Neuronal NF1/RAS regulation of cyclic AMP requires atypical PKC activation

Corina Anastasaki and David H. Gutmann

Department of Neurology, Washington University School of Medicine, St. Louis, MO, USA

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Neurofibromatosis type 1 (NF1; OMIM#162200) is a common neurodevelopmental disorder in which affected individuals are prone to learning, attention and behavioral problems. Previous studies in mice and flies have yielded conflicting results regarding the specific effector pathways responsible for NF1 protein (neurofibromin) regulation of neuronal function, with both cyclic AMP (cAMP)- and RAS-dependent mechanisms described. Herein, we leverage a combination of induced pluripotent stem cell-derived NF1 patient neural progenitor cells and Nf1 genetically engineered mice to establish, for the first time, that neurofibromin regulation of cAMP requires RAS activation in human and mouse neurons. However, instead of involving RAS-mediated MEK/AKT signaling, RAS regulation of cAMP homeostasis operates through the activation of atypical protein kinase C zeta, leading to GRK2-driven $G_{alpha}$ inactivation. These findings reveal a novel mechanism by which RAS can regulate cAMP levels in the mammalian brain.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is one of the most common single gene disorders in which individuals are prone to neurodevelopmental abnormalities. As such, over 60% of children with NF1 develop specific learning disabilities, difficulties with visual-spatial tasks, attention deficits and motor delays (1–5). Moreover, there is an increased prevalence of sleep disturbances (6), autism spectrum abnormalities (7) and impairments in social interactions (8). Collectively, these clinical observations suggest that the NF1 gene is a critical regulator of brain neuronal function.

The NF1 gene encodes a large 220 kDa cytoplasmic protein (neurofibromin) which contains a 300 amino acid domain that functions as a GTPase-activating protein (GAP) for p21-Ras (RAS) (9,10). In both human and mouse cells with a germline NF1 gene mutation, reduced neurofibromin expression is associated with increased RAS and RAS pathway activation (11,12). In support of a primary role for neurofibromin as a negative RAS regulator, pioneering work by Silva and colleagues has shown that neurofibromin controls mouse central nervous system (CNS) neuron function in vivo in a RAS-dependent manner. In these studies, genetic or pharmacologic inhibition of RAS activity ameliorated the learning and memory deficits observed in Nf1+/− mice (13,14).

In contrast, previous studies from our laboratory and others have demonstrated that CNS neuron axonal length, growth cone diameter and survival are dependent on neurofibromin positive regulation of cyclic AMP (cAMP) levels, which cannot be reversed by inhibition of RAS–MEK or RAS–PI3K downstream signaling (15–17). Moreover, additional investigations in NF1 mutant Drosophila have revealed that the observed learning deficits depend on neurofibromin regulation of cAMP homeostasis, which may operate in either a RAS-dependent (18,19) or RAS-independent (20,21) manner. Leveraging a combination of pharmacologic and genetic strategies in both human NF1 patient-derived induced pluripotent stem cell (iPSC)-neural progenitor cells (NPCs) and mouse Nf1+/− neurons, we establish that neurofibromin controls cAMP homeostasis in a RAS-dependent manner. In contrast to other cell types, RAS/cAMP regulation does not involve MEK/AKT signaling, but rather operates through the activation of atypical protein kinase C zeta (PKCζ), leading to GRK2-driven $G_{alpha}$ inactivation.

