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RESEARCH ARTICLE

Developmental nicotine exposure induced alterations in behavior and glutamate receptor function in hippocampus

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Abstract In the developing brain, nicotinic acetylcholine receptors (nAChRs) are involved in cell survival, targeting, formation of neural and sensory circuits, and development and maturation of other neurotransmitter systems. This regulatory role is disrupted when the developing brain is exposed to nicotine, which occurs with tobacco use during pregnancy. Prenatal nicotine exposure has been shown to be a strong risk factor for memory deficits and other behavioral aberrations in the offspring. The molecular mechanisms underlying these neurobehavioral outcomes are not clearly elucidated. We used a rodent model to assess behavioral, neurophysiological, and neurochemical consequences of prenatal nicotine exposure in rat offspring with specific emphasis on the hippocampal glutamatergic system. Pregnant dams were infused with nicotine (6 mg/kg/day) subcutaneously from the third day of pregnancy until birth. Results indicate that prenatal nicotine exposure leads to increased anxiety and depressive-like effects and impaired spatial memory. Synaptic plasticity in the form of long-term potentiation (LTP), basal synaptic transmission, and AMPA receptor-mediated synaptic currents were reduced. The deficit in synaptic plasticity was paralleled by declines in protein levels of vesicular glutamate transporter 1 (VGLUT1), synaptophysin, AMPA receptor subunit GluR1, phospho(Ser845) GluR1, and postsynaptic density 95 (PSD-95). These results suggest that prenatal nicotine exposure by maternal smoking could result in alterations in the glutamatergic system in the hippocampus contributing to the abnormal neurobehavioral outcomes.

Keywords Nicotine · Prenatal · Hippocampus · AMPA · Glutamate · Synapse

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Introduction

Tobacco smoking during pregnancy remains to be a significant public health issue in spite of several awareness programs and affects a large number of people in the US and worldwide. The proportion of pregnant women who smoke is estimated to be 20–25%, resulting in birth of several thousands of babies exposed to prenatal tobacco smoke annually in the US [1]. Prenatal exposure to tobacco smoke results in the developing fetus being exposed to several harmful toxic substances. The adverse effects caused by these toxic substances range from reduced placental function to long-term neurobehavioral alterations in the offspring [2, 3]. The neurobehavioral alterations are mainly due to nicotine, the major toxic compound in the tobacco smoke with psychoactive properties [4]. Nicotine is an agonist of nAChRs, which play important roles during brain development and plasticity [5, 6]. The cholinergic

innervation in the mammalian brain commences during the fetal development [7] and activation of nAChRs promotes synaptic connectivity during a critical period of prenatal development of hippocampus [8]. Therefore, premature activation of nAChRs, as might occur in cases of maternal smoking, could result in alterations in neurodevelopmental processes regulated by these receptors.

Chronic administration of nicotine during development has been reported to result in changes in nicotine binding in the brain [9, 10]. Apart from the molecular changes, prenatal nicotine exposure is believed to cause neurodegeneration and morphological changes in the hippocampus [11–13]. The hippocampus is a critical brain region involved in memory encoding and has also been implicated in disorders involving depression and anxiety [14]. Therefore, cellular disruptions observed in the hippocampus may play a key role in the neurobehavioral sequelae of prenatal nicotine exposure.

The excitatory neurotransmission in the hippocampus is mainly mediated by glutamate receptors that are essential mediators of LTP, which is now a widely accepted cellular correlate of memory encoding [15, 16]. During LTP, there is enduring enhancement of synaptic transmission in the synapses that undergo high-frequency stimulation. This enhancement of excitatory transmission is mainly achieved by recruitment of additional α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid (AMPA) receptors to the postsynaptic membranes. In a previous report, we demonstrated that in 2-month-old rats prenatally exposed to nicotine, the AMPA receptor-mediated synaptic currents were significantly reduced, suggesting long-lasting impairment in excitatory synaptic physiology [17]. Importantly, disruptions in the hippocampal glutamatergic system are attributed to mood disorders like anxiety and depression [18, 19] and several animal studies provided accounts on neurobehavioral outcomes of prenatal nicotine exposure [17, 20, 21]. However, there is a lack of understanding of the possible molecular mechanisms contributing to these behavioral outcomes and if they are specifically related to hippocampus and its major excitatory neurotransmitter system. We hypothesized that aberrations in the hippocampal glutamatergic system underlie the neurobehavioral alterations arising from prenatal nicotine exposure. In this study, we used a rat model of prenatal nicotine exposure, utilizing subcutaneous nicotine infusion with osmotic mini pumps in pregnant dams [22], with the mode and dose of delivery that has been shown to be a close match of conditions found in human pregnant smokers [23]. Neurobehavioral outcomes in young offspring as well as the possible changes in relevant synaptic proteins and synaptic function in glutamatergic terminals were analyzed. The new findings reported in this study provide evidence that hippocampal glutamatergic system is impaired and potentially contributes to the neurobehavioral alterations in prenatally nicotine-exposed rats.

Experimental procedures

Animals, surgical procedures, and chemicals

Female Sprague–Dawley rats weighing 200–250 g (Charles River Laboratories, Wilmington, MA) were anesthetized with isoflurane on day 3 of pregnancy and implanted with subcutaneous osmotic mini pumps (Alzet, Model 2004, Cupertino, CA) to deliver (−)-nicotine (6 mg/kg/day, free base) or physiological saline. On the day of parturition after all pups were born, osmotic mini pumps were removed under brief isoflurane anesthesia and the wound was closed with surgical clips. The day after parturition was considered day 1 (PND 1) and litters were culled to ten pups per dam with, if possible, an equal sex ratio. All animals were kept in a room with 22–24°C and 12 h light/dark cycle with unlimited access to food and water. Experiments were performed in 3-week-old animals and from hippocampi collected from animals of this age. All experiments were conducted in experimentally naive animals; animals were not subjected to more than one behavioral test and for Western-blot experiments, the hippocampi were collected from animals that were not subjected to behavioral tests. Electrophysiological experiments were done on a separate set of animals. Experiments were performed in accordance with the Principles of Animal Care (NIH Publication 85–23, 1985) and the protocol approved by Auburn University Institutional Animal Care and Use Committee. Unless specified, all the chemicals were purchased from Sigma (St. Louis, MO).

