Original Article

Cross-regulation between colocalized nicotinic acetylcholine and 5-HT_3 serotonin receptors on presynaptic nerve terminals

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Aim: Substantial colocalization of functionally independent α4 nicotinic acetylcholine receptors and 5-HT_3 serotonin receptors on presynaptic terminals has been observed in brain. The present study was aimed at addressing whether nicotinic acetylcholine receptors and 5-HT_3 serotonin receptors interact on the same presynaptic terminal, suggesting a convergence of cholinergic and serotonergic regulation.
Methods: Ca^{2+} responses in individual, isolated nerve endings purified from rat striatum were measured using confocal imaging.
Results: Application of 500 nmol/L nicotine following sustained stimulation with the highly selective 5-HT_3 receptor agonist *m*-chlorophenylbiguanide at 100 nmol/L resulted in markedly reduced Ca^{2+} responses (28% of control) in only those striatal nerve endings that originally responded to *m*-chlorophenylbiguanide. The cross-regulation developed over several minutes. Presynaptic nerve endings that had not responded to *m*-chlorophenylbiguanide, indicating that 5-HT_3 receptors were not present, displayed typical responses to nicotine. Application of *m*-chlorophenylbiguanide following sustained stimulation with nicotine resulted in partially attenuated Ca^{2+} responses (49% of control). Application of *m*-chlorophenylbiguanide following sustained stimulation with *m*-chlorophenylbiguanide also resulted in a strong attenuation of Ca^{2+} responses (12% of control), whereas nicotine-induced Ca^{2+} responses following sustained stimulation with nicotine were not significantly different from control.
Conclusion: These results indicate that the presynaptic Ca^{2+} increases evoked by either 5-HT_3 receptor or nicotinic acetylcholine receptor activation regulate subsequent responses to 5-HT_3 receptor activation, but that only 5-HT_3 receptors cross-regulate subsequent nicotinic acetylcholine receptor-mediated responses. The findings suggest a specific interaction between the two receptor systems in the same striatal nerve terminal, likely involving Ca^{2+}-dependent intracellular pathways that regulate these signaling systems at one or more levels.

Keywords: presynaptic calcium regulation; nicotinic receptors; serotonin receptors
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Introduction

Neurotransmitter receptors reside on presynaptic nerve terminals at synapses in brain, where they likely act to regulate neurotransmission. In the striatum, a key component of the psychomotor system[1] functioning in reward-related dopaminergic signaling[2] that is regulated by nicotine[3], presynaptic nicotinic acetylcholine receptors (nAChRs) colocalize with 5-HT_3 serotonin receptors as functionally and physically independent pathways[4]. This suggests convergence of nicotinic cholinergic and serotonergic pathways at the level of the individual presynaptic terminal.

Both presynaptic nAChRs and 5-HT_3 receptors induce robust increases in presynaptic Ca^{2+} on activation[4–6], which, in turn, trigger the release of neurotransmitter[7, 8]. Increases in $[Ca^{2+}]_i$ have also been found to regulate desensitization of the receptors[9, 10], possibly involving calcineurin[11]. Cross-regulation via receptor-mediated increases in $[Ca^{2+}]_i$ might be one means by which convergent serotonergic and nicotinic pathways could interact. Cross-talk has been noted for a wide variety of neurotransmitter receptors colocalized on presynaptic terminals[12–16] and this possibility was explored for colocalized nAChRs and 5-HT_3 receptors on striatal ter-

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minals in the present study.

5-HT_3 receptors and nAChRs are members of the Cys-loop ligand-gated ion channel superfamily[17, 18]. They respond to selective agonist activation by conducting cations, including, most notably, Ca^{2+} [19, 20]. In striatal terminals, increased $[Ca^{2+}]_i$ on activation of nAChRs[5] or 5-HT_3 receptors[6] appear to be mainly the result of Ca^{2+} entry via the receptor-channels, as the Ca^{2+} responses were largely insensitive to voltage-gated Ca^{2+} channel (VGCC) blockade or predepolarization. However, dependence of nicotine-induced dopamine release in striatal terminals on VGCCs has been noted[21–23], and additionally, presynaptic responses mediated by nAChRs in other brain regions, such as hippocampus, display a mixed dependence on VGCCs and Ca^{2+} influx via the receptor channel[22, 24, 25]. In any case, the robust increases in $[Ca^{2+}]_i$ resulting from presynaptic receptor activation in striatal nerve terminals are relatively sustained, and as such, may trigger activation of Ca^{2+}-dependent intracellular regulators, including CaM-dependent kinase, ERK, and calcineurin[26–28]. These intracellular regulators may provide the means for receptor-receptor cross-talk at the level of the presynaptic terminal. Here, we demonstrate cross-regulation between presynaptic 5-HT_3 receptors and nAChRs in rat striatal nerve terminals.