RESULTS

Neurofibromin controls cAMP generation in CNS neurons via $G_{alpha}$ activation

Similarly to previous reports using striatal and retinal ganglion neurons (15,16), mouse primary Nf1+/− hippocampal neurons exhibit shorter axonal lengths relative to their wild-type (WT) counterparts (46% reduction; Fig. 1A) concomitant with...
reduced intracellular cAMP levels (45% reduction; Fig. 1B). To
gain insights into the mechanism underlying neurofibromin cAMP regulation in human NF1 patients, we employed iPSC
technology to reprogram and subsequently differentiate NF1 patient as well as sex- and age-matched control skin fibroblasts into NPCs (Anastasaki et al., manuscript in preparation). Similar to the mouse Nf1+/− neurons, NPCs from patients with NF1 (hNf11-NPCs) also have reduced cAMP levels relative to age- and sex-matched control NPCs (47% reduction; Fig. 1C). Next, we examined the activation of the two major heterotrimeric Gα proteins, Gαi (inhibitory) and Gαs (activating), responsible for regulating adenylyl cyclase (AC) activation and cAMP production following G protein-coupled receptor (GPCR) stimulation. While there was no significant change in Gαi activity (Fig. 1D), Nf1+/− hippocampal neurons exhibited decreased Gαs activity (42% reduction; Fig. 1E) compared with WT controls. These data suggest that neurofibromin promotes cAMP production through Gαs activation.

Neurofibromin-regulated Gαs activation is RAS-dependent
Since neurofibromin functions as an endogenous inhibitor of RAS activity by catalyzing the conversion of RAS from its active GTP-bound to its inactive GDP-bound form (13,22), we next examined RAS-GTP levels in Nf1+/− hippocampal preparations and hNf1-NPCs. In these experiments, Nf1+/− mouse hippocampal neurons had 2.5-fold increased RAS activity relative to WT neurons (Fig. 1F), whereas hNf1-NPCs had 3-fold higher RAS activity relative to age- and sex-matched control NPCs (Fig. 1G).

To determine whether RAS is responsible for neurofibromin regulation of cAMP-dependent axonal length, we employed a combination of pharmacologic and genetic approaches. First, we inhibited RAS function with the farnesyltransferase inhibitor lovastatin (Supplementary Material, Fig. S1A), previously employed to reverse the cognitive deficits in Nf1 mutant mice (14). Following continuous lovastatin exposure in vitro, the attenuated Gαs activity, lower cAMP levels and reduced axonal lengths observed in Nf1+/− neurons were corrected (Fig. 2A and B). Secondly, since hippocampal neurons only express Nras and Kras, but not Hras (Supplementary Material, Fig. S1B–D), we focused on genetically engineered mouse strains in which one allele of the Nras (LSL-NrasG12D) or Kras (LSL-KrasG12D) genes were inactivated (23,24). Intercrossing Nf1+/− mice with LSL-NrasG12D or LSL-KrasG12D mice yielded Nf1+/− mice with reduced Nras or Kras expression, respectively. Consistent with the lovastatin results, neurons from Nf1+/− mice with reduced Nras (Nf1+/−;LSL-NrasG12D; Fig. 2C–D) or Kras (Nf1+/−;LSL-KrasG12D; Supplementary Material, Fig. S1E and F) expression exhibited Gαs activity, cAMP levels and axonal lengths similar to those observed in WT mice. Collectively, these data establish RAS as a primary regulator of CNS neuron cAMP generation.

Neurofibromin regulates atypical PKC activity in a RAS-dependent manner
To further define the mechanism underlying RAS regulation of Gαs activation, we initially examined the activity of the major downstream RAS effectors. Employing both embryonic Nf1+/− mouse hippocampi and hNf1-NPCs, no differences in AKT, ERK, p38 MAPK or JNK activation were identified (Supplementary Material, Fig. S2). In contrast, there was a 2-fold increase in atypical PKCζ Thr-403 phosphorylation in Nf1+/− primary hippocampal neuron cultures in vitro (Fig. 3A) as well as in adult Nf1+/− mice in vivo relative to their WT

