Early Stress Prevents the Potentiation of Muscarinic Excitation by Calcium Release in Adult Prefrontal Cortex

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Abstract

Background—The experience of early stress contributes to the etiology of several psychiatric disorders and can lead to lasting deficits in working memory and attention. These executive functions require activation of the prefrontal cortex (PFC) by muscarinic M1 acetylcholine (ACh) receptors. Such G_{α_q} -protein coupled receptors trigger the release of calcium (Ca^{2+}) from internal stores and elicit prolonged neuronal excitation.

Methods—In brain slices of rat PFC, we employed multiphoton imaging simultaneously with whole-cell electrophysiological recordings to examine potential interactions between ACh-induced Ca^{2+} release and excitatory currents in adulthood, across postnatal development, and following the early stress of repeated maternal separation, a rodent model for depression. We also investigated developmental changes in related genes in these groups.

Results—Acetylcholine-induced Ca^{2+} release potentiates ACh-elicited excitatory currents. In the healthy PFC, this potentiation of muscarinic excitation emerges in young adulthood, when executive function typically reaches maturity. However, the developmental consolidation of muscarinic ACh signaling is abolished in adults with a history of early stress, where ACh responses retain an adolescent phenotype. In prefrontal cortex, these rats show a disruption in the expression of multiple developmentally regulated genes associated with G_{α_q} and Ca^{2+} signaling. Pharmacologic and ionic manipulations reveal that the enhancement of muscarinic excitation in the healthy adult PFC arises via the electrogenic process of sodium/ Ca^{2+} exchange.

Conclusions—This work illustrates a long-lasting disruption in ACh-mediated cortical excitation following early stress and raises the possibility that such cellular mechanisms may disrupt the maturation of executive function.

Keywords

Acetylcholine; calcium imaging; development; maternal separation; muscarinic M1 receptor; slice electrophysiology

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The prefrontal cortex (PFC) is one of the last brain regions to mature (1–3), and accordingly, peak performance of executive function is only achieved in mid to late adolescence (4–6). Executive function is essential for performing complex tasks (7–9). Disruptions in PFC activity and impairments in executive function are observed in several psychiatric disorders (8,10), including depression (11,12), schizophrenia (13–15), bipolar disorder (16), and attention-deficit/hyperactivity disorder (17), all of which may have developmental origins (18–22). Brain regions requiring protracted development may be especially vulnerable to the effects of early stress (23), which in itself is a risk factor for PFC dysfunction, cognitive deficits, and psychiatric illness (23–25). Yet, much remains unknown about the normal maturation of the PFC and the cellular mechanisms underlying its vulnerability to disruption.

Acetylcholine (ACh) modulation of the PFC is essential for executive functions such as working memory and attention (26–29) and dysregulation of the cholinergic system has been implicated in the executive deficits prevalent in psychiatric disorders (29,30). At the cellular level, the ability of PFC neurons to sustain persistent activity forms the basis of working memory (31) and involves muscarinic acetylcholine receptors (32–34). Layer V pyramidal cells of the PFC receive a dense cholinergic innervation arising from the basal forebrain (35,36) and respond to ACh principally via M1 muscarinic receptors (37). Activation of these G_{α_q} -coupled receptors exerts robust excitatory actions (37–40) in addition to releasing calcium (Ca^{2+}) from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores (41). Although the ionic mechanisms underlying these responses have been extensively studied, they remain incompletely understood. In particular, the role of agonist-induced Ca^{2+} release in shaping muscarinic excitation is not known. Although intracellular Ca^{2+} likely plays an important role in shaping persistent activity (33,42,43), no study to date has simultaneously examined the release of intracellular Ca^{2+} together with the magnitude and timing of muscarinic excitation. Furthermore, it is unknown how these aspects of PFC cholinergic signaling change developmentally or if they are vulnerable to disruption by environmental factors such as early stress.

Using multiphoton Ca^{2+} imaging with concurrent whole-cell electrophysiological recordings in brain slices of PFC, we demonstrate for the first time that agonist-induced Ca^{2+} release from intracellular stores significantly potentiates the excitatory effects of muscarinic ACh receptors. We further provide mechanistic insight into the source of these excitatory effects. Importantly, we show that such potentiation of muscarinic receptor activity is associated with key developmental stages for executive function and is subject to disruption by early stress.

