Serotonin modulates multiple calcium current subtypes in commissural interneurons of the neonatal mouse

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Abbinanti MD, Harris-Warrick RM. Serotonin modulates multiple calcium current subtypes in commissural interneurons of the neonatal mouse. J Neurophysiol 107: 2212–2219, 2012. First published January 25, 2012; doi:10.1152/jn.00768.2011.—Calcium currents are critical to the intrinsic properties of neurons and the networks that contain them. These currents make attractive targets for neuro-modulation. Here, we examine the serotonergic modulation of specific calcium current subtypes in neonatal (P0-5) intersegmental commissural interneurons (CINs), members of the hindlimb locomotor central pattern generator in the mouse spinal cord. Previous work in our lab showed that serotonin (5-HT) excited CINs in part by reducing a calcium current and thus indirectly reducing the calcium-activated potassium current (Diaz-Rios et al. 2007). We have determined which calcium currents are targets of serotonin modulation. Utilizing whole cell voltage clamp and toxins to specific calcium current subtypes, we found that N- and P/Q-type currents comprise over 60% of the overall calcium current. Blockade of each of these subtypes alone with either ω-conotoxin GVIA or ω-agatoxin TK was unable to occlude 5-HT’s reduction of the calcium current. However, coapplication of both blockers together fully occluded 5-HT’s reduction of the calcium current. Thus, 5-HT decreases both N- and P/Q-type calcium current to excite neonatal CINs.

spinal cord; interneuron; locomotion

IN THIS STUDY, WE EXAMINE the subtypes of calcium currents that are modulated by serotonin (5-HT) in intersegmental commissural interneurons (CINs) of the neonatal mouse spinal cord. CINs are components of the rodent hindlimb locomotor central pattern generator network; they coordinate left-right hindlimb alternation by sending axons across the midline of the spinal cord to provide excitatory and inhibitory input to cells in the opposite hemicord, including inhibitory interneurons and motor neurons (Butt et al. 2002; Eide et al. 1999; Kiehn and Butt 2003; Nishimaru et al. 2006; Quinlan and Kiehn 2007; Zhong et al. 2006a, 2006b). Selective deletion of subpopulations of CINs results in decreased left-right coordination (Lanuza et al. 2004).

Descending modulatory input, and specifically 5-HT, is critically important for activation of the locomotor network. Serotonergic neurons projecting to the spinal cord increase firing during locomotion (Fornal et al. 1985). Serotonin can activate fictive locomotion in the neonatal mouse (Nishimaru et al. 2000). While NMDA alone can activate fictive locomotion in the rat, it cannot do so in the presence of 5-HT antagonists (MacLean et al. 1998). Stimulating brainstem serotonergic cells activates locomotor-like activity that can be blocked by 5-HT₂A and 5-HT₇ receptor antagonists (Liu and Jordan 2005).

Application of 5-HT during NMDA-induced fictive locomotion in the rat improved left-right and flexor-extensor coordination (Pearlstein et al. 2005, 2011). Thus, it is important to identify the targets of 5-HT action in the locomotor network and the mechanisms by which 5-HT works on them.

Calcium currents play important roles in a wide range of neuronal and network processes (Harris-Warrick 2002). I_Ca(V) is essential to neurotransmitter release and plays a role in shaping the intrinsic properties of neurons (Buschges et al. 2000; Catterall 1998; Li and Bennett 2003; Richter et al. 1993). Because of their crucial roles, calcium currents make attractive targets for 5-HT modulation (El Manira et al. 1997; Hounsgaard and Kiehn 1989). Previous electrophysiological and voltage-clamp studies in our lab showed that 5-HT increases excitability in neonatal CINs in part by decreasing I_Ca(V) thus indirectly decreasing I_K(Ca) (Diaz-Rios et al. 2007).

Apatin, a blocker of the SK-type calcium-activated potassium current, mimicked and occluded 5-HT’s effects to reduce the spike afterhyperpolarization and enhance neonatal CIN excitability. However, the identity of the calcium current subtypes modulated by 5-HT were not identified (Diaz-Rios et al. 2007).

We used whole cell voltage-clamp experiments and selective antagonists to specific calcium current subtypes to determine which subtypes are targets of 5-HT modulation in ascending and descending CINs. We found that 5-HT reduces both the P/Q- and N-type calcium current in PO-5 CINs.

