Prenatal Stress Affects Network Properties of Rat Hippocampal Neurons

Gayane Grigoryan and Menahem Segal

Background: Long-term effects of stress during pregnancy on brain and behavior have been analyzed extensively in recent years. One major problem with these studies is the inability to separate between the net effects of the prenatal stress (PS) and the effects of the stressed mother and siblings on the newborn animals.

Methods: To address these issues, we studied morphological and electrophysiological properties of neurons in dissociated cultures of the hippocampus taken from newborn PS rats. We complemented these studies with experiments on behaving rats and recordings from slices taken from PS rats and their control rats.

Results: While the density of cultured neurons was not different between PS and control rats, there were fewer glutamic acid decarboxylase-positive neurons in the former cultures. Additionally, cells taken from PS pups developed more extensive dendrites than control animals. These differences were correlated with a higher rate of synchronous activity in the PS cultures and a lower rate of spontaneous miniature inhibitory postsynaptic current activity. There were no differences in the excitatory synaptic currents or the passive and active properties of the recorded neurons in the two groups. Young PS rats were more motile in open field and elevated plus maze than control rats, and they learned faster to navigate in a water maze. Slices taken from hippocampus of PS rats expressed less paired-pulse inhibition than slices from control rats.

Conclusions: These results indicate that PS affects network properties of hippocampal neurons, by reducing gamma-aminobutyric acidergic inhibition.

Key Words: Culture, GABA, hippocampus, network bursts, prenatal stress, spatial memory

Evidence for long-term effects of prenatal environment on postnatal brain functions has been accumulating rapidly in recent years (1–3). Thus, exposure of pregnant dams to stressful stimulation can cause changes in neurotransmitter metabolism, brain morphology, and ability to cope with cognitive and emotional tasks in the juvenile and even in adult life (4–7). In fact, it has been suggested that prenatal stress (PS) can facilitate emergence of neuropsychiatric conditions such as depression and schizophrenia. The molecular mechanisms underlying these long-term changes in neuronal functions are beginning to unravel, and studies using gene arrays report on gene families that are altered by prenatal stress (8). One of the causes for this lasting alternation involves maturation and functions of growth factors, such as brain-derived neurotrophic factor (BDNF) (9,10). One of the many functions attributed to BDNF involves regulation of gamma-aminobutyric acid (GABA)ergic interneurons (11), and indeed, the density of nitric oxide-synthesizing neurons (12), a subtype of GABAergic neuron, is reduced in stressed individuals (13). While there is a growing understanding of the epigenetic control of gene expression and the role of prenatal stress hormones in this function, there is a paucity of information concerning the cellular and electrophysiological mechanisms underlying these long-term changes. A major concern is that when animals that have experienced prenatal stress are examined, it is difficult to discern between the direct effect of the stress in utero and altered developmental interaction with the stressed mother and siblings. One possible direct approach to examining the effect of PS on neuronal properties is to sacrifice the pups at birth and grow their neurons under controlled conditions (14). In the present study, we examined properties of 1- to 3-week-old cultured rat hippocampal neurons taken from control and PS male pups at postnatal day 1. We wish to report that the neurons that are taken from PS pups differ in several important morphological, electrophysiological, and behavioral attributes from control neurons.

Methods and Materials

All the methods are detailed in Supplement 1 and will be mentioned herein only briefly.

Animals

Animal handling was done in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Weizmann Institute. Prenatal stress was induced by exposing pregnant Wistar dams of a local breeding colony (between gestation days 14 and 21) to three daily stressful experiences including forced swim in a water bucket (20 minutes, gestation days 14 and 18), restraining tube (40 minutes, days 15 and 19), and placement on an elevated platform (30 minutes, day 16s and 20). This protocol was adapted from Weinstock (1). Experiments were conducted with at least five different litters, only with male rats.

Behavioral Tests

The elevated plus maze (EPM) and the open field (OF) tests were used to assess the effect of PS on anxiety and exploratory behavior, respectively, in 1-month-old male rats. The Morris water maze (MWM) was used to assess spatial learning. All behavioral tests were conducted in a noise-isolated, brightly lit room. Both OF and EPM were placed in the center of the room to provide similar levels of illumination to all parts of the apparatus.