Methods and Materials

Animals

Sprague Dawley rats were used for all experiments, which were approved by the University of Toronto Animal Care Committee or the Tata Institute of Fundamental Research Animal Ethics Committee. The characterization of the normal interaction between the ACh-elicited Ca^{2+} release and the ACh-elicited inward current (I_{ACh}) was conducted in 31 young adult

male rats (age: 60 ± 13 days, range 40–95 days). The second set of experiments examined how the above interaction matures and whether it is susceptible to disruption by early stress. For these experiments, we used repeated maternal separation to elicit early stress (44,45), and control animals were born and raised within the same animal room during the same time period. Recordings were performed in adolescence (postnatal day [P]30–P45; $n = 5$ control rats, $n = 4$ early stress [ES]), young adulthood (P60–P100; $n = 9$ control rats, $n = 6$ ES), or adulthood (P130–P175; $n = 6$ control rats, $n = 8$ ES). The third set of experiments employed the same maternal separation paradigm and examined gene expression in the PFC at either P21 ($n = 7$ –8 control rats, $n = 8$ –9 ES) or P60 ($n = 7$ control rats, $n = 7$ –8 ES).

Early Stress Paradigm

Pregnant primiparous dams delivered pups within the animal housing facility and litters were randomly assigned to ES or control groups on P1. Pups in the ES litters were separated from their dams for a period of 3 hours at the same time each day from P2 to P14. Control litters were left undisturbed during this time. All litters were handled briefly at 3- to 4-day intervals to allow for cage cleaning and weighing. Once weaned, all pups were housed in same-sex sibling groups of two to three rats.

Brain Slice Preparation and Recording Conditions

Each brain was rapidly cooled with 4°C oxygenated sucrose artificial cerebrospinal fluid (254 mmol/L sucrose substituted for sodium chloride). Coronal slices (400 μm) of the PFC were cut on a Dosaka Linear Slicer (SciMedia, Costa Mesa, California) and were transferred to 30°C oxygenated artificial cerebrospinal fluid (containing, in mmol/L: 128 sodium chloride, 10 D-glucose, 26 sodium bicarbonate, 2 calcium chloride, 2 magnesium sulfate, 3 potassium chloride, 1.25 monosodium phosphate, pH 7.4) in a prechamber (Automatic Scientific, Berkeley, California) and allowed to recover for at least 1.5 hours before the beginning of an experiment. Slices were placed in a chamber on the stage of an upright microscope for whole-cell recordings. Artificial cerebrospinal fluid was bubbled with 95% oxygen and 5% carbon dioxide and flowed over the slice at 30°C with a rate of 3 mL to 4 mL per minute.

Electrophysiological Recordings and Multiphoton Ca^{2+} Imaging

Layer V pyramidal neurons were patched under visual control using infrared differential interference contrast microscopy in the cingulate and prelimbic regions. Intracellular patch solution contained (in mmol/L): 120 K-gluconate, 5 potassiumchloride, 2 magnesium chloride, 4 dipotassium adenosine triphosphate, .4 disodium-guanosine triphosphate, 10 disodium-phosphocreatine, and 10 4-(2-hydroxyethyl)piperazin-1-yl ethanesulfonic acid (HEPES) buffer (adjusted to pH 7.33 with potassium hydroxide). The high-affinity Ca^{2+} dye Oregon Green BAPTA-1 (OGB-1, 100 $\mu\text{mol/L}$; Molecular Probes, Life Technologies, Burlington, Ontario, Canada) was included in the pipette along with the Ca^{2+} insensitive dye Alexa-594 hydrazide (20 $\mu\text{mol/L}$; Molecular Probes), which was used for the visualization of the neuron and subsequent morphological reconstruction (Supplementary Methods & Materials in Supplement 1). Currents were recorded with an Axopatch 200b (Molecular Devices, Sunnyvale, California), acquired and low-pass filtered at 2 kHz with pClamp10.2/ Digidata1440 (Molecular Devices).

Multiphoton imaging was performed using a Ti:sapphire laser (Newport, Irvine, California) tuned to wavelength 800 nm and an Olympus (Richmond Hill, Ontario, Canada) Fluoview FV1000 microscope with a 60× water-immersion .90 numerical aperture objective. The emitted fluorescence was separated into green (OGB-1 signal) and red (Alexa-594 hydrazide signal) channels with a dichroic mirror at 570 nm and filtered (green barrier filter: 495–540 nm; red barrier filter: 570–620 nm) before detection. Images were acquired at a rate of ~10 frames per second and analyzed with Fluoview software (Olympus). A pansomatic area of interest was selected for analysis and green fluorescence increases were calculated relative to baseline fluorescence (dF/F_0). Calcium responders ($ACh_{Ca^{2+}}$) were identified as cells where the ACh-elicited Ca^{2+} increase was at least five times the standard deviation of the baseline fluorescence signal; whereas, cells that lacked such a response to ACh were considered nonresponders ($ACh_{No\ Ca^{2+}}$). Pseudocoloring was achieved post hoc for illustrative purposes with look-up tables adjusted to the maximal signal bandwidth in Fiji (ImageJA v.1.45b; <http://fiji.sc/Fiji>).