Behavioral tests

Open-field tests were performed as described elsewhere [24]. Each rat was individually placed gently into the center of an enclosed open-field arena (46 × 41 cm; San Diego Instruments, San Diego, CA) facing away from the observer and allowed to explore it for 10 min. Exploration and arousal were measured as number of grids crossed and rearing using a photobeam activity system. Elevated plus-maze tests were preformed in an apparatus consisting of four arms (10 × 50 cm) with two of the contra-positioned arms being closed with opaque walls and the other two being open. All arms were interconnected with a 10 × 10 cm platform elevated 60 cm above the ground. Individual rats were placed at the distal end of one open arm, facing away and released, and the latency to enter last the third of a closed arm was measured. This latency, referred to as transfer latency (TL), was chosen to preferably test anxiety than to include simple exploratory activities [25]. Forced swim tests were performed to assess depressive-like behaviors as previously described [26–28], with some modifications. Rats were forced to

swim for a 10-min period in a vessel containing 40-cm-deep water maintained at 25 ± 1°C. The time taken (latency) to reach the first floating state (immobile state except for small limb movements to keep afloat) and the total time spent on floating state were measured. Hippocampus-based spatial memory was examined subjecting rats to a two-trial Y-maze task as described previously [29]. The Y-maze apparatus was of uniform beige color and consisted of three arms with an angle of 120° between them. The maze was set in a separate room with minimal lighting, and visual cues were placed around it. The two trials of the test were separated by a 3-h inter-trial interval to assess spatial recognition memory. During the first trial (acquisition), rats were allowed to explore two arms of the maze; the starting arm in which they were initially placed, and a second arm to which we refer to as the other arm; the third (novel) arm was closed. During the second trial (retention test), rats were placed back in the starting arm and allowed to explore for 5 min with free access to all three arms (the novel arm was opened). The total number of entries and time spent in each arm were measured. Motor coordination was assessed with a rotorod test. Rats were trained to maintain their balance on a rod rotating with a speed of 20 rpm for 10 min. Animals that fell from the rod were repositioned onto it. The training was followed by three experimental trials to assess motor coordination. Each trial lasted 10 min and the speed was set at 20 rpm reached within the first min, 80 rpm reached at a rate of 8 rpm/min, and 80 rpm reached at a rate of 20 rpm/min for the first, second, and third trials, respectively. The time taken for each rat to fall was calculated. Grip strength was assessed by wire hang test, in which the pup was made to hang by its forepaws on a horizontal wire (~2 mm in diameter), 30 cm above a soft pad. The duration the animal was able to hang was recorded [30–32].

Preparation of synaptosomes

Synaptosomes from the hippocampi were prepared as previously described [33]. In brief, hippocampi were homogenized in ice-cold modified KREBS (mKREBS) buffer containing (in mM): 118.5 NaCl, 4.7 KCl, 1.18 $MgSO_4$, 2.5 $CaCl_2$, 1.18 KH_2PO_4, 24.9 $NaHCO_3$, 10 dextrose supplemented with 10 μg/ml adenosine deaminase, 0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.1 mg/ml aprotinin, and 5 mM benzamide. The buffer was bubbled with 95:5% CO_2/O_2 mixture to adjust the pH to 7.4. Homogenate was initially filtered through three layers of nylon filter (100 μm pore) and then through a low-protein binding filter. The filtered particulate was spun at 1,000 g at 4°C and the pellet (synaptosomes) was resuspended in mKREBS buffer and stored at −80°C until use.

Electrophysiology

Coronal sections (350 μm) of hippocampus were prepared in ice-cold dissection buffer containing (in mM): 85 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 4 $MgSO_4$, 0.5 $CaCl_2$, 75 sucrose, 2 kynurenic acid, 0.5 ascorbate and 25 glucose, bubbled with 95% O_2/5% CO_2 and pH adjusted to 7.4. Slices were then incubated for 1 h in artificial cerebrospinal fluid (ACSF) supplemented with or without 2 mM kynurenic acid and containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.0 NaH_2PO_4, 26.0 $NaHCO_3$, and 11.0 dextrose bubbled with 95% O_2/5% CO_2, pH 7.4. Following incubation, electrophysiological recordings were performed in a recording chamber with continuous perfusion of ACSF bubbled with 95%CO_2/5%O_2. Field excitatory postsynaptic potentials (fEPSPs) from Schaffer collateral/commissural-CA1 (SC) synapses were recorded by stimulating CA1 stratum radiatum with bipolar electrodes and placing a recording glass electrode (1–4 MΩ) filled with ACSF ~200 μm from the stimulating electrode. The frequency of the test stimulation was every 20 s. Stimulation and acquisition of fEPSPs were controlled by LTP software program [34]; stimulus intensity was adjusted using a A365 stimulus isolator (WPI, Sarasota, FL), and amplified with Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA). For stimulus response curves, current intensity was increased from 0 to 300 μA at steps of 50 μA. In LTP experiments, after at least 15 min of stable baseline recording, three high-frequency stimulations (HFS, 100 Hz) were delivered every 20 s at an intensity that evoked 50% of the slope at which the population spike appeared. LTP was measured 55–60 min post-HFS. Whole-cell current recordings were performed in CA1 pyramidal neurons voltage clamped at −80 mV. The patch pipette (6–10 MΩ) was filled in an internal solution comprising (in mM): 100 K-gluconate, 0.6 EGTA, 5.0 $MgCl_2$, 2.0 Na-ATP, 0.3 Na-GTP and 40 HEPES, pH 7.4 and the slices were perfused continuously with ACSF supplemented with 50 μM picrotoxin, 100 μM DL-2-amino-5-phosphonopentonoic acid, and 1 μM tetrodotoxin to isolate AMPA receptor-mediated miniature excitatory postsynaptic current (mEPSCs). The currents were low-pass filtered (2 Hz), digitized, amplified (Axopatch 200B, Molecular Devices), acquired with the pCLAMP 8 program (Molecular Devices) and stored on disks for offline analysis. Series resistance was monitored continuously during the recordings and to be included for analysis the holding currents should be greater than the baseline noise levels but less than −150 pA and the series resistance typically 20–30 MΩ but not showing more 15% drift during the course of experiment [35]. At the end of each experiment, specific AMPA receptor antagonist (±)-4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbonyl-6,7

methylenedioxyphthalazine (SYM 2206, 30 μM; Tocris, Ellisville, MO) was added to the ACSF to verify if the mEPSCs were completely blocked.

Single channel recordings from synaptic AMPA receptors were performed as described previously [36]. In brief, a phospholipid bilayer, composed of 1,2 diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) dissolved in anhydrous hexane at a concentration of 1 mg/ml (Aldrich Chemical Co., Milwaukee, WI), was formed at the tip of a polished borosilicate glass pipette (100 MΩ). The extracellular bath solution (~300 μl) contained (in mM): 125 NaCl, 5 KCl, 1.25 NaH_2PO_4, and 5 Tris HCl. The pipette solution consisted of (in mM): 110 KCl, 4 NaCl, 2 $NaHCO_3$, 1 $MgCl_2$, 0.1 $CaCl_2$, and 2 3-N-Morpholino propanesulfonic acid (pH adjusted to 7.4). After forming a stable membrane, 3–5 μl suspension of the synaptosomes was delivered to the bath solution. After the addition of AMPA (290 nM), voltage was applied to evoke single-channel activity. Single-channel currents were filtered at 2 kHz and digitized at 5 kHz (Mini-digi, Molecular Devices) with pCLAMP 9 software (Molecular Devices) and saved on a computer hard disk. Only the data exhibiting long stretches of single-channel current transition without base line drifts were chosen for quantitative analysis.