Materials and methods

Chemicals Fluo-3/AM was purchased from Molecular Probes (Eugene, OR, USA). The adhesive matrix Cell-Tak was from BD Sciences (Bedford, MA, USA). Percoll was originally from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Ultrapure sucrose was from ICN Biomedicals (Aurora, OH, USA). HEPES (ULTROL grade) was from Calbiochem (San Diego, CA, USA). (–)Nicotine and 1-(*m*-chlorophenyl)biguanide (mCPBG) were from RBI-Sigma (St Louis, MO, USA). All other chemicals were of the highest reagent grade.

Animals Adult, male Sprague-Dawley rats were obtained from Taconic Farms at 6–8 weeks of age.

Purification of isolated presynaptic nerve terminals Intact isolated presynaptic nerve terminals (synaptosomes) were purified as described previously[5]. In brief, striata from adult Sprague-Dawley rats were dissected into ice-cold 0.32 mol/L sucrose. The tissue was rapidly homogenized in ice-cold 0.32 mol/L sucrose with a glass-Teflon tissue grinder. Synaptosomes were isolated using the Percoll step gradient method[29]. The purified synaptosomes were washed with oxygenated HEPES-buffered saline (HBS, pH 7.4) containing 142 mmol/L NaCl, 2.4 mmol/L KCl, 1.2 mmol/L K_2HPO_4, 1 mmol/L $MgCl_2$, 5 mmol/L *D*-glucose, and 10 mmol/L HEPES, containing 1 mmol/L Ca^{2+}. The protocol used for this study was approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee.

Measurement of relative Ca^{2+} levels Fluo-3 was loaded into the purified synaptosomes suspended in HBS containing 1 mmol/L Ca^{2+}, using the acetoxymethyl ester derivatives (AM) of the dye at 5 μmol/L final concentration, for 60 min at 37 °C as previously described[5]. The dye-loaded synaptosomes were then washed by centrifugation and resuspended in HBS. The preparations were plated onto coverslips coated with Cell-Tak and then inserted into a rapid-exchange Warner perfusion system mounted on a Nikon Diaphot microscope attached to a Nikon PCM 2000 laser-scanning confocal imaging system. Fluorescent images were recorded in response to excitation at 488 nm. During the confocal imaging, the preparations were under constant perfusion at 3–5 mL/min with HBS. Images were typically collected at 4-s intervals, with the first 5 consecutive images collected as a baseline. Each experiment corresponds to sequential images collected using a single preparation subjected to various conditions and/or reagents. Nicotine and mCPBG were used at maximal concentrations[5, 6].

Data analysis The quantification of fluorescence intensities associated with individual synaptosomes recorded in digitized images was calculated quantified using OPTIMAS image analysis software (Optimas Co) and then corrected for photobleaching (typically <3%, based on baseline images). Response to depolarization evoked by elevated extracellular K^+ was used as a criterion for synaptosomal viability for each preparation. Data are presented as normalized responses (F/F_0, where F_0 is the fluorescence intensity associated with a given structure at t_0). Comparisons were made for peak responses. All experiments were independently replicated at least 3 times. All averaged data are from pooled experiments and are presented as means±SEM. Statistical comparisons of averaged peak amplitudes were made by Student's *t*-test, with $P<0.05$ as minimal for significant difference.

