no effect on P_2X_3 currents obtained from isolated DRG sensory neurons although both the membrane clustering of TRPV1 and the capsaicin-activated currents were significantly reduced by 1 or 10 mM MβCD (Liu et al., 2006). It is unclear why the sensitivities of P_2X_3 to MβCD are different in trigeminal vs. DRG neurons. Taken together, these results indicate that the ion channels regulating neuronal excitability could be localized

to membrane rafts and that their association with different membrane components can directly affect their activity.

Conclusion

NGF plays a key role in the initiation of the inflammatory response. The levels of NGF are significantly elevated in the skin after blister formation or in pleural exudates after exposure to carrageenan (Weskamp and Otten, 1987). Upon activation, a number of immuno-competent cells release NGF (Ehrhard et al., 1993; Leon et al., 1994; Thacker et al., 2007). Associated with the inflammatory response is the activation of small diameter sensory neurons which, in turn, contributes to increased sensitivity, vasodilatation, and plasma extravasation. NGF is known to heighten the nociceptive sensitivity (Lewin et al., 1993, Woolf et al., 1995) as well as enhance the excitability of small diameter sensory neurons (Shu and Mendell 1999; Zhang et al., 2002). Our present results indicate that the signaling cascades regulating the heightened sensitivity of sensory neurons may occur in membrane raft domains. Important future studies will begin to identify and establish the protein-protein interactions that are critical in $p75^{NTR}$-mediated regulation of neuronal excitability and which occur inside or outside membrane raft signaling domains.

Acknowledgments

YHZ and RK performed the experiments; RK and GDN designed the studies and wrote the text; all authors approved the final manuscript. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 RR015481-01 from the National Center for Research Resources, NIH. This work was supported by NIH NINDS R01 NS046084.

Abbreviations

APs	action potentials
DRG	dorsal root ganglia
MβCD	methyl-β-cyclodextrin
MPL	1-methyl-2-pyrrolidinone
NGF	nerve growth factor
$p75^{NTR}$	p75 neurotrophin receptor
RM ANOVA	repeated-measures analysis of variance

References

Arcaro A, Aubert M, Espinosa del Hierro ME, Khanzada UK, Angelidou S, Tetley TD, Bittermann AG, Frame MC, Seckl MJ. Critical role for lipid raft-associated Src kinases in activation of PI3K-Akt signalling. Cell Signal. 2007; 19:1081–1092. [PubMed: 17275257]

Ausili A, Torrecillas A, Aranda FJ, Mollinedo F, Gajate C, Corbalán-García S, de Godos A, Gómez-Fernández JC. Edelfosine is incorporated into rafts and alters their organization. J Phys Chem B. 2008; 112:11643–11654. [PubMed: 18712919]

Bickel PE, Scherer PE, Schnitzer JE, Oh P, Lisanti MP, Lodish HF. Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. J Biol Chem. 1997; 272:13793–13802. [PubMed: 9153235]

Bilderback TR, Grigsby RJ, Dobrowsky RT. Association of p75(NTR) with caveolin and localization of neurotrophin-induced sphingomyelin hydrolysis to caveolae. J Biol Chem. 1997; 272:10922–10927. [PubMed: 9099750]

Bock J, Szabó I, Gamper N, Adams C, Gulbins E. Ceramide inhibits the potassium channel Kv1.3 by the formation of membrane platforms. Biochem Biophys Res Commun. 2003; 305:890–897. [PubMed: 12767914]

Brown DA, London E. Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol. 1998; 14:111–136. [PubMed: 9891780]

Brown DA, London E. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J Biol Chem. 2000; 275:17221–17224. [PubMed: 10770957]

Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell. 1992; 68:533–544. [PubMed: 1531449]

Chi XX, Nicol GD. Manipulation of the potassium channel Kv1.1 and its effect on neuronal excitability in rat sensory neurons. J Neurophysiol. 2007; 98:2683–2692. [PubMed: 17855588]

Christian AE, Haynes MP, Phillips MC, Rothblat GH. Use of cyclodextrins for manipulating cellular cholesterol content. J Lipid Res. 1997; 38:2264–2272. [PubMed: 9392424]