Western blot

Experiments were performed in either whole hippocampal homogenates or in synaptosomal homogenates. For quantifying receptor subunits and postsynaptic density-95 (PSD-95) synaptosomes were used. For all other intracellular proteins, whole homogenates were used. Synaptosomes and whole hippocampi were homogenized with lysis buffer (Cell Signaling, Danvers, MA) and the protein content of the homogenate was estimated using a Bio-Rad assay. Equal aliquots of each homogenate were mixed with NuPage LDS sample buffer (Invitrogen, Carlsbad, CA) and NuPage reducing agent (Invitrogen) and boiled for 5 min. Samples were then subjected to SDS-PAGE and the proteins were transferred to Bio-Rad polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% nonfat dry milk in phosphate buffered saline (PBS) containing 1% Tween (PBS-T) and then incubated with the following antibodies overnight at 4°C: rabbit anti-vesicular glutamate transporter 1 (VGLUT1, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-synaptophysin (1:1,000; Sigma), mouse anti-postsynaptic density 95 (PSD-95, 1:1,000; Millipore), mouse anti-GluR1 (1:1,000; Millipore), mouse anti phospho (Ser845) GluR1 (1:500; Millipore) and mouse anti-β-actin (1:2,000; Sigma). After incubation with primary antibodies, the membranes were washed with appropriate secondary antibodies (Millipore) at room temperature for 1 h. After three washes in PBS-T (each 5 min), the blots were developed with enhanced chemiluminescence kit (Millipore). Density of immunoreactivity for each band was measured with Bio-Rad's Quantity One software and values were normalized to the β-actin levels of corresponding lanes.

Data analysis

Two-way ANOVA with Tukey post hoc test was used to analyze behavioral data (treatment × gender) and fEPSP data (treatment × stimulus intensity). Since there was no significant gender variation, treatment mean values are presented. The mEPSC and LTP data were analyzed with one-way ANOVA. The mEPSC data were analyzed with the Mini Analysis program (Synaptosoft, Fort Lee, NJ). The mEPSCs included for analysis had a monotonic 10–90% rise time of <6 ms and exponential decay time <25 ms. The vast majority of the mEPSCs had rise times and decay times that were considerably less than these upper limits. The mEPSC amplitude (A) was measured from the baseline with the amplitude threshold for detection of mEPSCs was set above the noise level, at four times the SD of mean noise level, and individual events were confirmed visually. The decay times were best fitted with two terms with the function: $y = A1 \times \exp(-x/\tau 1) + A2 \times \exp(-x/\tau 2)$ [37]. The single-channel open probability was estimated as $Po = R_o/(R_c + R_o)$, where R_c and R_o represent the areas under the current-amplitude histogram corresponding to close and open states, respectively [38]. The all points-current amplitude histograms were constructed and fitted with two-term Gaussian method using the Microcal Origin program (OriginLab Corp., Northampton, MA) to identify individual conductance levels of channel open and close states. The single-channel conductance was computed by plotting current as a function of membrane voltage, according to the equation $g = I/(V - V_o)$, where I is the single channel current, V is the voltage, and V_o is the reversal potential [38]. Log-transformed open and close time histograms were fitted best with the exponential log probability function and variable metric method using pCLAMP 9.0 program. Data were analyzed with ANOVA. Two-tailed Student's t test was used for analysis of Western-blot data. Analyses were conducted using SigmaStat software and statistical significance was accepted at $p \leq 0.05$.

Results

Elevated levels of anxiety and depression and impaired memory in rats exposed to prenatal nicotine

Smoking during pregnancy is associated with the risk of behavioral alterations in children, which may also include changes in anxiety. It has been previously reported that

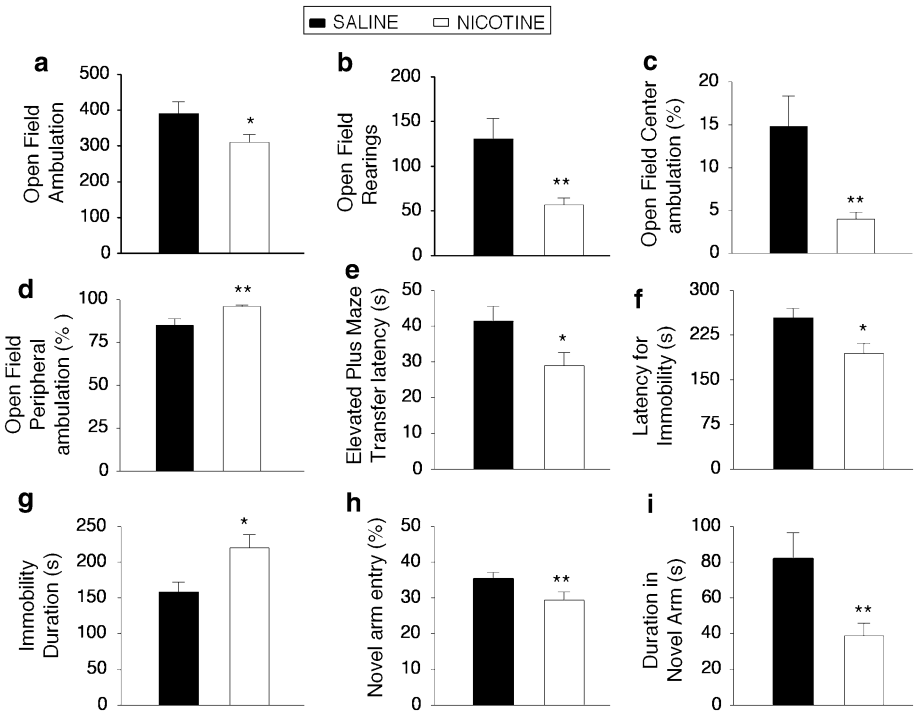

Fig. 1 Behavioral comparison of prenatally nicotine-exposed and control rats in open-field, elevated plus-maze, forced-swim, and Y-maze tests. Panels show that nicotine exposure resulted in increased anxiety and depression and reduced memory. *Shaded bars* represent the control/saline group data and the *open bars* represent the nicotine group data. **a** Reduced ambulation in terms of the number of grids crossed implicating reduced distance traveled ($n = 12$). **b** Reduced number of rearings ($n = 12$). **c** Reduced ambulation in the center of the field expressed as percentage of total ambulation ($n = 12$). **d** Increased ambulation in the periphery of the open field in the nicotine-exposed rats ($n = 12$). **e** Lower transfer latency in nicotine-exposed rats in elevated plus-maze test ($n = 10$). **f** Nicotine-exposed rats showed significantly less latency time to reach first immobile state in forced swim test ($n = 9$). **g** Significantly higher duration of time spent in immobile state without active swimming ($n = 9$). **h** Reduced number of entries into the novel arm in the Y-maze test ($n = 9$). **i** Reduced time spent in the novel arm ($n = 9$); (*$p < 0.05$, **$p < 0.01$; two-way ANOVA)