Neurofibromin regulates cAMP in a RAS/Gαs-dependent manner.
(A) Quantification of hippocampal neuron axons lengths by Smi-312 immunostaining. Nf1+/− mouse hippocampal neuron axons are significantly shorter than WT neurons (P < 0.001; n = 200). (B and C) Measurement of cAMP generation in mouse hippocampal neurons and human NF1 patient-derived NPCs (hNf11-NPCs). (B) Nf1+/− neurons have lower cAMP levels relative to their WT counterparts (P = 0.0002; n = 5). (C) hNf11-NPCs have reduced cAMP levels compared with age- and sex-matched controls (P < 0.0001; n = 3). (D and E) Quantification of Gαi and Gαs activation of mouse embryonic hippocampal preparations. (D) Nf1+/− mouse hippocampal preparations show no difference in Gαi activation relative to WT neurons (P = 0.7638; n = 5). (E) Nf1+/− mouse embryonic hippocampal preparations exhibit significantly lower Gαs activity (Gαs-GTP) than their WT counterparts (P = 0.001; n = 8). (F and G) Measurement of RAS activation in mouse neurons and human NPCs. (F) Nf1+/− mouse hippocampal neurons exhibit higher levels of RAS activation than control (CTRL) NPCs (P = 0.0002; n = 3). Data are presented as means ± SEM (n ≥ 3). **P < 0.001; Student’s t-test.
counterparts (Fig. 3B; Supplementary Material, Fig. S3A). Importantly, 2.5-fold increased PKCζ phosphorylation was also observed in hNF1-NPCs relative to age- and sex-matched controls (Fig. 3D), thereby implicating PKCζ as a potential novel effector of neurofibromin/RAS signaling in the brain.

Since previous studies have shown that RAS and PKCζ physically interact to result in RAS-mediated atypical PKC activation (25), we sought to determine whether RAS activation was required for PKCζ phosphorylation. Consistent with a model in which RAS regulates PKCζ function, both pharmacologic and genetic reduction of RAS activity restored mouse Nf1−/− neuron PKCζ activation to WT levels (Fig. 3E and F). Together these experiments establish RAS as a critical regulator of PKCζ activity.

**RAS inhibits Goα-GTP through PKCζ phosphorylation**

We next sought to determine whether PKCζ activation is responsible for regulating cAMP homeostasis in Nf1−/− CNS neurons and hNF1-NPCs. First, we employed two independent small molecule inhibitors of PKCζ activity (PKCζ pseudosubstrate, PKCζ-ps and PITenin7, PIT7). Following PKCζ-ps or PIT7 treatment, PKCζ activity was decreased in both Nf1−/− hippocampal neurons (2.2- and 2-fold reduction, respectively; Supplementary Material, Fig. S3B and D) and hNF1-NPCs (2.5- and 1.8-fold reduction, respectively; Supplementary Material, Fig. S3C and E). Moreover, after PKCζ-ps (Fig. 4A, B and D) or PIT7 (Supplementary Material, Fig. S3D and F) administration, Goα activity, cAMP levels and axonal lengths in Nf1−/− neurons were indistinguishable from WT controls. Similarly, in hNF1-NPCs, PKCζ-ps (Fig. 4C) or PIT-7 (Supplementary Material, Fig. S3E) treatment increased cAMP levels by 2-fold.

Secondly, to establish PKCζ as a key regulator of cAMP-driven axonal length, we reduced PKCζ protein expression in Nf1−/− neurons using two independent siRNA constructs (46 and 54% reduction in protein expression; Supplementary Material, Fig. S3G). In these experiments, both siRNA constructs restored Nf1−/+ neuron axonal lengths to WT levels (Fig. 4E). These results demonstrate that PKCζ activation is necessary for Goα-modulated cAMP homeostasis in mouse and human CNS neuronal cultures.

Thirdly, to mimic the increased PKCζ activation observed in Nf1−/− neurons, WT neurons were treated with phosphatidic acid (PA) dioleoyl to activate PKCζ (2-fold). Following PA administration, WT neurons had lower cAMP levels and shorter axonal lengths (Supplementary Material, Fig. S3H and I). Taken together, these data reveal that neurofibromin-regulated cAMP generation requires PKCζ function in mammalian neurons.