MATERIALS AND METHODS

Slice preparation. Experiments were performed on PO-5 B6 mice. The animal protocol was approved by the Cornell University Institutional Animal Care and Use Committee and is in accordance with National Institutes of Health guidelines. Mice were killed by rapid decapitation, and the spinal cord was removed from the animal by ventral laminectomy in ice-cold (4°C), oxygenated (95% O₂:5% CO₂) sucrose-based aCSF containing (in mM) 188 sucrose, 25 Na-glucose, 26 NaHCO₃, 25 NaCl, 10 MgSO₄, 1.2 NaH₂PO₄, and 1.9 KCl (pH = 7.4). CINs were retrogradely labeled by making small slits in the contralateral hemicord rostral and caudal to the L2 region and placing crystals of different fluorescent dextran amines in each slit (3,000 MW Texas Red dextran amine or 3,000 MW fluorescein dextran amine; Invitrogen, Carlsbad, CA).

Labeled spinal cords were incubated in ice-cold sucrose-based aCSF for 30–45 min to allow for dye diffusion to CIN cell bodies. Transverse slices (250-μm thick) were made with a vibrating microtome (Leica Microsystems, Wetzlar, Germany). Slices were placed in 30°C normal mouse aCSF (adapted from Carlin et al. 2000) containing (in mM): 119 NaCl, 1.9 KCl, 26 NaHCO₃, 1.2 NaH₂PO₄, 10 MgSO₄, 1 CaCl₂, and 10 Na-glucose (pH = 7.4) and were allowed to come to room temperature (~20–22°C) over 1 h.

Whole cell voltage-clamp recordings. At room temperature, slices were transferred to the recording dish mounted to the stage of a Zeiss
Axioskop 2 FS Plus upright microscope and perfused with (3 ml/min) oxygenated normal aCSF. CINs were identified as Texas Red or fluorescein-labeled cells contralateral to the dye application sites under epifluorescent illumination and visualized using differential interference contrast optics; dually labeled CINs, which send bifurcating axons to both the rostral and caudal contralateral cord, were excluded, as they do not participate in fictive locomotion and are not sensitive to serotonin (Zhong et al. 2006b). Electrodes were pulled on a Narishige vertical puller (Narishige International USA, East Meadow, NY) from thick-wall borosilicate glass (WPI, Sarasota, FL) with resistances of 3–6 MΩ. A seal resistance of at least 1 GΩ was obtained before patch rupture in all recordings. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. The voltage-clamp experiment pipette solution was adapted from Carlin et al. (2000) and contained (in mM) 100 Cs-methane-sulfonate, 30 tetraethylammonium chloride (TEA-Cl), 1 MgCl2, 10 EGTA, 5 NaCl, 1 CaCl2, 10 HEPES, 3 ATP-Mg, and 0.3 GTP-Li (pH 7.3). Voltage-clamp experiments used barium to substitute for calcium in a HEPES-buffered, sulfate-free extracellular solution containing (in mM): 105 NaCl, 1.9 KCl, 10 HEPES, 2 MgCl2, 2.5 BaCl2, 10 d-glucose, 30 TEA-Cl, 4 4-aminopyridine (4-AP), 2 CsCl, 1 kynurenic acid, 0.001 tetrodotoxin (TTX), 0.009 strychnine, and 0.01 picrotoxin (pH = 7.4). After complete superfusion of extracellular solution into the bath, perfusion was stopped to allow application of aliquots of calcium channel blockers. Stopping the perfusion did not alter the quality of the calcium current measurements or the rate of rundown during the time of channel blocker application. In some experiments, 100% O2 was blown over the surface of the solution during the time the perfusion was stopped, to maintain oxygenation of the slice. However, this did not affect the quality of the recordings. Series resistance and capacitance was compensated by at least 70%. Leak currents were corrected online using P/4 leak subtraction. Recordings were made using a Multiclamp 700A amplifier and Digidata 1322A digitizer (Axon Instruments, Foster City, CA) using Clampex (pClamp 9, Axon Instruments). Data were filtered at 2 kHz and digitized at 5 or 10 kHz.