young adult rats that are exposed to nicotine prenatally show elevated anxiety [17]. In this study, we chose a younger age group, as information on young ages is scant. We analyzed the behavior of young rats in two different tests commonly used to evaluate anxiety in rodents, the open-field and the elevated plus-maze tests. Nicotine-exposed animals showed reduced ambulation (crossed less grids) in the open-field test (Fig. 1a; control 390.0 ± 29.76, nicotine 309.1 ± 25.05; $p < 0.05$, $n = 12$). The number of rearings was also reduced in the nicotine group (Fig. 1b; control 130.58 ± 16.67, nicotine 56.47 ± 7.95; $p < 0.01$, $n = 12$). Enhanced levels of anxiety could contribute to reduced exploratory activity in the open-field task and, therefore, we analyzed ambulation in the center of the field and in the periphery (thigmotaxis). Ambulation in the center of the field and periphery were analyzed separately and we found decreased center ambulation (control 14.84 ± 3.54%, nicotine 3.96 ± 0.81%) and increased peripheral ambulation (control 85.15 ± 3.55%, nicotine 96.03 ± 0.81%) in the rats exposed to nicotine (Fig. 1c, d; $p < 0.01$, $n = 18$). Reduced activity in the center of the field and elevated thigmotaxis are suggestive of increased anxiety in rodents in the open-field test paradigm [39]. Another method of testing increased anxiety is the elevated plus-maze test, and in this test we found that rats exposed to nicotine showed reduced transfer latency (Fig. 1e; control 41.36 ± 4.09 s, nicotine 28.96 ± 3.62 s; $p < 0.05$, $n = 10$). These results show that prenatal nicotine exposure resulted in elevated anxiety in the offspring.

Since clinical studies have suggested a link between maternal substance abuse and mood disorders including depression [40], we next studied whether prenatal nicotine exposure would result in depressive-like behavior in the offspring. Rats exposed to nicotine prenatally reached an immobile state sooner, resulting in reduced latency to reach an immobile state in the forced-swim test (Fig. 1f; control 253.56 ± 16.28, nicotine 194.36 ± 15.96 s; $p < 0.05$, $n = 9$). They also spent more time in the immobile state than controls (Fig. 1g; control 158.08 ± 13.70 s, nicotine 220.07 ± 18.41 s; $p < 0.05$, $n = 9$). These results indicate that nicotine exposure during fetal development could result in depressive behavior in the young offspring.

Cognitive deficits in offspring with prenatal nicotine exposure have been demonstrated by clinical and animal

studies [40]. Since the mammalian hippocampus is one of the primary regions involved in learning and memory processes, we tested the hippocampal-based spatial memory performance using a Y-maze apparatus. Results of this study suggest that number of visits to the novel arm (Fig. 1h; control 35.36 ± 1.72%, nicotine 29.34 ± 2.35%; $p < 0.01$, $n = 9$) and the total duration of time spent in the novel arm (Fig. 1i; control 82.03 ± 14.48 s, nicotine 38.66 ± 7.32 s; $p < 0.01$, $n = 9$) were reduced in nicotine-exposed rats. These differences were not due to the nicotine-exposed animals exhibiting less exploratory behavior or mobility than the control animals; rather, it seems that the nicotine-exposed animals did not differentiate the novel from the other, previously visited, arm. Control animals data showed more entries into the novel than the other arm (novel arm = 35.36 ± 1.72%, other arm = 31.4 ± 0.71%; $p < 0.05$, $n = 9$), which is consistent with increased exploration of the novel area. In contrast, nicotine-exposed rats did not show a difference between novel arm and other arm entries (novel arm = 29.34 ± 2.35%, other arm = 35.33 ± 2.27%; $p > 0.05$, $n = 9$), which can be interpreted as a lack of recognition of the novelty of the previously unavailable arm (novel arm). Because the novel and other arm only differed in their spatial attributes, these results suggest that the nicotine-exposed rats did not distinguish the novel arm from spatial cues and therefore had spatial memory deficits. To ascertain whether the reduced activities in these behavioral experiments are indeed due to the changes in specific behaviors tested and not due to any impairment in the motor function, coordination, or strength, we performed a wire-hang test and a rotorod test. Results of these experiments show that there are no differences between the nicotine and control groups in latencies to fall in the wire-hang test as well as the rotorod test (Fig. 2; $n = 10$, $p > 0.05$).

Prenatal nicotine exposure results in diminished LTP in the rodent hippocampus

Since some forms of synaptic plasticity such as LTP are now widely accepted as a cellular correlate of memory encoding, we studied LTP in SC synapses. The LTP was measured at 55–60 min following HFS to induce conditioning in SC synapses in hippocampal slices from young nicotine-exposed and control rats (Fig. 3a). The average slope, as a percentage of baseline over 55–60-min-interval post-HFS showed diminished LTP maintenance in prenatally nicotine-exposed rats (Fig. 3a–c; 106 ± 5.63%; $p < 0.05$, $n = 6$), whereas the controls showed stable maintenance of LTP (Fig. 3; 132.69 ± 6.32%; $p < 0.05$, $n = 6$). These data demonstrate that prenatal nicotine exposure is associated with impaired synaptic plasticity, suggesting that this impairment may account for the memory deficits.

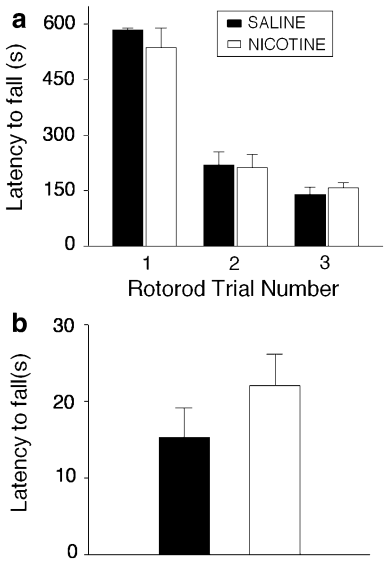

Fig. 2 Prenatal nicotine exposure does not impair locomotor activity or muscle strength. **a** Mean latency time before falling off the rod in rotorod test did not show any significant difference. **b** There was no significant difference in mean latency time in the wire-hang task. *Shaded bars* indicate control data and the *open bars* indicate nicotine group data ($p > 0.05$, $n = 10$; ANOVA)

Altered basal synaptic transmission in the hippocampus of rats exposed to nicotine prenatally

To investigate whether LTP impairment is due to pre- and/or post synaptic deficiencies, we analyzed the presynaptic fiber volley (FV) amplitude and slope of EPSPs from SC synapses at different input current intensities. Summation of action potentials arriving at the CA1 region following SC stimulation are represented by FVs and therefore FVs provide a measure of number of presynaptic neurons recruited [41]. We analyzed the amplitude of FVs across different stimulation intensities and found that FV amplitudes from the hippocampi of prenatally nicotine-exposed rats were less than that of controls (Fig. 4a; $p < 0.05$, $n = 6$), suggesting that the number of active afferent axons are reduced by nicotine exposure during prenatal development. Prenatal nicotine-exposed rats also showed reduced fEPSP slopes across the range of stimulus intensities used (Fig. 4b; $p < 0.05$, $n = 6$) and these results when combined with the analysis of FV amplitude versus fEPSP slope (Fig. 4c) suggest that prenatal nicotine exposure leads to reduction of fEPSPs elicited by the same number of axons, i.e., to impairment in the synaptic strength per axon.