**PKCζ regulates cAMP homeostasis through GRK2**

Based on these findings, we explored the possibility that PKCζ blocks Goα activation by modulating GRK function. GPCR signal transduction is controlled by GPCR kinases (GRKs),...
such that agonist-bound GPCRs are phosphorylated by GRKs to cause receptor de-sensitization (26). Since chronic PKC activity can activate GRK2 (27), we hypothesized that PKCζ blocks Ga activation by modulating GRK2-dependent GPCR signaling. Consistent with this hypothesis, we found 2- and 3-fold increases in GRK2 phosphorylation (at Ser-29 and Ser-685, respectively) in both Nf1+/− mouse CNS neurons and hNF1-NPCs (Fig. 5A, Supplementary Material, Fig. S4C and D). In addition, there was increased total GRK2 expression in Nf1+/− mouse CNS neurons (2-fold) and hNF1-NPCs (2-fold; Fig. 5A). Since GRK2-mediated GPCR phosphorylation uncouples Ga from AC, we reasoned that PKCζ downstream signaling might activate GRK2 to attenuate Ga activation. To evaluate this, primary mouse CNS neuronal cultures and hNF1-NPCs were treated with PKCζ-ps. Following PKCζ inhibition, GRK2 phosphorylation was reduced by 2-fold (near WT levels; Fig. 5B), indicating that PKCζ signaling is necessary for GRK2 activation.

Lastly, to determine whether GRK2 mediates neurofibromin/RAS/PKCζ regulation of Ga, cAMP levels and axonal length, we employed the GRK2 inhibitor, βARK1 inhibitor (GRK2-inh). Following GRK2-inh treatment of either Nf1+/− mouse neurons or hNF1-NPCs, GRK2 phosphorylation was decreased by 2- or 2.3-fold, respectively (Supplementary Material, Fig. S4A and B). Moreover, GRK2 inhibition normalized Nf1+/− mouse neuronal Ga, cAMP levels and axonal lengths (Fig. 5C-E) as well as hNF1-NPCs cAMP levels (Fig. 5D). Together, these findings demonstrate that neurofibromin/RAS regulate mammalian CNS neuron cAMP homeostasis through GRK2-mediated attenuation of GPCR-Ga activation (Fig. 5F).

**DISCUSSION**

The majority of what is known about neurofibromin regulation of cAMP regulation derives from studies in Drosophila. In the fly, Nf1 loss leads to a neuromuscular junction overgrowth phenotype (28,29), olfactory learning and memory defects (21,30,31), reduced lifespan (32) and somatic growth deficits (17,19). The use of human NF1 gene mutants lacking a functional RAS regulatory GAP-related domain (GRD) have revealed that some of these
First, our findings reconcile a series of seemingly contradictory reports demonstrating that RAS inhibition, using either farnesyltransferase inhibitors (14) or genetic knockdown (13), restores *Nf1* mutant mouse neuronal dysfunction, but that impaired cAMP generation in *Nf1*+/− mouse neurons could not be rescued by PI3-Kinase or MEK pharmacologic inhibition (13). The discovery that neurofibromin regulates cAMP in a RAS-dependent manner, but involving a distinct downstream effector pathway (PKCζ-GRK2) separable from RAS/PI3K and RAS/MEK signaling, brings some mechanistic clarity to this issue.

Secondly, the studies in CNS neurons reported herein also demonstrate that neurofibromin regulation of RAS downstream signaling can be cell type-specific. In this regard, RAS activation resulting from reduced or absent neurofibromin expression in neuronal cells (33), leukemic cells (34), some NPCs (35), hematopoietic cells (36) and osteoblasts (37) operates through a MEK-dependent pathway. In astrocytes and Schwann cells, *Nf1* loss leads to both MEK/ERK and AKT/mTOR hyperactivation to result in dysregulated cell growth (38–40). However, abnormal neurofibromin function in microglia (41) and osteoblast progenitors (42) involves JNK signaling. As such, the observation that neurofibromin/RAS transmits its regulatory signal through an atypical PKC in CNS neurons and in human patient-derived NPCs provides further experimental evidence for the use of distinct RAS downstream effectors in different cell types. This cell type specificity should be considered when extrapolating results from other tissues relevant to therapeutic drug design for NF1 patient neuronal dysfunction.