Intrinsic cell properties were assessed in current clamp mode. Calcium responders ($ACh_{Ca^{2+}}$ cells) were not significantly different from $ACh_{No\ Ca^{2+}}$ cells as assessed by membrane potential ($ACh_{Ca^{2+}}$: -80 ± 2 mV, $n = 16$; $ACh_{No\ Ca^{2+}}$: -81 ± 1 mV, $n = 14$; $p = 0.9$), input resistance ($ACh_{Ca^{2+}}$: 118 ± 12 M Ω , $n = 16$; $ACh_{No\ Ca^{2+}}$: 144 ± 14 M Ω , $n = 14$; $p = .2$), and membrane capacitance ($ACh_{Ca^{2+}}$: 180 ± 10 pF, $n = 16$; $ACh_{No\ Ca^{2+}}$: 167 ± 9 pF, $n = 14$; $p = .4$). Examination of cholinergic currents was performed in voltage-clamp mode at a liquid junction potential-corrected holding potential of -75 mV.

Pharmacology

Acetylcholine chloride and caffeine were obtained from Sigma (Oakville, Ontario, Canada). Pirenzepine, thapsigargin, and KB-R7943 were obtained from Tocris (R&D Systems Inc., Minneapolis, Minnesota). All drugs were bath applied.

Quantitative Polymerase Chain Reaction

We assessed the developmental expression of genes involved in Ca^{2+} signaling using quantitative polymerase chain reaction (qPCR). Control and ES animals were killed by decapitation and the PFC dissected in ice-cold phosphate buffered saline. RNA was extracted from tissue samples using Trizol reagent (Sigma) and 2 μ g from each sample was reverse-transcribed (high-capacity complementary DNA reverse transcription kit, Applied Biosystems, Life Technologies, Carlsbad, California). The synthesized complementary DNA was subjected to qPCR with primers specific to the genes of interest and data analyzed using the C_t method described previously (46) with normalization to the endogenous housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). Results were compared with age-matched control animals to examine the influence of early stress on gene expression within a particular age or to the P21 control group to assess the influence of early stress on the developmental profile of gene expression. Results were expressed as fold change $\pm$ SEM.

Statistics

Results are expressed as mean $\pm$ SEM, and all statistical comparisons were made at a significance level of .05 (Prism versions 5.0d/6.0, GraphPad Software, La Jolla, California). Average current and fluorescent increases were generated with Axograph X (Axograph Scientific, Sydney, Australia). Gene expression analysis experiments with two groups were analyzed using the unpaired Student *t* test. To analyze the influence of ES on the developmental expression profile of genes involved in Ca^{2+} signaling, data were subjected to two-way analysis of variance, followed by a Bonferroni post hoc test for group comparisons.

Results

ACh-Elicited Somatic Ca^{2+} Release Potentiates Excitatory Muscarinic Currents

The stimulation of muscarinic receptors can elicit IP_3 -induced somatic Ca^{2+} increases (47) that initiate gene transcription (48–50). Initial experiments in layer V pyramidal neurons revealed that bath application of ACh (1 mmol/L, 15 sec) elicited Ca^{2+} release as measured by increased OGB-1 fluorescence ($\text{dF}/\text{F}_{\text{ACh}}$; Figure 1). When observed, this somatic increase was seen in multiple regions of interest, as illustrated on the $\text{dF}/\text{F}_{\text{ACh}}$ overlay in Figure 1. Accordingly, we selected a pansomatic region of interest for our investigation of the relative magnitude and timing of the two distinct responses to acetylcholine: the increase in Ca^{2+} within the soma ($\text{dF}/\text{F}_{\text{ACh}}$) and the excitatory inward current (I_{ACh}).