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA. Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science. 1994; 265:1596–1599. [PubMed: 8079174]

Ehrhard PB, Erb P, Graumann U, Otten U. Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. Proc Natl Acad Sci USA. 1993; 90:10984–10988. [PubMed: 7902578]

Gnanasekaran A, Sundukova M, van den Maagdenberg AM, Fabbretti E, Nistri A. Lipid rafts control P2X3 receptor distribution and function in trigeminal sensory neurons of a transgenic migraine mouse model. Mol Pain. 2011; 7:77. [PubMed: 21958474]

Golub T, Wacha S, Caroni P. Spatial and temporal control of signaling through lipid rafts. Curr Opin Neurobiol. 2004; 14:542–550. [PubMed: 15464886]

Hajdú P, Varga Z, Pieri C, Panyi G, Gáspár R Jr. Cholesterol modifies the gating of Kv1.3 in human T lymphocytes. Pflügers Arch. 2003; 445:674–682. [PubMed: 12632187]

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. Pflügers Arch. 1981; 391:85–100. [PubMed: 6270629]

Hancock JF. Lipid rafts: contentious only from simplistic standpoints. Nat Rev Mol Cell Biol. 2006; 7:456–462. [PubMed: 16625153]

Harding AS, Hancock JF. Using plasma membrane nanoclusters to build better signaling circuits. Trends Cell Biol. 2008; 18:364–371. [PubMed: 18620858]

Holzer P. Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. Pharmacol Rev. 1991; 43:143–201. [PubMed: 1852779]

Higuchi H, Yamashita T, Yoshikawa H, Tohyama M. PKA phosphorylates the p75 receptor and regulates its localization to lipid rafts. EMBO J. 2003; 22:1790–1800. [PubMed: 12682012]

Huang CS, Zhou J, Feng AK, Lynch CC, Klumperman J, DeArmond SJ, Mobley WC. Nerve growth factor signaling in caveolae-like domains at the plasma membrane. J Biol Chem. 1999; 274:36707–36714. [PubMed: 10593976]

Jacobson K, Mouritsen OG, Anderson RG. Lipid rafts: at a crossroad between cell biology and physics. Nat Cell Biol. 2007; 9:7–14. [PubMed: 17199125]

Khanna R, Myers MP, Lainé M, Papazian DM. Glycosylation increases potassium channel stability and surface expression in mammalian cells. J Biol Chem. 2001; 276:34028–34034. [PubMed: 11427541]

Kiyokawa E, Baba T, Otsuka N, Makino A, Ohno S, Kobayashi T. Spatial and functional heterogeneity of sphingolipid-rich membrane domains. J Biol Chem. 2005; 280:24072–24084. [PubMed: 15840575]

Lang DM, Lommel S, Jung M, Ankerhold R, Petrausch B, Laessing U, Wiechers MF, Plattner H, Stuermer CA. Identification of reggie-1 and reggie-2 as plasma membrane-associated proteins

which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. J Neurobiol. 1998; 37:502–523. [PubMed: 9858255]

Leon A, Buriani A, Dal Toso R, Fabris M, Romanello S, Aloe L, Levi-Montalcini R. Mast cells synthesize, store, and release nerve growth factor. Proc Natl Acad Sci USA. 1994; 91:3739–3743. [PubMed: 8170980]

Levitan I, Christian AE, Tulenko TN, Rothblat GH. Membrane cholesterol content modulates activation of volume-regulated anion current in bovine endothelial cells. J Gen Physiol. 2000; 115:405–416. [PubMed: 10736308]

Lewin GR, Ritter AM, Mendell LM. Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. J Neurosci. 1993; 13:2136–2148. [PubMed: 8478693]

Lindsay RM. Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. J Neurosci. 1988; 8:2394–2405. [PubMed: 3249232]

Lingwood D, Simons K. Lipid rafts as a membrane-organizing principle. Science. 2010; 327:46–50. [PubMed: 20044567]

Liu M, Huang W, Wu D, Priestley JV. TRPV1, but not P2X, requires cholesterol for its function and membrane expression in rat nociceptors. Eur J Neurosci. 2006; 24:1–6. [PubMed: 16800863]