Results

Immunostaining of individual isolated terminals from rat striatum revealed that over 90% of the terminals expressing α4 containing nAChRs also express 5-HT_3 receptors, whereas terminals expressing non-α4 containing nAChRs displayed no overlap in immunostaining for 5-HT_3 receptors[4]. The colocalized nAChRs and 5-HT_3 receptors were shown to be functionally independent on the nerve terminals via

pharmacological characterization of agonist-induced Ca^{2+} responses[4]. However, the possibility remained that sustained activation of one receptor could affect signaling by the other receptor. Following a positive test stimulation with 100 nmol/L mCPBG, prolonged incubation of striatal synaptosomes with mCPBG (40 min) resulted in substantial attenuation of Ca^{2+} responses evoked subsequently with 500 nmol/L nicotine (Figure 1), when compared to control nicotine-induced Ca^{2+} responses evoked following the same long incubation but without mCPBG (28%±3% of control; $P<0.05$). The regulation of nAChR-mediated Ca^{2+} responses following 5-HT_3 receptor activation developed by 1 min, but increased over tens of minutes (Figure 2). For the subset of synaptosomes that did not initially respond to mCPBG, prolonged incubation with mCPBG had no effect on subsequent stimulation by nicotine (Figure 1, lower traces). The presence of this subset of synaptosomes is consistent with previous findings that <50% of striatal terminals responding to nicotine with increased $[Ca^{2+}]_i$ also responded to subsequent stimulation with the 5-HT_3 agonist mCPBG[4].

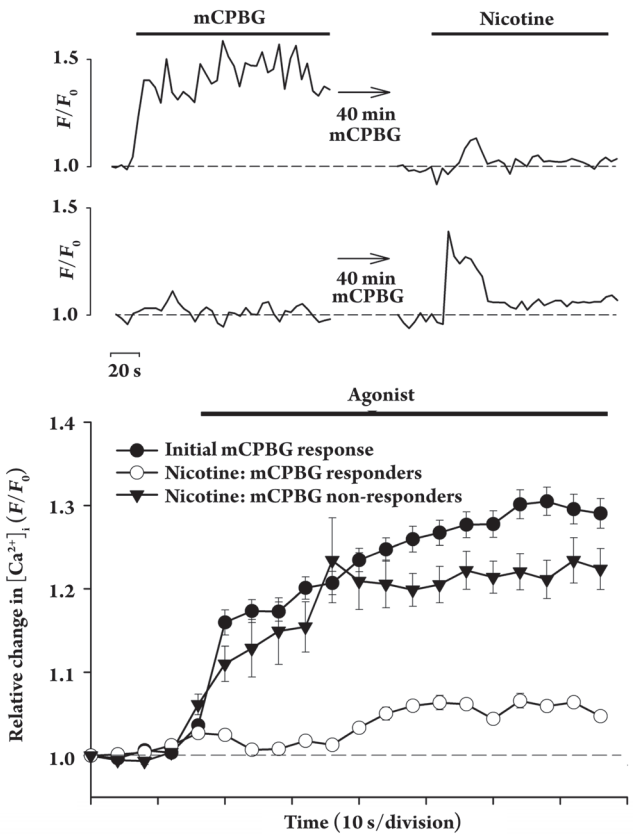

Figure 1. Cross-regulation of presynaptic nAChRs on individual isolated terminals from rat striatum by colocalized 5-HT_3 receptors. Successive stimulation of striatal synaptosomes with the highly selective 5-HT_3 receptor agonist mCPBG at 100 nmol/L followed by stimulation with 500 nmol/L nicotine after an intervening incubation with 100 nmol/L mCPBG. Ca^{2+} responses in two individual synaptosomes measured using confocal imaging are shown in the sequences of traces at top, selected as representative of responders and non-responders for mCPBG stimulation which subsequently responded to nicotine stimulation[4]. Averaged initial responses to mCPBG (n=71) and subsequent responses to nicotine in synaptosomes first responding to mCPBG (mCPBG responders; n=71) or not responding to mCPBG (mCPBG non-responders (n=24) are displayed as means±SEM. Relative $[Ca^{2+}]_i$ is expressed as F/F_0, where F_0 represents the fluorescent intensity of the individual synaptosome at t_0.