Electrophysiologically, an I_{ACh} was observed in all cells (-73 ± 7 pA, $n = 30$); however, the $\text{dF}/\text{F}_{\text{ACh}}$ varied among neurons: monophasic $\text{dF}/\text{F}_{\text{ACh}}$ were observed in 13 (43%) of 30 cells (Figure 2A), multi-peaked $\text{dF}/\text{F}_{\text{ACh}}$ in 3 (10%) of 30 cells (Figure 2B), and no $\text{dF}/\text{F}_{\text{ACh}}$ in 14 (47%) of 30 cells ($\text{ACh}_{\text{No Ca}^{2+}}$; Figure 2C). In addition to the prolonged excitatory inward I_{ACh} , a subpopulation of $\text{ACh}_{\text{Ca}^{2+}}$ neurons showed a transient outward current (Figure 2A), a previously described M1 muscarinic receptor-mediated phenomenon (37,51). Yet, examining the relationship between the magnitudes of $\text{dF}/\text{F}_{\text{ACh}}$ and I_{ACh} , we found that increased Ca^{2+} was associated with greater muscarinic excitation. Peak I_{ACh} amplitudes were significantly larger in $\text{ACh}_{\text{Ca}^{2+}}$ cells (Figure 2D, unpaired *t* test, $p = .02$) and were significantly correlated with $\text{dF}/\text{F}_{\text{ACh}}$ (Figure 2E, $R^2 = .41$, $p = .01$). While neuromodulatory responses of PFC layer V pyramidal neurons have been shown to exhibit cell type specificity (52), $\text{ACh}_{\text{Ca}^{2+}}$ and $\text{ACh}_{\text{No Ca}^{2+}}$ cells were neither electrophysiologically (see Methods and Materials) nor morphologically distinct (Figure S1 and Table S1 in Supplement 1).

Both $\text{dF}/\text{F}_{\text{ACh}}$ and I_{ACh} were abolished by the M1 muscarinic receptor antagonist pirenzepine (Figure 3A,B; 500 nmol/L, 10 min), consistent with a genetic deletion study that has demonstrated a predominant role for this receptor subtype in mediating cholinergic responses in PFC layer V pyramidal cells (37). Furthermore, Ca^{2+} store depletion achieved by bath application of the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin (10 $\mu\text{mol/L}$) almost completely eliminated $\text{dF}/\text{F}_{\text{ACh}}$ (Figure 3C; $\text{dF}/\text{F}_{\text{ACh}}$ reduction: $91 \pm 5\%$, $n = 7$), confirming the involvement of intracellular release from the endoplasmic reticulum following M1 receptor activation. Consistent with the hypothesis that Ca^{2+} release potentiates I_{ACh} , thapsigargin treatment led to a significant I_{ACh} reduction (Figure 3D; matched-pair *t* test, $p = .02$; percent reduction: $27 \pm 10\%$, $n = 7$), with the

remainder of I_{ACh} likely reflecting the direct effects of muscarinic M1 receptors on ion channels (38–40,53–55).

Of note, $ACh_{No\ Ca^{2+}}$ neurons (that did not initially show a dF/F_{ACh}) could be primed before ACh application by a depolarizing pulse (to 0 mV), which would replenish intracellular Ca^{2+} stores by means of voltage-gated Ca^{2+} entry (56). Importantly, successful priming resulted in a potentiated I_{ACh} in 9 of 14 cells (Figure 3E,F; matched-pair t test, $p = .002$). The potentiated I_{ACh} observed following priming was not likely to be attributed to the priming event per se, given that I_{ACh} was unchanged in the 5 of 14 cells where priming was ineffective at eliciting a subsequent dF/F_{ACh} (data not shown).

We next tested whether recruiting an additional mechanism of Ca^{2+} release would alter I_{ACh} . For this experiment, we used caffeine, a ryanodine receptor agonist that can elicit Ca^{2+} release from intracellular stores (57) (data not shown). The presence of caffeine (20 mmol/L) significantly prolonged the dF/F_{ACh} (decay time $_{ACh}$: $2.3 \pm .8$ sec, decay time $_{ACh + caff}$: 10.8 ± 2.9 sec; matched-pair t test, $p = .03$, $n = 9$) and significantly enhanced the I_{ACh} amplitude (peak I_{ACh} : -75 ± 6 pA, peak $I_{ACh + caff}$: -111 ± 15 pA; matched-pair t test, $p = .02$, $n = 9$) and I_{ACh} area under the curve (area- I_{ACh} : 4545 ± 551 pA*sec, area- $I_{ACh + caff}$: 6556 ± 874 pA*sec; matched-pair t test, $p = .01$; $n = 9$). These data emphasize that intracellular Ca^{2+} is an important modulator of excitatory muscarinic responses.