Liu P, Anderson RG. Compartmentalized production of ceramide at the cell surface. J Biol Chem. 1995; 270:27179–27185. [PubMed: 7592974]

Macdonald JL, Pike LJ. A simplified method for the preparation of detergent-free lipid rafts. J Lipid Res. 2005; 46:1061–1067. [PubMed: 15722565]

Martens JR, Navarro-Polanco R, Coppock EA, Nishiyama A, Parshley L, Grobaski TD, Tamkun MM. Differential targeting of Shaker-like potassium channels to lipid rafts. J Biol Chem. 2000; 275:7443–7446. [PubMed: 10713042]

Martens JR, O'Connell K, Tamkun M. Targeting of ion channels to membrane microdomains: localization of KV channels to lipid rafts. Trends Pharmacol Sci. 2004; 25:16–21. [PubMed: 14723974]

Martens JR, Sakamoto N, Sullivan SA, Grobaski TD, Tamkun MM. Isoform-specific localization of voltage-gated K+ channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae. J Biol Chem. 2001; 276:8409–8414. [PubMed: 11115511]

Mollinedo F, de la Iglesia-Vicente J, Gajate C, Estella-Hermoso de Mendoza A, Villa-Pulgarin JA, Campanero MA, Blanco-Prieto MJ. Lipid raft-targeted therapy in multiple myeloma. Oncogene. 2010; 29:3748–3757. [PubMed: 20418917]

Nguyen DH, Taub D. CXCR4 function requires membrane cholesterol: implications for HIV infection. J Immunol. 2002; 168:4121–4126. [PubMed: 11937572]

Paratcha G, Ibáñez CF. Lipid rafts and the control of neurotrophic factor signaling in the nervous system: variations on a theme. Curr Opin Neurobiol. 2002; 12:542–549. [PubMed: 12367633]

Peres C, Yart A, Perret B, Salles JP, Raynal P. Modulation of phosphoinositide 3-kinase activation by cholesterol level suggests a novel positive role for lipid rafts in lysophosphatidic acid signalling. FEBS Lett. 2003; 534:164–168. [PubMed: 12527380]

Petruska JC, Napaporn J, Johnson RD, Gu JG, Cooper BY. Subclassified acutely dissociated cells of rat DRG: histochemistry and patterns of capsaicin-, proton-, and ATP-activated currents. J Neurophysiol. 2000; 84:2365–2379. [PubMed: 11067979]

Pike LJ, Miller JM. Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. J Biol Chem. 1998; 273:22298–22304. [PubMed: 9712847]

Pristerà A, Baker MD, Okuse K. Association between tetrodotoxin resistant channels and lipid rafts regulates sensory neuron excitability. PLoS One. 2012; 7:e40079. [PubMed: 22870192]

Romanenko VG, Fang Y, Byfield F, Travis AJ, Vandenberg CA, Rothblat GH, Levitan I. Cholesterol sensitivity and lipid raft targeting of Kir2.1 channels. Biophys J. 2004; 87:3850–3861. [PubMed: 15465867]

Romanenko VG, Rothblat GH, Levitan I. Modulation of endothelial inward-rectifier K+ current by optical isomers of cholesterol. Biophys J. 2002; 83:3211–3222. [PubMed: 12496090]

Shaw AS. Lipid rafts: now you see them, now you don't. Nat Immunol. 2006; 7:1139–1142. [PubMed: 17053798]

Shogomori H, Brown DA. Use of detergents to study membrane rafts: the good, the bad, and the ugly. Biol Chem. 2003; 384:1259–1263. [PubMed: 14515986]

Shu X-Q, Mendell LM. Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. Neurosci Lett. 1999; 274:159–162. [PubMed: 10548414]

Simons K, Gerl MJ. Revitalizing membrane rafts: new tools and insights. Nat Rev Mol Cell Biol. 2010; 11:688–699. [PubMed: 20861879]

Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 2000; 1:31–39. [PubMed: 11413487]

Singer SJ, Nicolson GL. The fluid mosaic model of the structure of cell membranes. Science. 1972; 175:720–731. [PubMed: 4333397]

Thacker MA, Clark AK, Marchand F, McMahon SB. Pathophysiology of peripheral neuropathic pain: immune cells and molecules. Anesth Analg. 2007; 105:838–847. [PubMed: 17717248]