From the Department of Neurobiology, The Weizmann Institute, Rehovot, Israel.

Address correspondence to Menaham Segal, Ph.D., The Weizmann Institute, Department of Neurobiology, Rehovot 75100, Israel; E-mail: Menahem.segal@weizmann.ac.il.

Received Jul 30, 2012; revised Jan 24, 2013; accepted Feb 8, 2013.

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2013.02.003

Cultures

Cultures were prepared as detailed elsewhere with male pups decapitated on day of birth (15).

Electrophysiology

Hippocampal cultures at 7 to 14 days in vitro (DIV) were transferred to a recording chamber placed on the stage of an inverted Olympus IX70 microscope (Center Valley, Pennsylvania) and washed with a standard recording medium, containing (in mmol/L) sodium chloride (NaCl), 129; potassium chloride (KCl) 4; calcium chloride, 2; magnesium chloride, 1; glucose, 10; HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10; pH 7.4, 320 mOsm. Neurons were recorded with patch pipettes containing (in mmol/L): K-gluconate, 136; KCl, 10; NaCl, 5; HEPES, 10; ethylene glycol tetraacetic acid, .1; guanosine 5′-triphosphate sodium salt, .3; Mg-adenosine-5′ trisphosphate, 1; phosphocreatine, 5; pH 7.2, with a resistance in the range of 5 to 8 MΩ. Signals were amplified with MultiClamp 700B and recorded with pClamp9 (Axon Instruments, Foster City, California).

When recording spontaneous miniature excitatory postsynaptic currents (mEPSCs), .5 μmol/L tetrodotoxin (TTX) and 10 μmol/L bicuculline were added to the medium. For recording of miniature inhibitory postsynaptic currents (mIPSCs), .5 μmol/L TTX and 20 μmol/L 6,7-dinitroquinoxaline-2,3-dione were added to the recording medium, and a cesium chloride-based intracellular solution was used. Neurons were clamped at −60 mV.

Morphological Analysis and Immunochemistry

The morphological analysis was conducted with 7 to 21 DIV cultures. Cultures were placed on the stage of an inverted Zeiss confocal laser-scanning microscope (LSM-510; Zeiss Inc., Jena, Germany). High-resolution, thin optical sections of the cells were obtained from green fluorescent protein (GFP)-transfected neurons. Offline analysis included three-dimensional reconstruction of the neuron dendrites and spines. Image analysis was conducted using LSM image analysis software (Zeiss Inc.). For immunocytochemistry, cells were fixed in 4% paraformaldehyde for 30 minutes, blocked in serum, and stained overnight with the primary antibody (mouse anti-NeuN, 1:1000) (Millipore, Billerica, Massachusetts) and/or rabbit anti-glutamic acid decarboxylase (GAD) (Novus Biological LLC, Littleton, Colorado), and then with Alexa-conjugated fluorescent secondary antibodies (1:200). Cells were visualized on the confocal microscope.

Calcium Imaging and Analysis

Cultures were incubated for 1 hour at room temperature with the standard recording medium containing 2 μmol/L Fluo-4AM (Molecular Probes, Eugene Oregon). Cells were imaged thereafter on the stage of the Olympus microscope, equipped with a Till Photonics (Grafelfing, Germany) light source and an Andor Technology Ixon CCCD camera (Belfast, Ireland). Cells were imaged at a rate of five frames per second with a 488-nm light. Each field of the culture (40X lens, 190 μm^2) was imaged for 2 minutes and four to six fields were imaged in each glass coverslip (16). Axon Imaging Workbench (AIW V5.2; Axon Instruments Inc., Santa Clara, California) was used for data acquisition and analysis. Regions of interest including neuronal somata were measured.