AMPA receptor-mediated miniature synaptic currents are decreased in prenatal nicotine-exposed rats

The AMPA receptors are the major contributors of excitatory neurotransmission in the hippocampus and alterations

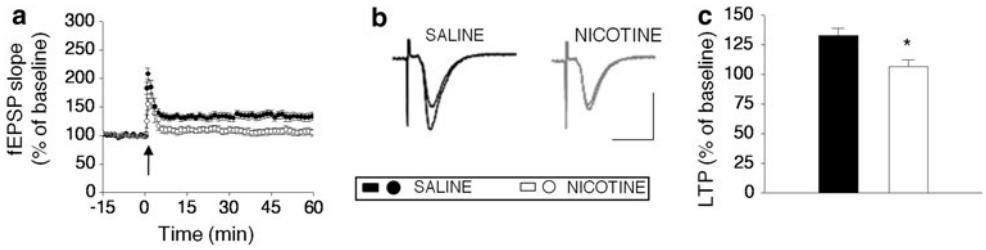

Fig. 3 Impairment in synaptic plasticity in the form of LTP in prenatally nicotine-exposed rats. **a** LTP was induced with three 1 s HFS at 100 Hz (*arrow*); the normalized fEPSPs slopes show significant differences between nicotine (*open circles*) and controls (*shaded circles*). **b** Representative traces at baseline overlaid on that at 60 min after HFS are shown for control saline (*black*) and nicotine (*gray*) groups. *Calibration bars*: equal 0.8 mV and 7 ms. **c** At 50 or 55–60 min, LTP was significantly lower in nicotine-exposed rats (*open bar*) compared to controls (*shaded bar*). (*$p < 0.05$, $n = 6$, ANOVA)

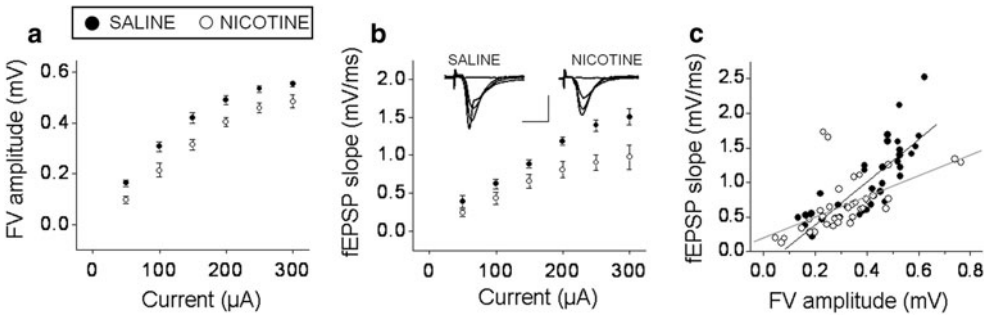

Fig. 4 Prenatal nicotine exposure affected basal synaptic transmission in the hippocampal Schaffer collateral-CA1 synapses. *Shaded circles* represent the saline/control group data and the *open circles* and *bars* represent the nicotine group data. **a** Plot of presynaptic fiber volley amplitude (FV) versus stimulus intensity. FVs of nicotine rats were significantly reduced than that of controls (*$p < 0.05$, $n = 6$, two-way ANOVA). **b** Input/output plot of fEPSP slope versus stimulus intensity. Representative traces of overlaid fEPSPs elicited by 100, 200, and 300 μA stimulation, which depict differences in I/O responses of nicotine (*right*) and control (*left*) groups are shown. Calibration: 1 mV, 5 ms (*$p < 0.05$, $n = 6$, two-way ANOVA). **c** Input/output plot of fEPSP slope versus FV. *Lines* represent the best-fit linear regressions (control: *back line*, $y = 3.0x$; nicotine: *gray line*, $y = 1.49x$); when slope of each animal data were compared nicotine data was significantly less than that of controls ($p < 0.01$, $n = 6$, ANOVA)

in the synaptic expression of these receptors as well as changes in their biophysical properties may affect the excitatory transmission in the hippocampus. In order to test whether decreased synaptic AMPA expression and/or function contributed to the postsynaptic deficits, we analyzed the action potential independent, spontaneous neurotransmitter release-elicited AMPA receptor-mediated mEPSCs in the CA1 pyramidal neurons. We found that the current amplitude (control 14.17 ± 1.29 pA, nicotine 10.11 ± 0.19 pA) as well as frequency of mEPSCs (interevent intervals: control 8.51 ± 1.54 s, nicotine 14.53 ± 2.17 s) were reduced in nicotine-exposed animals (Fig. 5a–d; $p < 0.05$, $n = 9$). The decay phase of the mEPSCs was best fitted with two time constants and the decay times of the nicotine group ($\tau 1$ 6.53 ± 0.41 s, $\tau 2$ 14.74 ± 0.24 s) were less than that of the control group ($\tau 1$ 9.31 ± 0.69 s, $\tau 2$ 17.65 ± 0.87 s) (Fig. 5e; $p < 0.05$, $n = 9$). These results suggest that postsynaptic expression/function of AMPA receptors as well as the presynaptic glutamate release were both impaired in prenatally nicotine-exposed rats.

Single-channel properties of synaptic AMPA receptor are altered in rats exposed to nicotine prenatally

The decreased mEPSC amplitude in nicotine-exposed pups can be due to altered single-channel properties of synaptic AMPA receptors. The mean amplitude of single-channel current is a product of single-channel open probability and single-channel conductance [42]. The sum of mean current amplitude of synaptic receptors activated by vesicular release of glutamate represents mEPSCs. In order to determine whether reduction in single-channel open probability and/or conductance contributed to the decrease in AMPA receptor-mediated mEPSC in nicotine-exposed pups, we performed single-channel measurements of synaptic AMPA receptor currents utilizing synaptosomes. Analysis of single-channel currents revealed that channel open probability was reduced in nicotine-exposed rats, (0.10 ± 0.027) compared to the controls (0.35 ± 0.043) (Fig. 6a–c; $p < 0.05$, $n = 5$). Channel open probability of AMPA receptors is positively correlated with phosphorylation of the subunit GluR1 at its serine 845 residue [43]. Therefore we compared the protein