Ca^{2+} -Potentiation of I_{ACh} Develops in Young Adulthood and Is Disrupted by Early Stress

Peak performance of ACh-dependent executive functions is only achieved as the PFC matures (2–6) and this late maturation is thought to enhance vulnerability to early stress (23,58). Therefore, we next examined the relationship of the I_{ACh} and dF/F_{ACh} across a broader developmental period and tested whether it is altered by the early stress of repeated maternal separation (Figure 4; Figure S2 in Supplement 1) (44,45). In this independent replication, we found that peak I_{ACh} were significantly greater in cells where somatic Ca^{2+} release could be detected, both in young and mature adult control animals. However, the potentiation of I_{ACh} by dF/F_{ACh} was absent in adolescents (Figure 4B; Figure S2 in Supplement 1), although the proportion of $ACh_{Ca^{2+}}$ cells did not differ significantly with developmental stage ($\chi^2_{df=2} = 4.4$, $p = .1$). Thus, it appears that the potentiation of muscarinic excitation by ACh-induced Ca^{2+} release is a phenomenon that emerges in early adulthood.

Following the experience of early stress, this expected developmental emergence of the Ca^{2+} potentiation of I_{ACh} was absent (Figure 4B; Figure S2 in Supplement 1). Yet, the proportion of $ACh_{Ca^{2+}}$ cells between control animals and those that had undergone the experience of early stress remained unchanged in the adolescent (Fisher's exact test, $p = 1.00$), young adult (Fisher's exact test, $p = .4$), and mature adult (Fisher's exact test, $p = 1.00$). Interestingly, these developmental changes susceptible to early stress were accompanied by marked differences in the timing but not the amplitude of the individual dF/F_{ACh} responses (Figure S3 in Supplement 1).

Early Stress Produces Developmentally Specific Changes in PFC Gene Expression

We hypothesized that differences in the timing of dF/F_{ACh} in development and early stress reflect differences in $G\alpha_q$ - and Ca^{2+} -mediated signaling. To test this hypothesis, we performed qPCR for a number of genes that had previously been shown to be dysregulated in the PFC in adult animals with a history of early stress (45). As shown in Figure 5 and Table S2 in Supplement 1, we found that early stress significantly altered the pattern of developmental expression of genes associated with Ca^{2+} -mediated signaling, including a voltage-gated Ca^{2+} channel subunit, Ca^{2+} -dependent enzymes, Ca^{2+} -sensitive adhesion molecules, and notably, two members of the potassium (K^+)-dependent sodium (Na^+)/ Ca^{2+} exchanger (NCKX) family, *Slc24a2* and *Slc24a6*. Many of the genes in Figure 5 have been shown to structurally or functionally interact with the IP_3 receptor (59–66) and together may contribute to the complex developmental regulation of ACh-induced Ca^{2+} release and its dysregulation following early stress.

Na^+/Ca^{2+} Exchange Potentiates Muscarinic Excitation in Healthy Adult Control Animals

We next sought to further elucidate the mechanism whereby the dF/F_{ACh} may potentiate I_{ACh} in healthy adult control animals. While mindful that optimal muscarinic function likely depends on a multitude of $G\alpha_q$ - and Ca^{2+} signaling-related genes, the differences in NCKX expression patterns raised the interesting possibility that Na^+/Ca^{2+} exchange might contribute to muscarinic function in the PFC. Specifically, we hypothesized that electrogenic Na^+/Ca^{2+} exchange might be responsible for the potentiation of I_{ACh} by dF/F_{ACh} in the PFC of adult control animals. While this phenomenon has not been investigated in the cerebral cortex, it has been suggested to contribute to muscarinic signaling in the medial septum (67) and the tuberomammillary nucleus (68). Molecular mediators of Na^+/Ca^{2+} -exchange include two families of electrogenic exchangers that increase cellular excitation in the process of removing Ca^{2+} : the Na^+ -dependent Ca^{2+} exchanger (NCX), which couples the extrusion of one Ca^{2+} in exchange for three Na^+ ions, and the Na^+ - and K^+ -dependent Ca^{2+} exchanger (NCKX), which couples the extrusion of one Ca^{2+} and one K^+ in exchange for four Na^+ ions (69,70). These proteins are highly expressed in the brain (70,71) and the cerebral cortex (72).

To evaluate the possibility of Na^+/Ca^{2+} exchanger involvement in muscarinic potentiation, we examined the relative timing of I_{ACh} and the decay of dF/F_{ACh} , a measurement of Ca^{2+} clearance and potential indicator of the extrusion of Ca^{2+} . For this analysis, we averaged the traces obtained in $ACh_{Ca^{2+}}$ ($n = 16$) and $ACh_{No\ Ca^{2+}}$ neurons ($n = 14$) of adult control animals from our original dataset (Figure 2). This analysis highlights that the presence of a dF/F_{ACh} yielded a supplemental I_{ACh} , in addition to the basal I_{ACh} observed in $ACh_{No\ Ca^{2+}}$ neurons (Figure S4 in Supplement 1). Interestingly, this supplemental I_{ACh} coincided with the decay, or clearance, of the dF/F_{ACh} (Figure S4 in Supplement 1), which is consistent with a contribution of electrogenic exchangers to PFC cholinergic modulation.