Vacca F, Amadio S, Sancesario G, Bernardi G, Volonté C. P2X3 receptor localizes into lipid rafts in neuronal cells. J Neurosci Res. 2004; 76:653–661. [PubMed: 15139024]

van der Luit AH, Budde M, Ruurs P, Verheij M, van Blitterswijk WJ. Alkyl-lysophospholipid accumulates in lipid rafts and induces apoptosis via raft-dependent endocytosis and inhibition of phosphatidylcholine synthesis. J Biol Chem. 2002; 277:39541–39547. [PubMed: 12183451]

Vial C, Evans RJ. Disruption of lipid rafts inhibits P2X1 receptor-mediated currents and arterial vasoconstriction. J Biol Chem. 2005; 280:30705–30711. [PubMed: 16006561]

Weskamp G, Otten U. An enzyme-linked immunoassay for nerve growth factor (NGF): a tool for studying regulatory mechanisms involved in NGF production in brain and in peripheral tissues. J Neurochem. 1987; 48:1779–1786. [PubMed: 3572400]

Wong W, Schlichter LC. Differential recruitment of Kv1.4 and Kv4.2 to lipid rafts by PSD-95. J Biol Chem. 2004; 279:444–452. [PubMed: 14559911]

Woolf CJ, Safieh-Garabedian B, Ma Q-P, Crilly P, Winter J. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience. 1994; 62:327–331. [PubMed: 7530342]

Wu C, Butz S, Ying Y, Anderson RG. Tyrosine kinase receptors concentrated in caveolae-like domains from neuronal plasma membrane. J Biol Chem. 1997; 272:3554–3559. [PubMed: 9013605]

Zamir O, Charlton MP. Cholesterol and synaptic transmitter release at crayfish neuromuscular junctions. J Physiol. 2006; 571:83–99. [PubMed: 16339182]

Zaremberg V, Gajate C, Cacharro LM, Mollinedo F, McMaster CR. Cytotoxicity of an anti-cancer lysophospholipid through selective modification of lipid raft composition. J Biol Chem. 2005; 280:38047–38058. [PubMed: 16155007]

Zhang YH, Chi XX, Nicol GD. BDNF enhances the excitability of rat sensory neurons through activation of the p75 neurotrophin receptor and the sphingomyelin pathway. J Physiol (Lond). 2008; 586:3113–3127. [PubMed: 18450779]

Zhang YH, Kays J, Hodgdon KE, Sacktor TC, Nicol GD. Nerve growth factor enhances the excitability of rat sensory neurons through activation of the atypical protein kinase C isoform, PKMζ. J Neurophysiol. 2012; 107:315–335. [PubMed: 21975456]

Zhang YH, Nicol GD. NGF-mediated sensitization of the excitability of rat sensory neurons is prevented by a blocking antibody to the p75 neurotrophin receptor. Neurosci Lett. 2004; 366:187–192. [PubMed: 15276244]

Zhang YH, Vasko MR, Nicol GD. Ceramide, a putative second messenger for NGF, modulates the tetrodotoxin-insensitive Na^+ current and delayed rectifier K^+ current in rat sensory neurons. J Physiol (Lond). 2002; 544:385–402. [PubMed: 12381813]

Zhuang L, Lin J, Lu ML, Solomon KR, Freeman MR. Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. Cancer Res. 2002; 62:2227–2231. [PubMed: 11956073]

Zidovetzki R, Levitan I. Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. Biochim Biophys Acta. 2007; 1768:1311–1324. [PubMed: 17493580]

Highlights

- NGF via its interaction with $p75^{NTR}$ augments the capacity of small diameter sensory neurons to fire action potentials
- We examined the idea that the interaction of $p75^{NTR}$ with its downstream signaling partner(s) may occur in membrane domains known as lipid rafts.
- Two raft disrupting agents, edelfosine and MβCD, blocked the increased excitability produced by NGF whereas adding back cholesterol in the presence of MβCD restored the sensitization produced by NGF.
- Isolation of raft and non-raft membranes demonstrated that $p75^{NTR}$ as well as the neuronal marker for membrane rafts, flotillin, were localized to raft fractions
- These results indicate that the loss of cholesterol from the neuronal membrane and disruption of raft structural integrity was key in promoting NGF-induced sensitization