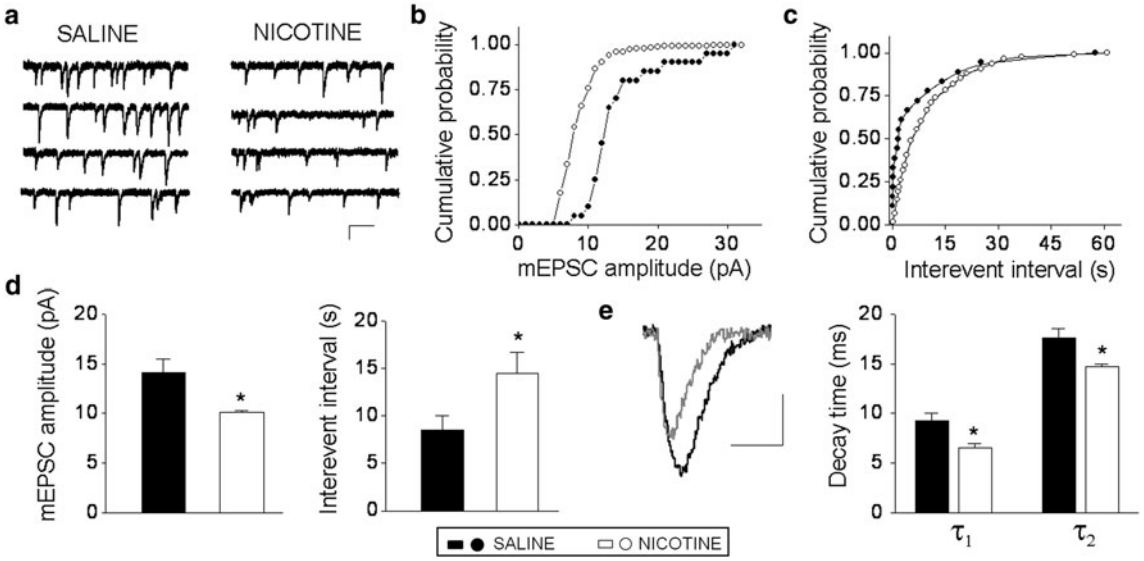

Fig. 5 AMPA receptor-mediated whole-cell synaptic currents were reduced in prenatal nicotine-exposed rats. Control/saline group data are represented by *shaded bars* and *circles* and the nicotine group data are represented by *open circles* and *bars*. **a** Sample traces of voltage clamp whole-cell currents recordings at −80 mV show AMPA receptor-mediated mEPSCs, which are reduced in amplitude and frequency in nicotine-exposed rats compared to the control/saline group. *Calibration bar* equals 15 pA and 150 ms. **b** Cumulative frequency plot for mEPSC amplitudes show that the curve of nicotine data is shifted left to that of the control data, suggesting that mEPSC amplitude is significantly less than that of controls. **c** Cumulative plot for interevent intervals shows that the distribution curve corresponding to the nicotine group is shifted to the right of control curve, implying reduced frequency of mEPSCs in the nicotine group. **d** Bar plot depicting the significantly less mean amplitude of mEPSCs and significantly higher interevent interval (lower frequency) in nicotine group than that of controls ($n = 9$, $^*p < 0.05$, ANOVA). **e** Decay times (τ) of mEPSCs were best fitted with two terms and the decay times were significantly reduced in the prenatal nicotine group. Sample mEPSCs of control (*black*) and nicotine (*gray*) groups. Calibration: 5 pA, 15 ms. Bar plot showing the reduced decay times in the nicotine group compared to the controls ($n = 9$, $^*p < 0.05$, ANOVA)

levels of ser845–GluR1 levels in the hippocampi of control and nicotine-exposed rats by quantitative Western blotting. Results showed that prenatal nicotine-exposed rats had lower levels of ser 845–GluR1 in their hippocampi (Fig. 6d; $p < 0.05$, $n = 5$). The open and closes dwell time data showed shorter open times (Fig. 6e–g; control: $\tau 1$ 5.41 ± 0.32 ms, $\tau 2$ 72.33 ± 1.76 ms, nicotine: $\tau 1$ 3.06 ± 0.40 ms, $\tau 2$ 36.13 ± 2.79 ms; $p < 0.05$, $p < 0.01$, $n = 5$) and longer close times (Fig. 6h–j; control: $\tau 1$ 1.40 ± 0.31 ms, $\tau 2$ 87.25 ± 6.19 ms, nicotine: $\tau 1$ 2.51 ± 0.21 ms, $\tau 2$ 217.59 ± 7.36 ms; $p < 0.05$, $p < 0.01$, $n = 5$). These results suggest that prenatal nicotine exposure could manifest in alteration of the intrinsic functional properties of single synaptic AMPA receptors contributing to altered amplitude and time course of mEPSCs.

Prenatal nicotine exposure results in reduced expression of synaptic proteins in the hippocampus

Certain synaptic proteins regulate presynaptic glutamate release and postsynaptic AMPA receptor expression. The VGLUT1 is a neurotransmitter vesicle protein that is involved in the translocation of glutamate into vesicles [44] and VGLUT1 has recently been shown to be critical for hippocampal LTP [45]. Another synaptic vesicle protein synaptophysin is also involved in regulation of LTP [46] and plays an essential role in presynaptic release [47]. The PSD-95, a member of the membrane-associated guanylate kinase family that is highly abundant in the postsynaptic density, regulates AMPA receptor expression at synapses and other aspects of synaptic transmission and plasticity [48–50]. The AMPA receptor subunit GluR1 is the most abundantly expressed subunit of this receptor. Therefore, in order to identify possible alterations in expression patterns of these proteins which may have contributed to the reductions in the synaptic transmission and plasticity, we performed Western-blot experiments. We found that levels of VGLUT1 (Fig. 7; $p < 0.05$, $n = 5$), synaptophysin (Fig. 7; $p < 0.01$, $n = 5$), GluR1 (Fig. 7; $p < 0.05$, $n = 5$), and PSD-95 (Fig. 7; $p < 0.01$, $n = 5$) were decreased in the hippocampus of the prenatal nicotine group. These results suggest that prenatal nicotine exposure alters the levels of pre- and postsynaptic proteins in the hippocampus, which may have caused the deficiencies in synaptic physiology.

Discussion

Smoking during pregnancy is associated with mood disorders and cognitive impairments in children. While there