To probe further the potential involvement of Na^+/Ca^{2+} exchange, we reduced the concentration gradient of Na^+ on which electrogenic Ca^{2+} extrusion depends. Eighty percent Na^+ substitution reduced I_{ACh} to a degree that suggests the Na^+ dependence of both the basal and supplemental I_{ACh} (Figure S5 in Supplement 1). Importantly, Na^+ substitution

prolonged the dF/F_{ACh} response, an effect enhanced with dual Na^+ and K^+ substitution (Figure S5 in Supplement 1). These observations further suggested that clearance of Ca^{2+} by electrogenic Na^+/Ca^{2+} exchange contributes to I_{ACh} following agonist-induced Ca^{2+} release in PFC and that members of the NCX and/or NCKX family may be involved. Accordingly, we found that a NCX and NCKX inhibitor (73), KB-R7943 (50 μ mol/L, 5 min), reduced the I_{ACh} by an average of $34 \pm 10\%$ (Figure S6 in Supplement 1) and that nonspecific inhibition of Na^+/Ca^{2+} exchange with nickel chloride (3 mmol/L) (67) reduced the I_{ACh} by an average of $63 \pm 7\%$. The schematic in Figure 6 shows the mechanism hypothesized to underlie muscarinic excitation of layer V PFC neurons in healthy adults.

Discussion

Here, we provide insights into the mechanisms and developmental changes of cortical muscarinic modulation and its vulnerability to disruption by early stress. First, we demonstrated that muscarinic excitation is potentiated by ACh-induced Ca^{2+} release. Second, we found that this phenomenon emerges in young adulthood as agonist-induced Ca^{2+} release becomes more precisely timed. Third, we discovered that the experience of early stress disrupts this developmental consolidation of muscarinic signaling, leading to the retention of the adolescent phenotype with potential implications for executive function. We further report that these changes in cellular function are compounded by developmental changes in the PFC expression of genes associated with Ca^{2+} signaling. We conclude by implicating Na^+/Ca^{2+} exchange-mediated electrogenesis in the potentiation of muscarinic excitation in healthy adult PFC.

Mechanisms of Muscarinic Modulation of the PFC Circuits of Executive Function

Acetylcholine modulation of the PFC is essential for executive functions such as working memory and attention (26–29) and disruption of such modulation is thought to contribute to the executive deficits prevalent in psychiatric disorders (29,30). Muscarinic ACh receptors are necessary to sustain persistent activity, which is thought to underlie working memory (32–34,74) and to stabilize attention circuitry (29). Here, we demonstrate for the first time that agonist-induced Ca^{2+} release from intracellular stores can significantly potentiate the excitatory effects of PFC muscarinic ACh receptors.

There has been considerable debate over the specific mechanisms underlying cortical muscarinic excitation. The inhibition of several subtypes of K^+ channels (38,39,53) and/or the activation of transient receptor potential-like, nonselective cation channels (40,54,75,76) have been implicated. The evidence provided in this study suggests that electrogenic clearance of dF/F_{ACh} contributes a supplemental excitatory I_{ACh} that lasts well beyond any transient inhibitory Ca^{2+} effects and clearly enhances the excitatory component of the muscarinic response. We suggest that in healthy adult PFC, members of the NCX and/or NCKX families potentiate muscarinic excitation by coupling extrusion of ACh-induced Ca^{2+} release to net Na^+ influx, as summarized in Figure 6. The NCX and NCKX proteins have been shown to play a major role in Ca^{2+} clearance (70,77–79) and their low affinity and high transport properties position them especially well for the clearance of larger cytosolic Ca^{2+} increases resulting from signaling events (80). Many different isoforms are expressed in

adult rat cerebral cortex, including NCX1.4, NCX1.5, NCX2, and NCX3 (72,81,82), as well as NCKX2, NCKX3, and NCKX4 (71,72,83). NCKX6 has also been shown to be expressed in brain (84). This apparent complexity may be increased further by the differential sensitivity of these exchanger isoforms to posttranslational modulation (70). Of note, a growing body of evidence suggests that the individual exchanger isoforms make unique contributions to normal cognition (85–87). Further studies will be necessary to elucidate the molecular mechanisms of ACh-induced $\text{Na}^+/\text{Ca}^{2+}$ exchange in the PFC and to investigate its role in shaping cellular and network activity in health and disease.