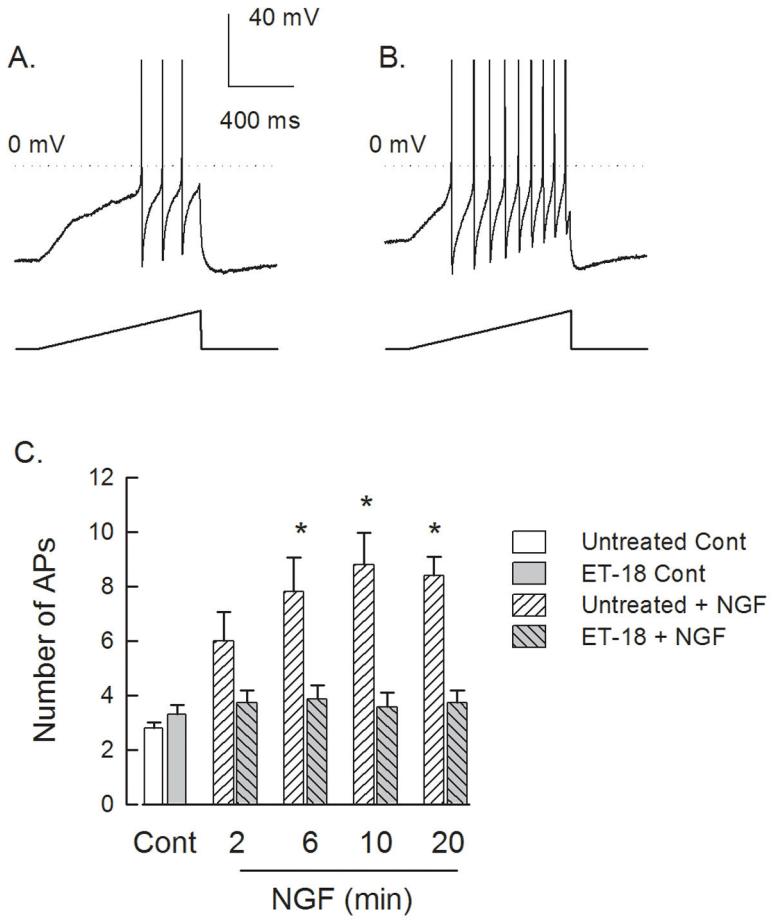

Figure 1.

Edelfosine blocks the enhanced action potential firing produced by NGF. Panel A shows a representative recording of AP firing evoked by the ramp of depolarizing current where this neuron fired 3 APs and had a resting membrane potential of −53.2 mV. Panel B shows that after a 6 min exposure to 100 ng/ml NGF, this neuron now fired 9 APs and was depolarized to −42.4 mV. Panel C summarizes the sensitization produced by NGF as recorded from 5 neurons. In parallel sets of neurons, pretreatment with 10 μM edelfosine (ET-18) blocked the ability of NGF to augment excitability (n=7, P=0.28 vs. ET-18 control, RM ANOVA). Asterisks represent a significant difference vs. control (P<0.001 vs. untreated control, RM ANOVA, Holm-Sidak test).