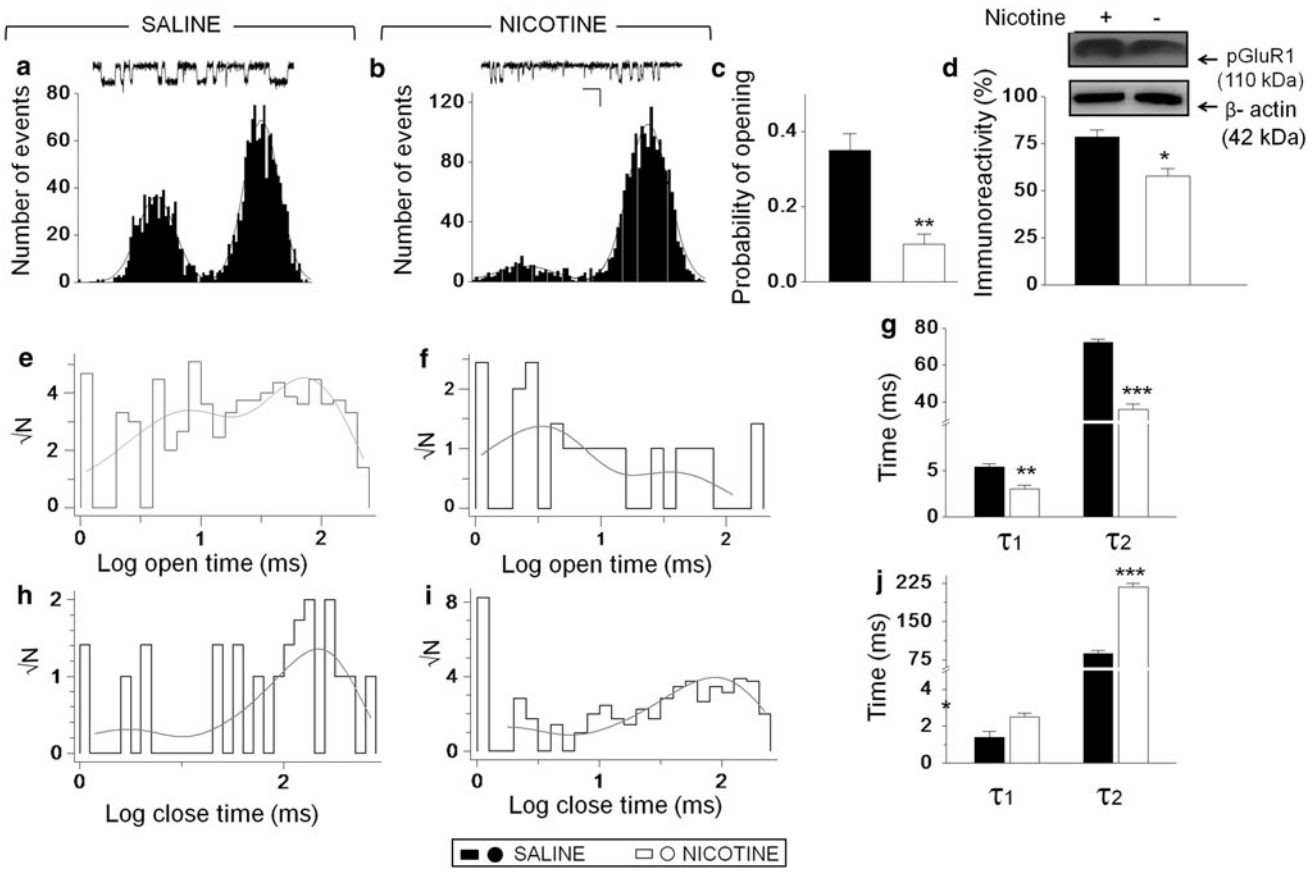

Fig. 6 Single-channel properties of synaptic AMPA receptors were altered in rats exposed to nicotine prenatally. Control/saline group data are represented by *shaded bars* and the nicotine group data are represented by *open bars*. **a** Sample trace of single-channel currents from control animals shown above the amplitude histogram. **b** Sample trace and corresponding amplitude histogram of nicotine-exposed animal data. **c** Bar plot showing significantly reduced channel open probability in nicotine-exposed rats compared to the controls. **d** Reduced protein expression of phospho-GluR1(ser 845) in the hippocampus of nicotine-exposed rats. **e** Log-transformed open time frequency distribution of control single channel recordings were best fitted by two time constants. **f** Nicotine-exposed rat data for open times also fitted best with two terms. **g** Bar plot illustrating significantly reduced open times ($\tau 1$ and $\tau 2$) in nicotine-exposed rats compared to controls. **h** Log-transformed channel close time distributions of control data. **i** Corresponding close time data of nicotine-exposed animals. Close time distributions of control and nicotine data were fitted with two time constants. **j** Bar plot showing significantly increased close times in nicotine-exposed animals compared to controls. ($n = 5$, $^*p < 0.05$, ANOVA)

are extensive behavioral analyses on this subject, the underlying molecular mechanisms are not fully explored. In the present study, we utilized a rodent model of prenatal nicotine exposure in which pregnant rats were infused with nicotine at the rate of 6 mg/kg/day through a subcutaneously implanted osmotic mini pump. This dose has been shown to be a close match to the condition in human smokers in terms of plasma nicotine levels and upregulation of nAChRs in the fetal brain. In rats, 6 mg/kg/day nicotine exposure resulted in plasma nicotine levels ranging from 25 to 75 ng/ml, with young rats showing levels close to the lower limit [22, 51, 52]. The upper limit was similar to the plasma nicotine level found in heavy smokers [53]. Since we used young female rats for nicotine exposure, the plasma nicotine levels were expected to be the levels found in moderate human smokers. In addition, the level of upregulation of nAChRs in the fetal rat brains when pregnant dams received this particular dose was similar to the level of nAChR upregulation in human fetal brains from smoking mothers [54, 55]. Nicotine in rodents has a short half-life and therefore the use of an osmotic mini pump would ensure a steady infusion. This also fits the human condition, as amniotic fluid maintains a reservoir of steady levels of nicotine so that the fetus is continuously exposed [56–58]. The results from our study for the first time demonstrate that the cognitive deficits observed in prenatal nicotine exposure are paralleled by altered glutamatergic hippocampal neurotransmission. Specifically, we show that LTP deficits resulted from altered pre- and postsynaptic impairments such as modified glutamate release and altered synaptic AMPA receptor expression and function. Taken together, this study provides new information on the potential mechanisms that may underlie the behavioral aberrations, particularly

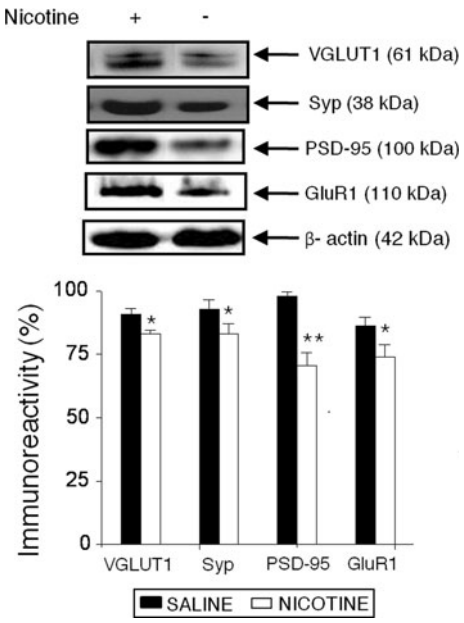

Fig. 7 Prenatal nicotine exposure results in reduced expression of key synaptic proteins. Levels of synaptic proteins from synaptosomal fractions. The expression of vesicular proteins VGLUT1 and synaptophysin (Syp) and postsynaptic marker PSD-95 is significantly reduced as assessed by Western blots. In addition, AMPA receptor subunit GluR1 is also reduced in prenatal nicotine-exposed rats. Signal densities were normalized to the β-actin levels for each lane. Representative bands are shown above the bar plot. Control/saline group data are represented by *shaded bars* and the nicotine group data are represented by *open bars* ($n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$, Student's *t* test)

cognition in the offspring subjected to gestational nicotine exposure.