Slice Electrophysiology

Transverse hippocampal slices (400 μm) were prepared from the hippocampi of 2- to 3-week-old male Wistar rats using a McIlwain tissue chopper (Gomshall, Surrey, United Kingdom). Slices were incubated for 1.5 hours in carbogenated (5% carbon dioxide and 95% oxygen) artificial cerebrospinal fluid at room temperature. The medium contained (in mmol/L) 124 NaCl, 2 KCl, 26 sodium bicarbonate, 1.24 monopotassium phosphate, 2.5 calcium chloride, 2 magnesium sulfate, and 10 glucose, at pH = 7.4. Recording was made from slices that were slightly submerged in a standard chamber at 33.8°C to 34.0°C with a flow rate of 2.5 mL per minute artificial cerebrospinal fluid.

Extracellular recordings of population spike (PopS) in stratum pyramidale of cornu ammonis (CA) 1 region of transverse hippocampal slices were made through a glass pipette containing .75 mol/L NaCl (4 MΩ). Responses were evoked by stimulation of the Schaffer collaterals using bipolar electrode positioned in stratum radiatum. Data acquisition and offline analysis were performed using pCLAMP 9.2 (Axon Instruments). All numerical data were expressed as mean ± SEM. Statistical comparisons were made with analysis of variance or *t* tests, as the case required.

Results

Behavior

The Elevated Plus Maze. Prenatal stress animals spent significantly more time in the open arm than control rats (.85 ± .31 min compared with .27 ± .09 min, $n = 20$ and $n = 15$, respectively, $p < .05$). That was correlated with an increase of their total activity on the EPM (8.05 ± 1.06 entries compared with 5.67 ± .83 entries, $p < .05$, in control rats), as well as with an increase in number of entries to the open (4.1 ± .54 entries compared with 3.2 ± .59 entries, $p < .05$, in control rats) and closed (4.05 ± .93 entries compared with 2.6 ± .63 entries, $p < .05$, in control rats) arms (Figure 1A).

Open Field. Normally, animals spend more time in the corners and the periphery than in the center, the most anxiogenic area. Prenatal stress rats crossed significantly more central squares than control rats (3.95 ± .85 crossings compared with 1.27 ± .42, $p < .001$, respectively) and more peripheral lines (37.2 ± 5.19 compared with 27.3 ± 3.84, respectively) (Figure 1B).

The Morris Water Maze. A second cohort of rats was used for the study of spatial learning in the MWM. One-month-old PS rats ($n = 11$) showed accelerated spatial learning compared with control rats ($n = 9$) with significantly shorter latencies on second (32.91 ± 4.96 compared with 55.42 ± 7.26, $p < .01$, $F = 6.92$, in control rats), third (25.11 ± 4.09 compared with 43.17 ± 6.35, $p < .01$, $F = 6.1$, in control rats), and fifth (24.86 ± 3.77 compared with 45.19 ± 6.13, $p < .004$, $F = 8.59$, in control rats) days of training sessions (Figure 1C1). Probe test showed a difference in preference for the platform quadrant in PS rats (39.24 ± 2.21 compared with 29.81 ± 7.33 in control rats) (Figure 1C2). Prenatally stressed animals showed a significantly lower latency to first platform quadrant crossover (7.27 ± 1.56 compared with 18.78 ± 3.73, $p < .01$, $F = 9.38$) (Figure 1C3).

This second cohort of rats was also tested in the OF and EPM. We were able to reproduce the results obtained in the first cohort of rats demonstrating that PS rats show higher numbers of line crossings of the center squares than control rats. There were no differences in numbers of fecal boluses (1.1 ± .46 compared with 1.2 ± .8 in control rats). In addition, they spent significantly more time in the open arm of the EPM than control rats (and no difference in numbers of fecal boluses: .18 ± .18 compared with .2 ± .2 in control rats, data not shown).

Morphology

Neuronal density was estimated in fixed NeuN immunostained neurons, in four different experiments (i.e., different litters) using

confocal microscopy. The number of cells was counted in four randomly selected fields of view in each glass coverslip. Altogether, the total number of cells per field was not different between control and PS cultures (19.5 ± 2.3 vs. 16.9 ± 1.15, $n = 16$ and $n = 15$ fields in control and PS, respectively; Figure 2A,B).