Thirdly, neurofibromin regulation of cAMP functions at the level of GPCR-Gα signaling to AC. In this manner,
pharmacologic treatments that activate AC ameliorate the neuronal defects observed in Nf1+/− mouse neurons and human NF1 patient-derived NPCs (15). However, rather than involving Ga activation, as observed in Nf1-deficient astrocytes (43,44), neurofibromin regulation of cAMP in CNS neurons involves GPCR suppression of Ga activation. Similarly, the engagement of Ga in neurons is also required for neurotransmitter-induced neurofibromin/cAMP generation and learning in flies (18,31). Neurofibromin control of cAMP in Drosophila operates in a RAS-dependent manner, thus reinforcing both cell type- and species-related differences in the mechanisms underlying neurofibromin/cAMP signaling. Further studies will be required to more precisely define how GPCR-Ga protein coupling to AC is regulated by neurofibromin in astrocytes and neurons in the CNS.

Fourthly, we show that neurofibromin control of cAMP generation involves RAS-mediated PKCζ engagement to modulate GPCR signaling through GRK2. Previous studies have demonstrated that RAS can activate PKCζ (45,46) as well as regulate vascular endothelial growth factor transcription in a PKCζ-dependent manner (47). In addition, PKCζ can physically bind to RAS (25,48), supporting a direct interaction underlying RAS/PKCζ activation. While atypical PKC molecules have not been previously shown to phosphorylate GRK2, our studies demonstrate that inhibition of PKCζ function impairs GRK phosphorylation and expression. Since GRK2 lacks a consensus PKC phosphorylation motif, additional investigations will be required to determine whether PKCζ directly phosphorylates GRK2 or operates indirectly through another, currently unidentified, kinase molecule. Similarly, while GRK2 can regulate cAMP homeostasis in cardiac fibroblasts (49), it is not known how GRK2 regulates GPCR function, which could operate at the level of desensitization or resensitization (50). Nonetheless, the fact that neurofibromin modulates cAMP generation downstream of cell type-specific GPCRs (20,51,52) responsive to distinct ligands and extracellular signals provides a novel way for neurofibromin to regulate a diverse number of GPCRs in distinct CNS cell populations and suggests previously unexplored strategies for correcting NF1-related CNS deficits.

Finally, the availability of human NF1-NPCs as a complementary resource to study CNS neuronal function in this common neurogenetic condition provides unprecedented opportunities to validate observations initially made in rodent systems relevant to future translation to the treatment of children and adults with NF1.

MATERIALS AND METHODS

Mice

Nf1+/− (53), LSL-KrasG12D (24), LSL-NrasG12D (23) mice were generated as previously described. All mice were
maintained on an inbred C57BL/6 background and used in accordance with an approved Animal Studies protocol at the Washington University School of Medicine. All mice had *ad libitum* access to food and water. Littermate controls were used for all experiments.

**Human iPSC generation and NPC differentiation**

Primary fibroblast cultures from individuals with an established diagnosis of NF1 (NIH Consensus Development Conference 1988) or age- and sex-matched controls were collected (54) reprogrammed into iPSCs as previously described (55). In brief, confluent fibroblast cultures were reprogrammed into iPSCs using Cyto-Tune technology (Invitrogen). Cultures were infected once with integration-free Sendai virus carrying the four Yamanaka stem cell reprogramming factors (*OCT4*, *KLF4*, *SOX2*, *C-MYC*) and cultured for ~6 weeks. iPSC colonies were isolated, and their pluripotency was confirmed by assessing their morphology and expression of stem cell markers (*OCT4*, *SSEA-3*, *TRA-1-60/81*). Chromosomal analysis ensured normal karyotype (Anastasaki et al., manuscript in preparation). Two clones from each iPSC line were cultured in Neural Induction Medium (NIM; STEMCell Technologies) as previously described (56), to form embryoid bodies (EBs) for 5 days. EB aggregates were then plated in NIM on adhesive plates pre-coated with poly-ornithine/laminin to allow for rosette formation. Once established, neural rosettes were collected, gently dissociated and re-plated in PLO/laminin-coated plates to differentiate into NPCs. A portion of the NPCs spontaneously differentiated into neurons, which were then analyzed by immunofluorescence. NPC pellets were snap-frozen in liquid nitrogen for western blot analysis.