Drugs. AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX), glycine antagonist strychnine, and sodium channel antagonist TTX were purchased from Tocris Bioscience (Ellisville, MO). Transient potassium current antagonist 4-AP, NMDA receptor antagonist d-(-)-2-amino-5-phosphophentonic acid (APV), calcium channel blocker cadmium chloride (CdCl2), NMDA and AMPA/kainate antagonist kynurenic acid, GABA antagonist picrotoxin, serotonin creatine sulfate (5-HT), and potassium channel antagonist TEA-Cl were purchased from Sigma. N-type calcium channel blocker ω-conotoxin GVIA and P/Q-type calcium channel blocker ω-agatoxin TK were purchased from Alomone Labs (Jerusalem, Israel), and aliquots were dissolved in recording solution before use.

Data analysis. Peak current amplitudes at each step were reported. We were able to resolve the effects of the drugs by determining the change in the current amplitude over time throughout the experiments, defined as the instantaneous change in current over time, dI/dt. Using these values, we were able to observe the rapid effects of the drugs on the current as well as monitor the steady-state rundown of the current throughout the experiment. By averaging the dI/dt value over time before the addition of toxins or 5-HT, we could quantify the amount of rundown occurring during each experiment. The average rate of rundown multiplied by the time from drug application to steady-state drug effect (determined by dI/dt returning to resting levels) was subtracted from the measured values, providing us with rundown-corrected values.

Data are presented as means ± SD. All tests of significance used the Student’s t-tests (paired or unpaired where appropriate) unless otherwise noted.

RESULTS

In the neonatal (P0-5) mouse spinal cord, 5-HT increases the excitability of ascending and descending CINs by decreasing calcium currents, which in turn appear to decrease calcium-activated potassium currents (Diaz-Rios et al. 2007). We sought to determine which specific subtypes of calcium currents are the targets of 5-HT’s reduction in CINs at this age.

Utilizing the whole cell voltage clamp, we observed an inward current in HEPES-buffered aCSF with calcium replaced by 2.5 mM barium, elicited by steps from −60 mV in the presence of blockers for sodium, potassium, and Ih currents (TTX, TEA-Cl, 4-AP, CsCl) (Carlin et al. 2000). This current was detectable at voltages more depolarized than −40 mV, and reached a maximum around 0 mV, as previously reported (Diaz-Rios et al. 2007) (Fig. 1).

In the following experiments, we examined the inward current activated by voltage steps every 30 s from −60 to 0 mV. This was done to be able to correct for any rundown of the barium current that occurred over time. We observed a peak inward barium current of at least 25 pA in 32 out of 36 CINs. The remaining 4 cells had less than 25 pA of current, which was difficult to resolve, so we excluded these cells. The peak amplitude of barium current at 0 mV was 212 ± 153 pA (n = 32). Although developmental increases in ICa densities have been reported from late embryonic to neonatal rat motorneurons (Gao and Ziskind-Conhaim 1998), we did not observe any significant differences in the amplitude of this current with age over the age range of mice we studied (P0-5). This current was completely blocked by 200 μM cadmium (for example, see Fig. 1).
A number of different subtypes of calcium current contribute to this barium current.

**Contribution of P/Q- and N-type calcium current in CINs.** We used ω-conotoxin GVIA to specifically block N-type calcium current (Carlin et al. 2000; Feldman et al. 1987). By monitoring the current response to voltage steps every 30 s, we could monitor the time course of the current amplitude before and during application of the toxin, and correct for rundown by looking for rapid changes in current when the drug is added (Fig. 2A). This was done by calculating the instantaneous change in current over time, dI/dt, during the experiment (Fig. 2D); the drug-induced reduction is clearly seen by the peak in dI/dt after the drug was added, which falls to the steady-state decline over a few minutes (Fig. 2D). Additionally, we calculated the rundown of the current in each experiment by averaging the instantaneous dI/dt over a period of time before the application of blockers and/or 5-HT. This allowed us to resolve what percentage of the reduction was due to the effects of the drug or to the relatively linear rundown of the current. In neonatal CINs, 1 μM ω-conotoxin GVIA was the saturating concentration as determined by the lack of additional effect by a second application of the conotoxin. ω-Conotoxin GVIA blocked 31 ± 8% of the total barium current (n = 9, P < 0.01) (Fig. 2, A–C). The effect of conotoxin was rapid, shown by the peak in the rate of current reduction (Fig. 2D), and the full effect was observed by 2–3 min after application (Fig. 2B).