The balance between excitatory and inhibitory neurons was assessed by measuring the proportion of GAD-positive neurons in populations of 14 DIV NeuN-positive cells (Figure 2C,D). As opposed to control cultures, where the GAD-positive neurons amounted to 37 ± .06% of the total NeuN population ($n = 12$ fields, two cultures), the GAD-positive neurons amounted only to 17 ± 1% of the total NeuN positive neurons in the PS neurons (11 fields, $t = 2.30$, $p < .03$). In addition, the GAD fluorescence intensity was significantly lower in the PS neurons than in the control neurons (53.8 ± 1.1 compared with 65.9 ± 1.5 arbitrary fluorescence units, $n = 56$ and $n = 41$ cells, respectively, $p < .01$).

The complexity of dendritic arborization was estimated using Sholl analysis (Figure 2E). The analysis was conducted with three age groups, 7, 14, and 21 DIV cultures, so as to assess possible age-dependent developmental changes in neuronal morphology. The analysis was conducted in three spheres of 50, 100, and 150 μm, centered on the neuronal somata, in three-dimensional reconstructed neurons. Due to resolution of the microscope, further spheres could not be studied systematically. In twelve 7 DIV GFP transfected PS neurons, there were significantly more dendrites at 150 μm away from the soma, compared with 12 GFP control neurons ($t = 2.40$. $p < .02$, three experiments in each group; Figure 2F). This difference was maintained in 14 DIV GFP transfected neurons (10 neurons in each group, two experiments, 5 neurons in each, $t = 2.56$, $p < .01$) (Figure 2F, middle). Interestingly, this difference was no longer seen in 21 DIV cultures (7 neurons in each group, one experiment) (Figure 2F, right). Interestingly, the dendritic density increased by more than fourfold between the 7 DIV and 21 DIV control cultures, indicating a continuous growth process during this time in culture (Figure 2F). These results indicate that PS neurons mature faster than control neurons but reach the same level of dendritic density within 3 weeks in culture.

To assess the likelihood that the difference in dendritic density is reflected in synaptic connectivity, the dendritic spine density in two dendritic segments, 40 to 50 μm long, in each of the 10 GFP-transfected cells used for the Sholl analysis, was measured (Figure 2G,H). For the initial analysis, no distinction was made between different shapes of spines, which were lumped together. The analysis indicates that there was no difference in spine density, being .28 ± .03 spines per μm in the control group ($n = 18$ segments) and .27 ± .02 spines per μm in the PS group ($n = 19$ segments) (Figure 2E,H, left). Further analysis, separating between spines having a distinct head and thin headless filopodia, was conducted in a subset of the analyzed dendrites (12 dendritic segments in each group). Similar proportion of spine/filopodia in the two groups, being 1.92 ± .47 in the control