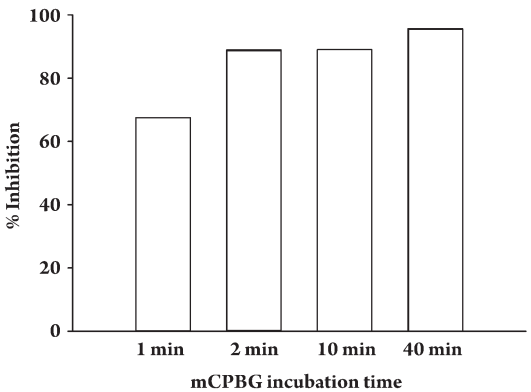

Figure 2. Time-course of cross-regulation of presynaptic nAChRs by colocalized 5-HT_3 receptors in isolated terminals from rat striatum. Data are expressed as the percent inhibition of averaged nicotine-induced peak Ca^{2+} responses (Nic) in striatal synaptosomes that had originally responding to 100 nmol/L mCPBG relative to the averaged nicotine-induced Ca^{2+} responses in those synaptosomes that did not respond to mCPBG, which are equivalent to control nicotine-induced responses. % Inhibition=[100–(Nic responses/control Nic responses ×100)]. n=2.

Likewise, following a test stimulation with nicotine, prolonged incubation of the striatal terminals with nicotine (40min) resulted in attenuation of mCPBG-evoked Ca^{2+} responses (Figure 3) only in those terminals initially responding to nicotine. However, the nicotine-induced attenuation of subsequent mCPBG-evoked responses was only partial (49%±6% of control; $P<0.05$) in comparison to the mCPBG-induced attenuation of subsequent nicotine-evoked responses, and the responses had rather slow kinetics.

Stimulation of presynaptic nAChRs in isolated terminals from striatum by 500 nmol/L nicotine results in inactivation after several mins[5], whereas stimulation of striatal presynaptic 5-HT_3 receptors by mCPBG leads to little apparent inactivation after tens of minutues[6]. For comparison, homologous regulation of the receptors was thus examined. Succes-

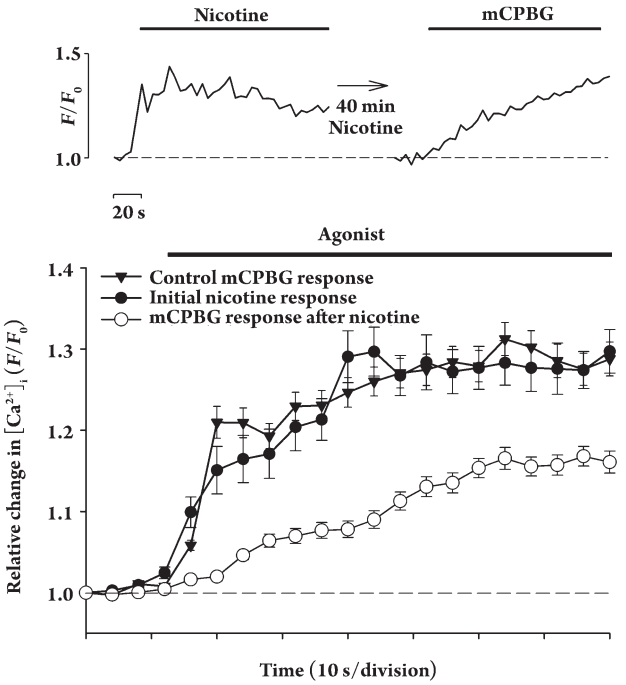

Figure 3. Cross-regulation of presynaptic 5-HT_3 receptors on individual isolated terminals from rat striatum by colocalized nAChRs. Successive stimulation of striatal synaptosomes with 500 nmol/L nicotine followed by stimulation with 100 nmol/L mCPBG after an intervening incubation with 500 nmol/L nicotine. Ca^{2+} responses in an individual synaptosome measured using confocal imaging are shown in the sequences of traces at top. Averaged responses to nicotine (n=33) and subsequent responses to mCPBG (n=33) are displayed as means±SEM. Averaged control responses to 100 nmol/L mCPBG (n=50) performed under the same conditions are shown for comparison. Only a subset (about 50%) of striatal synaptosomes that respond to nicotine also respond to mCPBG[4]. Relative $[Ca^{2+}]_i$ is expressed as F/F_0, where F_0 represents the fluorescent intensity of the individual synaptosome at t_0.