To determine the contribution of P/Q-type current to the overall calcium current in neonatal CINs, we utilized ω-agatoxin TK, a specific antagonist for P/Q-type calcium channels (Carlin et al. 2000; Teramato et al. 1995). We determined experimentally that 200 nM agatoxin was a saturating concentration by showing that additional doses of agatoxin did not further decrease the remaining current (data not shown); this concentration was also used to block P/Q-currents in older mouse spinal cord motor neurons (Carlin et al. 2000). ω-Agatoxin TK blocked 33 ± 13% of the total barium current (n = 12, P < 0.01) (Fig. 2, A, B, E). Our results suggest that P/Q- and N-type calcium current make up about two-thirds of the total current elicited by steps from −60 to 0 mV in P0-5 CINs (Fig. 2, C and E). We did not observe a difference in the contribution of P/Q- and N-type current with age within the neonatal age group (P0-P5).

5-HT’s effects persist after application of agatoxin or conotoxin alone. Using our experimental procedure, we confirmed the findings of Diaz-Rios et al. (2007) that 5-HT reduces IBa in most neonatal CINs (Fig. 3). 5-HT (10 μM) reduced the current by 24 ± 11% in 4 out of 5 neurons tested (P < 0.05). Additional 5-HT did not further reduce the current (Fig. 3B). If the 5-HT application was for a short period, the reduction was at least partially reversible on washout; however, longer applications were not reversible over the time of our recordings (Diaz-Rios et al. 2007). We were unable to see an effect of
5-HT in the fifth neuron, consistent with previous work showing that not all CINs respond to serotonin (Diaz-Rios et al. 2007). Diaz-Rios et al. (2007) reported a larger (49%) reduction of the barium current by 5-HT. However, they did not measure the current as often as we did, and perhaps overestimated the reduction by not correcting adequately for the baseline rundown of the current.

To determine if the reduction of IBa by 5-HT is mediated by the modulation of P/Q- or N-type calcium channels alone, we applied saturating concentrations of ω-agatoxin TK or ω-conotoxin GVIA followed by 10 μM 5-HT. Prior treatment with 200 nM ω-agatoxin TK, the specific P/Q blocker, did not occlude the reduction of the current in 3/4 CINs tested (Fig. 4, A and B). The effects of ω-agatoxin TK and 5-HT were faster than the effects of the rundown of the current (Fig. 4C). One CIN did not show a rapid reduction of the current by 5-HT during the application of ω-agatoxin TK, but this neuron may not have shown a response to serotonin in any case (Diaz-Rios et al. 2007). In these experiments, the first application of ω-agatoxin TK reduced IBa by 27 ± 5% (n = 4). Addition of 10 μM 5-HT, in the continued presence of ω-agatoxin TK, reduced the remaining current by another 14 ± 8% of the original current amplitude (n = 3, P < 0.05). This reduction in IBa amplitude occurred at a much faster rate than the rundown. This demonstrated that the P/Q-type current is not the sole target of 5-HT’s reduction of calcium current.

ω-Conotoxin GVIA, the blocker of N-type calcium channels, was also unable to completely occlude 5-HT’s reduction of calcium current in 4/4 CINs (Fig. 5). ω-Conotoxin GVIA (1 μM) reduced IBa before 5-HT application by 32 ± 9% (n = 4). In the continued presence of ω-conotoxin, 5-HT reduced the remaining IBa by 12 ± 5% of the original current amplitude (n = 4 P < 0.01; Fig. 5, A and B). As with all of these experiments, this reduction was observed as a rapid reduction in IBa amplitude at a faster rate than the rundown (Fig. 5C).