Overall, the further characterization of $\text{dF}/\text{F}_{\text{ACh}}$, its regulation, and its activity-dependence is a timely subject for further investigation. The observation of multi-peaked Ca^{2+} responses may reflect regenerative Ca^{2+} release, either due to the increased sensitivity of IP_3 receptors by Ca^{2+} or to the recruitment of local ryanodine receptors (88). Different $\text{dF}/\text{F}_{\text{ACh}}$ responses could reflect differences in muscarinic receptor phosphorylation state (89). Furthermore, the ability to elicit $\text{dF}/\text{F}_{\text{ACh}}$ in $\text{ACh}_{\text{No } \text{Ca}^{2+}}$ by priming the cells with a depolarizing pulse, replenishing intracellular Ca^{2+} stores by means of voltage-gated Ca^{2+} entry (56), suggests that the propensity for ACh to elicit a somatic Ca^{2+} response does not reflect cell type but possibly Ca^{2+} store readiness.

Gα_q-Coupled Receptor Signaling Pathway Complexity and Vulnerability

G-protein signaling involves a cascade of several second messengers, including Ca^{2+} (90), that can interact to yield responses on a variety of timescales (59,91,92). The complexity of muscarinic signaling, and of Gα_q-protein coupled receptors more generally, may help confer the flexibility necessary for executive function at the neuronal level (7). Disruptions in the phospholipase C pathway, activated by Gα_q-protein coupled receptors, have been implicated in the pathophysiology of mental illness (93). Here, we present evidence that the experience of early stress dysregulates Gα_q-coupled muscarinic signaling and alters the expression of a network of proteins that structurally and/or functionally interact with the IP_3 receptor (59–61), including calcineurin (60,62), the L-type Ca^{2+} channel β_2 subunit (63), CD44 (64,65), calpain 8 (60,66), ANKFY1 (65), and AKAP1 (59,90).

Interactions of Genes, Development, and Early Stress

Peak performance on tasks of executive function is only achieved as the PFC matures in late adolescence (1–6) and brain regions with such protracted development are especially vulnerable to early stress (23). Here, we investigated aspects of the normal maturation of muscarinic acetylcholine responses in the PFC. We found that intracellular Ca^{2+} release normally potentiates muscarinic excitation in adulthood but not in the adolescent period. This developmental profile was not observed in the brains of animals that have undergone the experience of early stress. Gene expression differences suggest that the timing of muscarinic-elicited Ca^{2+} dynamics is vulnerable to developmental disruptions of the molecular machinery associated with Ca^{2+} release-related microdomains (59–61,90).

The maintenance of a juvenile phenotype into adulthood following early stress is an interesting possibility; however, this notion appears overly simplistic. Our data show not only that early stress can lead to expression changes in genes relating to Gα_q Ca^{2+} signaling

but also that early life experience interacts with development (Table S2 in Supplement 1; Figure 5). In particular, the potentiation of muscarinic excitation by Ca^{2+} release appears to require the maturation of a network of $\text{G}\alpha_q$ - and Ca^{2+} signaling-related genes susceptible to modulation by early experience. Therefore, rather than the retention of a juvenile phenotype, it is the developmental trajectory of gene expression and cellular function that is altered following early stress.

In sum, this work illustrates cellular mechanisms and molecular pathways that may allow early stress to disrupt cognitive performance on tasks requiring mature executive function. Additionally, these findings elucidate a mechanism contributing to PFC muscarinic excitation that shows delayed developmental consolidation. It is hoped that a deeper understanding of the cholinergic modulation of PFC will ultimately help improve treatment strategies for cognitive deficits in neurological and psychiatric disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Acetylcholine (ACh)-elicited somatic calcium (Ca^{2+}) increases in layer V pyramidal cells of adult prefrontal cortex (PFC). Left, a schematic of the recording area is shown in gray. Right, bath application of 1 mmol/L ACh (15 sec, red) elicits pansomatic Ca^{2+} increases (dF/F_{ACh}). Various areas of interest (AOIs) are depicted on the red channel image and their respective Ca^{2+} signals are overlapped below. Changes in fluorescence measured in four smaller AOIs do not significantly differ from that measured in a single, pansomatic AOI. Sample Oregon Green BAPTA-1 (OGB-1) fluorescence increases from baseline (1), peak Ca^{2+} release (2), and washout (3) are pseudocolored for illustrative purposes. Scale bar, 10 μm . [Schematic reprinted from Paxinos and Watson (94), with permission from Elsevier, copyright 2007].