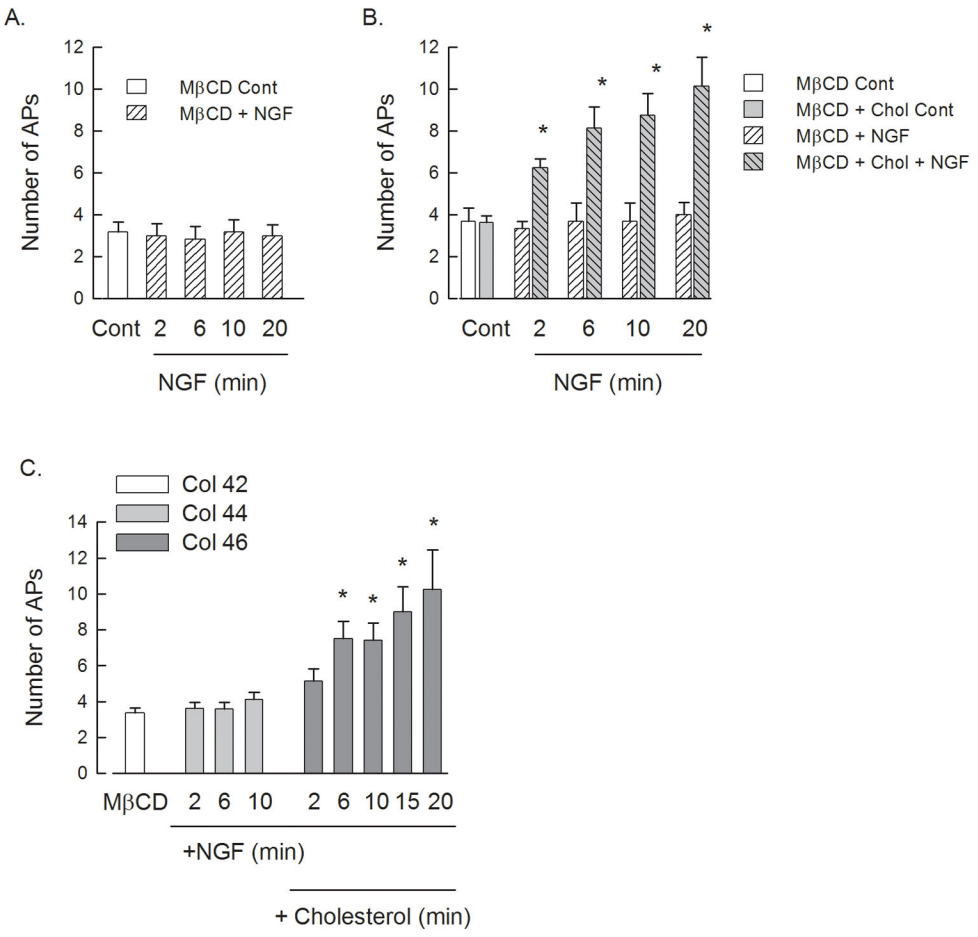

Figure 2.

MβCD inhibits NGF-induced sensitization. Panel A shows that a 30 min pretreatment with 10 mM MβCD blocks the capacity of 100 ng/ml NGF to augment AP firing (n=6, P=0.71 RM ANOVA). Panel B summarizes the effects of 1 mM MβCD and 1 mM MβCD + a saturating concentration of cholesterol on NGF-induced sensitization of AP firing. Treatment with 1 mM MβCD for 60 min blocked the sensitization produced by 100 ng/ml NGF (n=3, P=0.72 RM ANOVA). Treatment with 1 mM MβCD with saturating cholesterol did not alter the increase in AP firing produced by 100 ng/ml NGF (n=8). Asterisks represent P<0.001 vs. control (RM ANOVA, Holm-Sidak test). Panel C demonstrates that a 30 min pretreatment with 10 mM MβCD prevents the increase in AP firing produced by 100 ng/ml NGF. After a 10 min exposure to MβCD + NGF, the superfusate was changed to one containing MβCD + NGF + saturating cholesterol. After only 6 min exposure, there was a significant increase in AP firing compared to the 10 min time point for MβCD + NGF. The number of neurons was 8 for the MβCD control, MβCD + NGF at 2 and 10 min, and MβCD + NGF + cholesterol at 2 and 6 min; 7 for MβCD + NGF at 6 min and MβCD + NGF + cholesterol at 10 min; 4 for MβCD + NGF + cholesterol at 15 and 20 min. Asterisks represent P<0.001 vs. MβCD + NGF 10 min (ANOVA, Holm-Sidak test).