Maternal tobacco smoking has a strong correlation with birth defects, low body weight, and adverse neurobehavioral outcomes in children [40]. The neurobehavioral perturbations from smoking, such as depression and anxiety disorders, affects both the mother and her children [59–62]. Our results indicate that prenatal nicotine exposure results in elevated anxiety and depression in the young rat offspring. These results are in agreement with that of a recent study [63] that reported open-field hypoactivity in gestational nicotine-exposed rats. Other reports have also shown the presence of anxiety with elevated plus-maze test in older adolescent rats being about 40 days old [17, 21]. Consistent with our results, another recent study showed depressive-like behavior in prenatal nicotine-exposed animals using a learned helplessness paradigm [64].

Many clinical reports suggest that maternal smoking is a risk factor for deficient cognitive function in children [3, 65–68]. Our results indicate that spatial memory is impaired in prenatally nicotine-exposed young rats. This is in line with prenatal and postnatal nicotine effects on spatial and working memory that have been reported [21, 69, 70]. Of particular interest is the complex interplay between affective disorders and memory; increased anxiety or depression can induce memory deficits and, in turn, strong memory declines can lead to affective disorders. Indeed, the hippocampal formation along with other brain regions is associated with these behavioral processes [71]. Importantly, the spatial memory deficits observed were likely independent or additive with elevated anxiety and depression in the nicotine-exposed rats. This was evident in the Y maze, in which the nicotine-exposed animals were equally likely to enter the arm to which they had been previously exposed and the novel arm (control animals showed a marked preference for the novel arm). Thus, nicotine-exposed animals seemed unable to use spatial cues to discriminate the novel and the previously visited arm, and decreases in time spent in the novel arm were likely not due to the animals exhibiting decreased mobility or being reluctant to enter the novel arm. These observations are consistent with previous studies reporting memory deficits associated with prenatal nicotine exposure. These data along with the results of this study indicate that prenatal nicotine exposure results in complex behavioral modifications that include memory deficits, anxiety, and depression.

Our results with Y-maze experiments in the current study demonstrated impaired hippocampus-based spatial memory and a previous report showed that prenatal nicotine exposure causes morphological disruption in cellular layers of the hippocampus [13]. Therefore, we investigated LTP, basal synaptic transmission and synaptic AMPA receptor currents to elucidate if there is a decline in glutamatergic synaptic physiology at regional, cellular, and molecular levels that would shed light on the mechanistic basis for memory loss. Our results demonstrated impaired LTP, basal synaptic transmission, and AMPA receptor-mediated synaptic currents, which fits well with data from Western-immunoblot experiments showing decreases in the levels of VGLUT1, synaptophysin, GluR1, phospho(Ser845) GluR1, and PSD-95. In particular, the reduced expression of synaptosomal GluR1 in the nicotine-exposed rats suggests reduced levels of AMPA receptors in the postsynaptic site, which would explain the impairments in synaptic plasticity and physiology observed in this study. In the CA1 region LTP can be reliably induced from postnatal day 14 and the degree of LTP at PND15 and PND30 has been shown to be same [72, 73]. In addition, levels of AMPA receptors also reach a stable level at 2 weeks [74]. Therefore, it is reasonable to assume that behavioral changes are correlated well with physiological modifications such as LTP impairments and AMPA receptor deficits.

Alterations in intrinsic single-channel properties of synaptic AMPA receptors in the nicotine-exposed rats is a novel and interesting finding that adds support to the

deficient mEPSC properties. Since mEPSC amplitude and single-channel open probability are positively correlated [42], these results suggest that reduced single-channel open probability and open times coupled with increased close times may contribute to the deficits in single neuronal AMPA receptor currents and in turn to the reduced synaptic transmission in prenatally nicotine-exposed rats. Reduced single-channel AMPA receptor open probability in prenatal nicotine-exposed rats can be further explained by the diminished levels of GluR1 subunits phosphorylated at Ser845 as phosphorylation at this site enhances open probability [43]. The decrease in mEPSC amplitude can also result from decreased expression of synaptic AMPA receptors. In our study, the decreased GluR1 expression in the synaptosomes adds support to this notion. In addition, altered quantal glutamate content can influence the mEPSC amplitude. In this study, we noted a significant decrease in the vesicular protein VGLUT1 that would have possibly contributed to impaired packaging of glutamate. Therefore, we conclude that decreased mEPSC amplitude, which possibly contributed to impaired synaptic plasticity, resulted from decreased expression and function of synaptic AMPA receptors as well as altered quantal content of glutamate. The decrease in frequency of mEPSC in this study can be explained by: (i) a decrease in probability of glutamate release due to reduced presynaptic afferents as evidenced by decreased FV amplitude, (ii) impaired presynaptic release machinery as indicated by decreased presynaptic protein synaptophysin, and (iii) deficiency in vesicular packaging of glutamate due to decreased levels of VGLUT1.

It is currently not known how prenatal nicotine exposure affected single-channel properties, however, this could be most likely due to indirect effects of nicotine by modifying phosphorylation of amino acid residues in the receptor subunits as revealed in this study and/or by altering receptor associated auxiliary molecules that have the potential of determining the channel properties. The deficits in basal synaptic transmission and LTP could be associated with alterations in nAChR subtypes that lend a modulatory role synaptic transmission and plasticity [75, 76]. This notion is supported by a recent finding that mRNA levels of α7-nAChRs were reduced in the hippocampus of rats exposed to nicotine prenatally [20]. In summary, these findings strongly suggest that prenatal nicotine exposure results in potent glutamatergic impairment in the excitatory transmission in the hippocampus. Clinical and animal studies have suggested a central role for the glutamatergic system in psychiatric and cognitive functions and the glutamatergic system is a therapeutic target for ameliorating specific symptoms in these disorders [18, 19, 77]. In addition to its regulatory role in synaptic plasticity that underlies memory formation in the hippocampus, the glutamatergic system, that includes AMPA receptors, also plays an important role in affective disorders including anxiety and depression [78, 79]. Interestingly, reduced expression of VGLUT1 has been linked to enhanced anxiety, depressive behavior, and impaired recognition memory [80]. Consistently with our results showing decreased levels of synaptophysin and PSD-95 in the hippocampus of prenatal nicotine-exposed rats, other studies have shown decreased levels of these proteins in the hippocampi of depressed animals [81–83]. Overall, our findings implicate a critical role of glutamatergic neurotransmitter system in generating the behavioral alterations connected with prenatal nicotine exposure.

In conclusion, the current study used a rodent model for prenatal nicotine exposure and the results of this study show parallel striking similarities with neurobehavioral alterations observed in children exposed to tobacco smoke in utero. The most important finding of this study is the co-occurrence of memory deficits, anxiety, and depressive behavior in prenatal nicotine-exposed rats and the results of this study suggest a causal relationship between these neurobehavioral alterations and aberrations in glutamatergic synaptic physiology in the hippocampus. The assessment of key physiological processes, in prenatal nicotine-exposed hippocampus, that are believed to govern hippocampal memory formation and the changes that may impair memory processing would be of great interest as nicotinic cholinergic physiology is critical for learning and memory processing.

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