**Primary neuronal cultures**

Primary hippocampal neuronal cultures were generated from E12.5–13 mouse embryos as previously described (15). Lovastatin (6.25 μM; Sigma), PKCζ pseudosubstrate (PKCζ-ps), myristoylated (0.5 μM; Enzo Life Sciences), PI[3]T[7] (5 μM; Millipore), Phosphatidic Acid, dioleoyl (PA dioleoyl, 5 μM; Enzo Life Sciences) or βARK1 inhibitor (GRK2-inh, Methyl 5-[2-(5-nitro-2-furyl)vinyl]-2-furoate), 5 μM; Millipore) were added to the culture media 1 h after initial neuronal plating for the entire 3-day culture period. A minimum of three animals per genotype were used and experiments were repeated at least three times with identical results.

**Lentivirus generation and lentiviral infection of primary hippocampal neurons**

HEK-293T (293T) cells were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Pen/Strep solution (Gibco). Twenty-four hours before transfection, 293T cells were seeded at 300 000 cells/well in 6-well plates in antibiotic-free media. Three micrograms of total DNA was transfected per well (1.5 μg shPKCζ or pLKO-GFP control; 1.5 μg lentiviral packaging constructs) using Fugene HD reagent (Promega) following manufacturer's instructions. The media was replaced with fresh DMEM supplemented with 10% FBS and 1% antibiotics 12 h post-transfection. Viral preparations were collected at 48 and 72 h.

**Table 1. Primary antibodies**

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<tr>
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<th>Source</th>
<th>Host</th>
<th>Dilution</th>
<th>Application</th>
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<td>Mouse</td>
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<td>IP</td>
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WB, western blot; IHC, immunohistochemistry; ICC, immunocytochemistry; IP, immunoprecipitation.
post-transfection, aliquoted and frozen at –80°C. Viral titers of 10⁸–10⁹ were used for primary neuronal infections. The sequences of the siRNA constructs employed are shown below:

<table>
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<th>TRC identifier</th>
<th>Vector</th>
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<td>5′-GAAGTGCACA TCATTACGTGTT-3′</td>
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Primary hippocampal neurons were cultured in 10 cm diameter Poly-d-Lysine/Laminin-coated culture plates as previously described (15) for 1 day. Viral dilutions of 1:1000 v/v were prepared in Neurobasal media with added Polybrene (1:10 000 v/v), and were subsequently administered directly to the neurons for 3 h. Following infection, the media was completely aspirated and replaced with fresh Neurobasal media. The cultures were allowed to grow for a total of 3 days, at which time the cells were collected for further immunocytochemical or protein analysis.

Immunocytochemistry and immunohistochemistry
Immunocytochemistry was performed as previously described (15). Images were acquired on an inverted Olympus FV-500 confocal microscope and analyzed using ImageJ software (http://rsbweb.nih.gov/ij/; Wayne Rasband, National Institute of Mental Health, Bethesda, MD). Immunohistochemistry was performed on mice perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4) and post-fixed in 4% PFA prior to paraffin embedding, as previously described (57). Appropriate primary antibodies (Table 1) and secondary antibodies were used.

Western blotting
Western blotting was performed as previously described (15) using appropriate primary antibodies (Table 1), secondary horseradish peroxidase-conjugated antibodies (Sigma) and ECL (Fisher) chemiluminescence.

cAMP and activity assays
All assays were performed on dissected embryonic hippocampi, snap-frozen in liquid nitrogen. cAMP levels were quantitated from tissue homogenized in 0.1 M HCl, using a cAMP ELISA immunoassay kit (Enzo Life Sciences) following manufacturer’s instructions. Go α and Go β activity were determined using commercially available activation kits (NewEast Biosciences) following the manufacturer’s instructions. Active Ras (Ras-GTP) was detected by Ras-GTP immunoprecipitation using the Ras activation kit (Millipore) following manufacturer’s instructions.

Statistical analyses
All statistical analyses were performed using GraphPad Prism 5 software. Unpaired two-tailed Student’s T-tests were used for experiments analyzing data between two groups. One-way analysis of variance (ANOVA) with Bonferroni post-test correction analyses were employed for multiple comparisons.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. The authors declare that they have no conflict of interest.

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