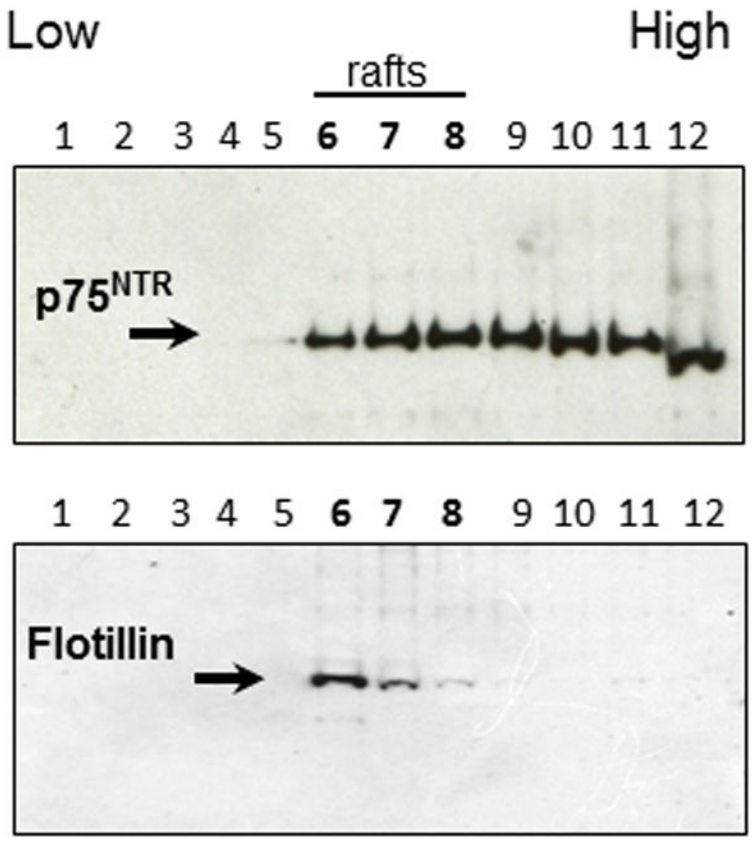

Figure 3.

$p75^{NTR}$ is detected in raft and non-raft membrane fractions. The top panel illustrates a representative Western blot showing that $p75^{NTR}$ is detected in density fractions 6–12. The bottom panel demonstrates that the protein flotillin, a marker for neuronal membrane rafts, is detected in fractions 6–8 and represents the raft fractions.

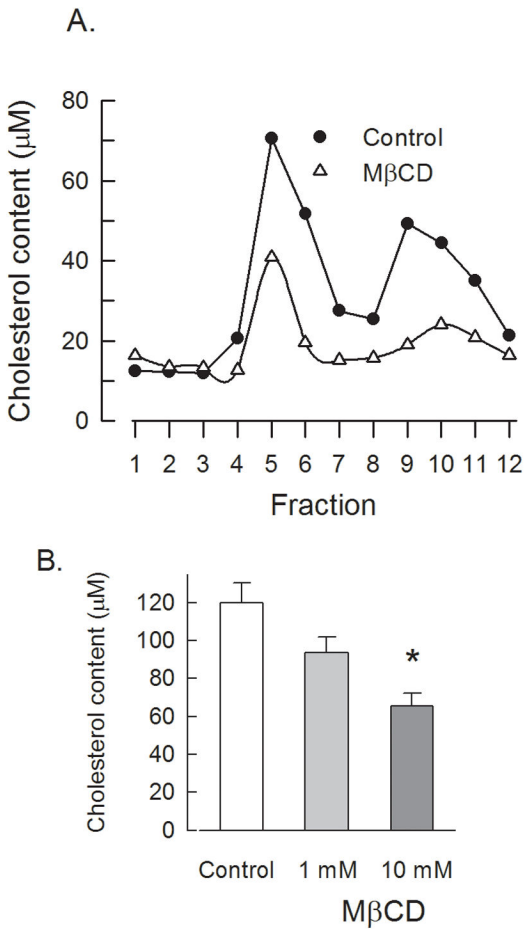

Figure 4.

MβCD depletes cholesterol from isolated sensory neurons. Panel A shows that exposure of isolated sensory neurons to 10 mM MβCD for 1 hr at 37° C reduces the cholesterol concentration in density fractions 4–12. Panel B summarizes the concentration-dependent effects of MβCD on the levels of cholesterol in isolated sensory neurons. Incubations were for 30 min at 37° C; n=4 for control and 10 mM MβCD, n= 3 for 1 mM MβCD. Asterisk represents $P<0.05$ ANOVA.