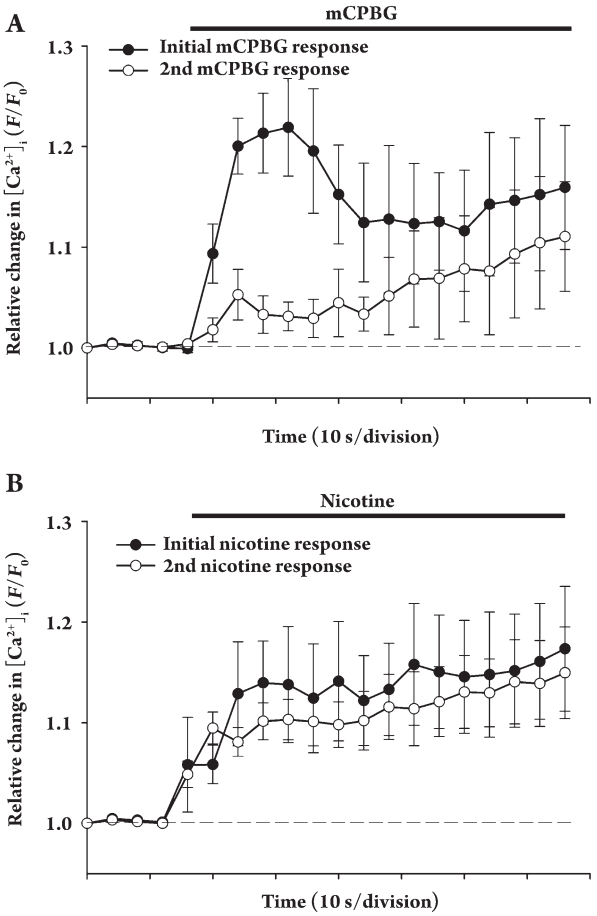

Figure 4. Presynaptic 5-HT_3 receptors on isolated terminals from rat striatum display sustained desensitization, whereas presynapic nAChRs do not. Averaged Ca^{2+} responses to successive stimulation of striatal synaptosomes with 100 nmol/L mCPBG (A; n=4) or with 500 nmol/L nicotine (B; n=8) are shown as means±SEM. A brief (several mins) intervening wash with HBS was performed between stimulations. The sustained desensitization of the 5-HT_3 receptor-mediated responses required the presence of external Ca^{2+} (not shown). Relative $[Ca^{2+}]_i$ is expressed as F/F_0, where F_0 represents the fluorescent intensity of the individual synaptosome at t_0.

sive stimulation with 100 nmol/L mCPBG resulted in very strong attenuation of the second Ca^{2+} response (12%±5% of control; P<0.05) after a short intervening wash to remove agonist (Figure 4A). In contrast, no significant attenuation was observed with successive stimulation with nicotine (Figure 4B).

Discussion

Prolonged activation of presynaptic 5-HT_3 receptors on isolated striatal nerve terminals was found to cross-regulate presynaptic nAChRs colocalized on the same terminal (Figure 5). This occurred even though successive stimulation with nicotine did not result in homologous desensitization of presynaptic Ca^{2+} responses, whereas successive stimulation with the 5-HT_3 receptor agonist did. Prolonged stimulation (min) of presynaptic 5-HT_3 receptors will result in a sustained increase in $[Ca^{2+}]_i$, owing to the very slow inactivation of this receptor when localized to presynaptic nerve terminals[6], which may activate any number of intracellular Ca^{2+}-dependent pathways. In contrast, stimulation of presynaptic nAChRs will result in inactivation[5], though the time course for inactivation is still relatively slow when compared to postsynaptic responses[30] and some degree of cross-regulation of colocalized 5-HT_3 receptor was observed. The latter observation is consistent with an extended incubation with nicotine being necessary for inducing desensitization[5, 31].

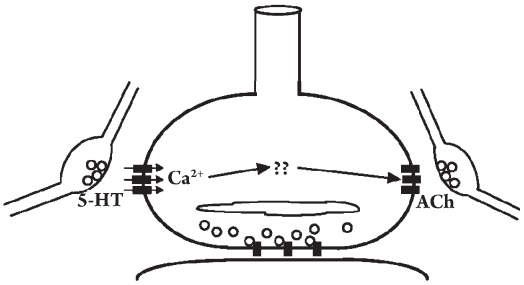

Figure 5. Cartoon of cross-regulation of presynaptic nAChRs by colocalized 5-HT_3 receptors. Prolonged activation of presynaptic 5-HT_3 receptors (left) evokes sustained elevations in $[Ca^{2+}]_i$ within the nerve terminal. Activation of colocalized presynaptic nAChRs (right) after prolonged activation of presynaptic 5-HT_3 receptors results in strongly attenuated Ca^{2+} responses as compared to control responses in the absence of pretreatment with nAChR agonist. It is postulated that the sustained increase in presynaptic $[Ca^{2+}]_i$ on 5-HT_3 receptor stimulation activates Ca^{2+}-dependent signaling pathways in the nerve terminal that regulate presynaptic nAChRs, effectively resulting in desensitization.