Figure 2.

Acetylcholine (ACh)-elicited somatic calcium (Ca^{2+}) increases potentiate muscarinic responses in layer V pyramidal cells of adult prefrontal cortex. Acetylcholine elicits three types of responses: **(A)** monopeak dF/F_{ACh} (47%), **(B)** multipeak dF/F_{ACh} (10%), and **(C)** nonresponse (47%, ACh_{No} Ca^{2+}). **(D)** Peak excitatory inward currents (I_{ACh}) are greater in cells where Ca^{2+} release is also stimulated. **(E)** Correlation of peak I_{ACh} and dF/F_{ACh} is significant ($p = .01$), with the exclusion of one outlier. * $p < .05$. ACh _{Ca^{2+}} , calcium responder.

Figure 3.

Muscarinic excitation (acetylcholine [ACh]-elicited inward current [I_{ACh}]) is potentiated by ACh-elicited calcium (Ca^{2+}) release from intracellular stores (dF/F_{ACh}). (**A, B**) Muscarinic M1 receptor antagonist pirenzepine (500 nmol/L, 10 min) abolished I_{ACh} and dF/F_{ACh} in layer V pyramidal cells of adult prefrontal cortex (lower and upper traces, respectively). The middle trace schematically depicts changes in holding potential. Note the brief depolarizing step at the end of the trace, which serves as a control. (**C, D**) Depletion of intracellular Ca^{2+} stores with thapsigargin (10 mmol/L, 10 min) attenuates dF/F_{ACh} and significantly reduces I_{ACh} in calcium responder cells. (**E, F**) Conversely, in calcium nonresponder cells, a depolarizing pulse (to 0 mV) delivered before ACh application could trigger Ca^{2+} entry and subsequent dF/F_{ACh} , presumably due to the replenishment of intracellular stores. TG, thapsigargin.

Figure 4.

The calcium (Ca^{2+}) potentiation of acetylcholine (ACh)-elicited inward current (I_{ACh}) emerges in young adulthood but is absent following early stress (ES). **(A)** Schematic of the experimental paradigm used to examine the developmental profile of I_{ACh} potentiation by dF/F_{ACh} and its susceptibility to early stress. The stress of maternal separation was used, where pups were separated from their dams for 3 hours daily from postnatal day (P)2 to P14. **(B)** Findings of I_{ACh} potentiation by dF/F_{ACh} were replicated in undisturbed young adult and adult control animals. Note that the phenomenon appears to be not yet present in the adolescent brain and rather emerges in early adulthood. This normal developmental emergence of dF/F_{ACh} potentiation of I_{ACh} is absent following early stress. * $p < .1$, ** $p < .01$, unpaired t tests between calcium responder and calcium nonresponder cells. See also Figure S2 in Supplement 1.

Figure 5.

Early stress (ES) produces changes in the expression of genes involved in $G\alpha_q$ -coupled receptor signaling pathways in both adolescent and adult brain. Functional analysis by reverse transcriptase polymerase chain reaction reveal gene expression differences plotted as fold change with respect to age-matched control animals in adolescent and adult brain. Note that different genes are altered in adolescence and adulthood, indicating an interaction of age and early experience. Slc24a6 and Slc24a2 are both members of the potassium-dependent family of sodium-calcium exchangers (NCKX). Also see Table S2 in Supplement 1 for quantification of the interaction. * $p < .1$, ** $p < .05$, *** $p < .001$. P, postnatal day; wrt, with respect to.

Figure 6.

Schematic to illustrate the hypothesis that prefrontal cortex excitatory muscarinic currents are potentiated via electrogenesis mediated by the sodium (Na⁺)-calcium (Ca²⁺) exchanger (NCX)- and Na⁺-potassium (K⁺)-Ca²⁺ exchanger (NCKX). Activation of M1 muscarinic acetylcholine (ACh) receptors leads to release of Ca²⁺ from intracellular stores and modulation of a Na⁺-dependent ion channel conductance to generate the primary ACh-elicited inward current. NCX and/or NCKX-mediated electrogenesis underlies the supplementary ACh-elicited inward current observed in the presence of dF/F_{ACh}. DAG, diacyl glycerol; IP₃, inositol 1,4,5-trisphosphate; M1R, M1 muscarinic acetylcholine receptor; NSCC, nonselective cation channel; PIP2, phospholipid phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C.