Blocking P/Q- and N-type calcium currents together occludes 5-HT’s effect. To determine if both P/Q- and N-type channels are targets of 5-HT modulation, we coapplied saturating concentrations of both ω-agatoxin TK and ω-conotoxin GVIA before applying 10 μM 5-HT. IBa was reduced by 56 ± 8% after coapplication of both 200 nM ω-agatoxin and 1 μM ω-conotoxin (n = 5; Fig. 6, A and B). After application of 5-HT, consistent with previous work showing that not all CINs respond to serotonin (Diaz-Rios et al. 2007). Diaz-Rios et al. (2007) reported a larger (49%) reduction of the barium current by 5-HT. However, they did not measure the current as often as we did, and perhaps overestimated the reduction by not correcting adequately for the baseline rundown of the current.
5-HT, no rapid reduction of current amplitude was seen \((n = 5; \text{Fig. 5C})\). This lack of a rapid decrease in response to 5-HT suggests that any “reduction” in the current amplitude under these conditions is a result of rundown and not modulation by 5-HT. After correcting for rundown as described above, no reduction by 5-HT was observed \((1.2 \pm 1.4\% \text{ reduction}; n = 5)\). Thus, a saturating block of both P/Q- and N-type calcium current occluded the effects of 5-HT on the current.

**DISCUSSION**

Multiple calcium current subtypes are present in neonatal CINs. Our previous study showed that 5-HT reduces high-voltage-activated (HVA) calcium current in neonatal mouse CINs (Diaz-Rios et al. 2007). Here we used pharmacological tools in a voltage-clamp study to distinguish the components of the HVA current in these neurons and determine which of these currents are affected by 5-HT. We demonstrated that there are multiple subtypes of HVA calcium currents present in P0-5 dCINs and aCINs. Of the HVA barium currents evoked by a voltage step from \(-60\) to 0 mV, \(\omega\)-agatoxin TK, a blocker of P/Q-type calcium current, blocked 31% of the current. The remaining 35% of the current is abolished by CdCl\(_2\) and may be made up of L-, R-, or even a component of the low-voltage-activated T-type calcium current. Because the major goal of this study was to identify 5-HT-sensitive currents, and these additional currents were not affected by 5-HT, we did not further differentiate them.

The contribution of different HVA currents to the total calcium current varies across neuron types and species. Some neurons express mostly agatoxin-sensitive P/Q-type calcium current. For example, in 1- to 3-wk-old rat Purkinje neurons, \(~80\%\) of HVA calcium current is P/Q-type (Regan 1991). In P3-6 rat hypoglossal motor neurons, 50% of HVA calcium current is blocked by agatoxin (P/Q-type), 33% by conotoxin (N-type), and 6% by nimodipine (L-type) (Umemiya and Berger 1994). Other neurons have variable amounts of P/Q current. P/Q-type calcium current contributed to between 14 and 45% of the total calcium current in a number of other P7-21 neurons in the hippocampus, visual cortex, dorsal root ganglion, and spinal cord (Mintz et al. 1992). Interestingly, in that study, spinal cord neurons expressed the highest agatoxin-sensitive current, with an average contribution (45%) similar to that measured in our study (33%). In contrast, in lamprey...
serotonin modulates N- and P/Q-type calcium currents capable of coupling with $I_{K(Ca)}$. Previous experiments in our lab showed that apamin, an SK blocker, mimics and occludes 5-HT’s effects on the AHP and excitability in P0-5 CINs. Our results showing that both N- and P/Q-type calcium currents alone each reduced the total effect of 5-HT to reduce the barium current, but was unable to eliminate serotonin’s effect. This suggests that multiple subtypes are being modulated. Coapplication of apamin and conotoxin did completely occlude 5-HT's reduction of calcium current (Fig. 6). This suggests that both N- and P/Q-type calcium currents (and not L-type or other calcium currents) are reduced by 5-HT in P0-5 spinal CINs. Similar effects have been demonstrated in a number of different preparations. In rat motor neurons, 5-HT reduces the amplitude of the AHP as well as the N- and P/Q-type calcium currents (Bayliss et al. 1995). In Xenopus larvae nonsensory spinal cord neurons, 5-HT reduced both N- and P/Q-type calcium currents via different 5-HT receptors (Sun and Dale 1998).

A variety of calcium currents are capable of regulating $I_{K(Ca)}$ in different neurons. For instance, N-, but not P/Q-, type calcium current is coupled to SK current in rat vagal neurons (Sah 1995). Blocking N-type calcium current with conotoxin mimics the effects of apamin in adult mouse thalamocortical neurons (Kasten et al. 2007). Hippocampal pyramidal neurons also show functional coupling between N-type calcium channels and BK channels (Loane et al. 2007). In striatal interneurons, blocking N-type calcium current blocked the apamin-insensitive medium afterhyperpolarization selectively, while the apamin-insensitive slow AHP was inhibited instead by L-type calcium current blockers (Goldberg and Wilson 2005). In deep cerebellar nuclei, N-type calcium channels are coupled to calcium-activated potassium channels, mediating the cells’ ability to fire in bursts (Alviña and Khodakhah 2008). Finally, blocking either N- or L-type calcium currents decreased BK current in neocortical pyramidal neurons (Sun et al. 2003).