Cross-talk in presynaptic nerve terminals has been observed for a number of different receptors in conjunction with nAChRs[12, 13, 15, 16]. Of particular note is the interaction between ionotropic P2X nucleotidic receptors and nAChRs on cholinergic terminals from rat midbrain, which was found to depend on CaM kinase II[32]. Activation of CaM kinase II and/or calcineurin by sustained increases in presynaptic $[Ca^{2+}]_i$ by 5-HT_3 receptors, due to their significant Ca^{2+} permeability[20], is an attractive possibility to explain mediation of cross-talk in the present study. However, a number of other intracellular regulators activated by Ca^{2+} may be candidates for mediating cross-talk between presynaptic 5-HT_3 receptors and nAChRs, including ERK and a direct action of CaM. In addition, long-term desensitization of muscle and *Torpedo* nAChRs, as opposed to rapid inactivation, was found to be regulated by protein kinase A[33, 34]. Increased Ca^{2+} could activate protein kinase A via stimulation of Ca^{2+}-regulated adenylyl cyclase[35]. Finally, direct interaction between colocalized receptors has been proposed for some receptor interactions; however, as the 5-HT_3 receptors and nAChRs function independently on the presynaptic terminals[4], this is an unlikely mechanism for cross-talk.

Striatal nerve terminals express several subtypes of α4 and α6 containing nAChRs[36, 37], including α4β2, α4α5β2, α2α4β2, α4α6β2, and α4α6β3β2. The α4 nAChR subunit was found to colocalize with 5-HT_3 receptors on these terminals, but not α5[4]. As 5-HT_3 receptors have been linked to the regulation of striatal dopamine release[38, 39], colocalization with α2 containing nAChRs is ruled out, because they are only expressed on nondopaminergic structures in the striatum[36]. The α6 containing nAChRs on striatal terminals appear to display the highest relative affinity for nicotine[37], and evoked Ca^{2+} responses in isolated striatal terminals was observed with relatively low concentrations (50–500 nmol/L) of nicotine[5]. Taken together, these observations would indicate the likelihood that the colocalized α4 containing nAChRs also contain α6. This postulate remains to be demonstrated. In addition, in view of the relatively slow apparent inactivation of striatal presynaptic nAChRs (min) compared to postsynaptic nAChRs, as noted previously, it would also be of interest to determine which β subunit is present in the receptor, as the particular β subunit will influence the rate of inactivation[40].

The functional consequences of cross-regulation between colocalized neurotransmitter receptors have yet to be elucidated. Where a negative effect occurs, here desensitization, it may be that sustained activation of one pathway may be acting to suppress the activity via the other pathway. Thus, intermittent or low frequency stimulation via both pathways may synergize, whereas high frequency, sustained stimulation of one pathway will dominate. As each pathway likely has characteristic patterns of activity, those patterns would be more efficiently transduced to downstream signals follow cross-regulation. Functional interaction of serotonergic and cholinergic signaling in the striatum has been described[41]. As cholinergic tone plays a key role in regulating the striatal GABAergic output in motor control, mainly via muscarinic receptors, it would be of interest to understand the role that regulation of nAChRs by serotonergic activity plays at dopaminergic presynaptic terminals in psychomotor output from the striatum.

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Author contribution

John DOUGHERTY carried out all of the experiments and analyzed the data; Robert NICHOLS designed and supervised all of the experiments and analysis, and wrote the paper.

Abbreviations

HBS, HEPES-buffered saline; VGCC, voltage-gated cal-

cium channel; nAChRs, nicotinic acetylcholine receptors; mCPBG, 1-(*m*-chlorophenyl)biguanide

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