Other studies implicate P/Q- or L-type calcium current in activating $I_{K(Ca)}$. In mouse motor nerve terminals, P/Q-type calcium current activates $I_{K(Ca)}$ (Protti and Uchitel 1997). In rat Purkinje cells, P/Q-type calcium current is coupled to both BK and SK current (Womack et al. 2004). Additionally, in hippocampal neurons, L-type calcium current is functionally coupled to SK channels while N-type calcium channels are coupled to SK channels in lamprey spinal neurons. Hill et al. (2003) demonstrated that N-type calcium current alone was being modulated by 5-HT receptors, thus reducing $I_{K(Ca)}$ and reducing the slow AHP. Finally, Berkefeld et al. (2006) demonstrated that calcium-activated potassium channels can form physical complexes with voltage-gated calcium channels in the rat brain. Our past and present results suggest that N- and P/Q-type channels are functionally coupled to SK channels and reduced by 5-HT in P0-5 CINs.

Why are multiple calcium current subtypes decreased by serotonin? Our results showing that both N- and P/Q-type calcium currents are modulated by 5-HT in neonatal CINs raises the question as to why more than one current would be modulated in a single cell. One answer to this may be rooted in development: different subtypes of calcium current are differentially expressed during development. For example, in embryonic Xenopus nonsensory spinal neurons, N-type current is the predominant calcium current subtype, with no detectable

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**Fig. 6.** Blocking N- and P/Q-type calcium channels together occludes 5-HT’s effects. A: current traces in response to steps from -60 to 0 mV in control conditions (a), during coapplication of 1 μM α-conotoxin GVIA and 200 nM α-agatoxin TK (b), and application of 5-HT in addition to conotoxin and agatoxin (c). B: peak current amplitudes measured every 30 s. Measurements from traces in A are indicated by lowercase letters (a, b, c). Black bars indicate application of noted drug. C: plot of instantaneous dI/dt. Note peaks just after application of conotoxin and agatoxin but no peak following application of 10 μM 5-HT.
P/Q-type current. However, by the larval stages, a P/Q-type current emerges (Sun and Dale 1998). At the rat neuromuscular junction, synaptic transmission is dependent on both N- and P/Q-type calcium channels from embryonic development up to P4. However, after P5, synaptic transmission becomes predominantly dependent on P/Q-type calcium currents (Rosato-Siri and Uchitel 1999). In the mouse spinal cord, new L-type calcium channel transcripts begin to be expressed only after P7 (Jiang et al. 1999). These studies demonstrate that the calcium channel profile is dynamic during both embryonic and postnatal development. Serotonergic modulation, which plays an important role throughout these developmental stages, may need to act on a number of different, seemingly redundant, targets to function consistently to excite the CINs. Perhaps the second messenger pathways of modulation that affect one calcium current subtype could also affect the other.

There is also evidence suggesting that different serotonin receptors can modulate different calcium channel subtypes. As discussed above, 5-HT reduces both N- and P/Q-type calcium currents in our preparation and in Xenopus larval spinal cord neurons (Sun and Dale 1998). Sun and Dale demonstrated that 5-HT$_{1A}$ receptors act to reduce agatoxin-sensitive (P/Q-type) channels while 5-HT$_{1B}$ receptors act to reduce conotoxin-sensitive (N-type) channels. This suggests that the effects of 5-HT can be fine-tuned at the cellular level by changing the relative expression profiles of calcium channel and/or 5-HT receptor subtypes. More research is required to determine which serotonin receptors are involved in which modulatory processes during postnatal development.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: M.D.A. and R.H.-W. conception and design of research; M.D.A. performed experiments; M.D.A. analyzed data; M.D.A. and R.H.-W. interpreted results of experiments; M.D.A. prepared figures; M.D.A. drafted manuscript; M.D.A. and R.H.-W. edited and revised manuscript; R.H.-W. approved final version of manuscript.

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