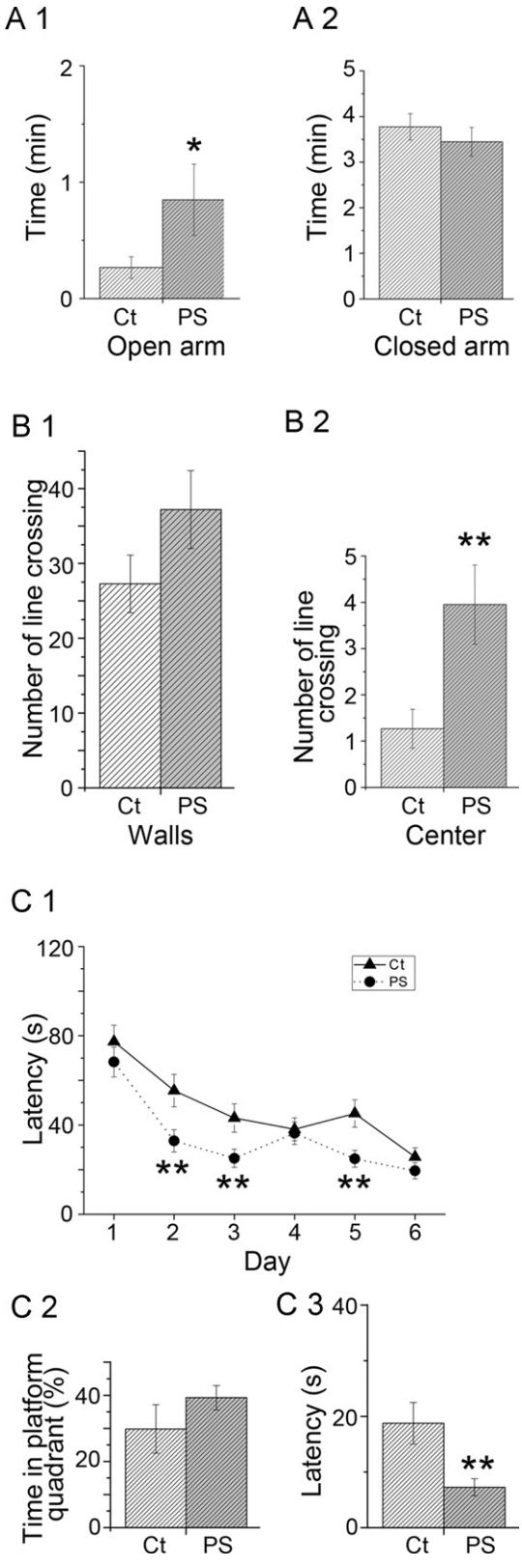

Figure 1. Behavioral analysis of the effects of prenatal stress (PS). **(A)** Elevated plus maze. The time spent in the open arms **(A1)** is significantly different between control rats (Ct) and PS rats, while there was no significant difference in the amount of time spent in the closed arms **(A2). (B)** Behavior of the rats in the open field. The PS rats spent significantly more time in the central part of the arena **(B2)**, while they were not different statistically in the total distance spent near the walls of the open field **(B1). (C)** Performance in the Morris water maze. **(C1)** Learning to navigate in the Morris water maze was faster across days of training in the PS compared with control rats, significantly in days 2, 3, and 5, while both groups reached the same performance level at the last day (day 6) of training. **(C2, C3)** Quadrant analysis, conducted 2 days after the last training session. The platform was removed, and the time spent in the relevant quadrant was not different between the groups **(C2)**, while the latency to reach the quadrant was shorter in the PS than in the control group **(C3)**. *$p < .05$; **$p < .01$.

group and 1.99 ± .42 in the PS group, were found (Figure 2H, right, showing the number of spines and filopodia in the two groups). Finally, to examine if the faster growth of PS dendrites compared with control dendrites at 7 DIV culture was accompanied by an increase in spine density, spines (including filopodia) were counted in 14 dendritic segments in control neurons and 13 segments in PS neurons. There was no difference in spine density between the two groups, also in the 7 DIV neurons, being .15 ± .014 and .146 ± .018 spines per μm dendrite, respectively. Interestingly, spine density grew by twofold in both groups between 1 and 2 weeks in culture.

Network Activity

Two-week-old cultures exhibited Fluo-4 fluorescence transients that corresponded to action potential discharges. Network activity was expressed as synchronous discharges of several neurons in the field of view (Figure 3). For quantification, the number of network bursts in 2-minute imaging sessions was counted, as well as the proportion of neurons, out of the total in the field of view, which participated in the network burst. Figure 3 demonstrates a highly significant difference in both parameters between the control and PS cultures ($n = 25$ and $n = 23$ fields, in six different experiments, respectively). These data indicate that PS cultures generate more network activity than control cultures.

In preliminary experiments, network activity was more intense also in 7 DIV PS cultures, but this difference was not significant statistically (two glasses, six fields, 1.66 ± .49 bursts per 2 minutes in control cultures, and 3.17 ± 1.01 in PS cultures).

The differences in network activity may reflect either intrinsic excitability of neurons or synaptic properties in the two culture

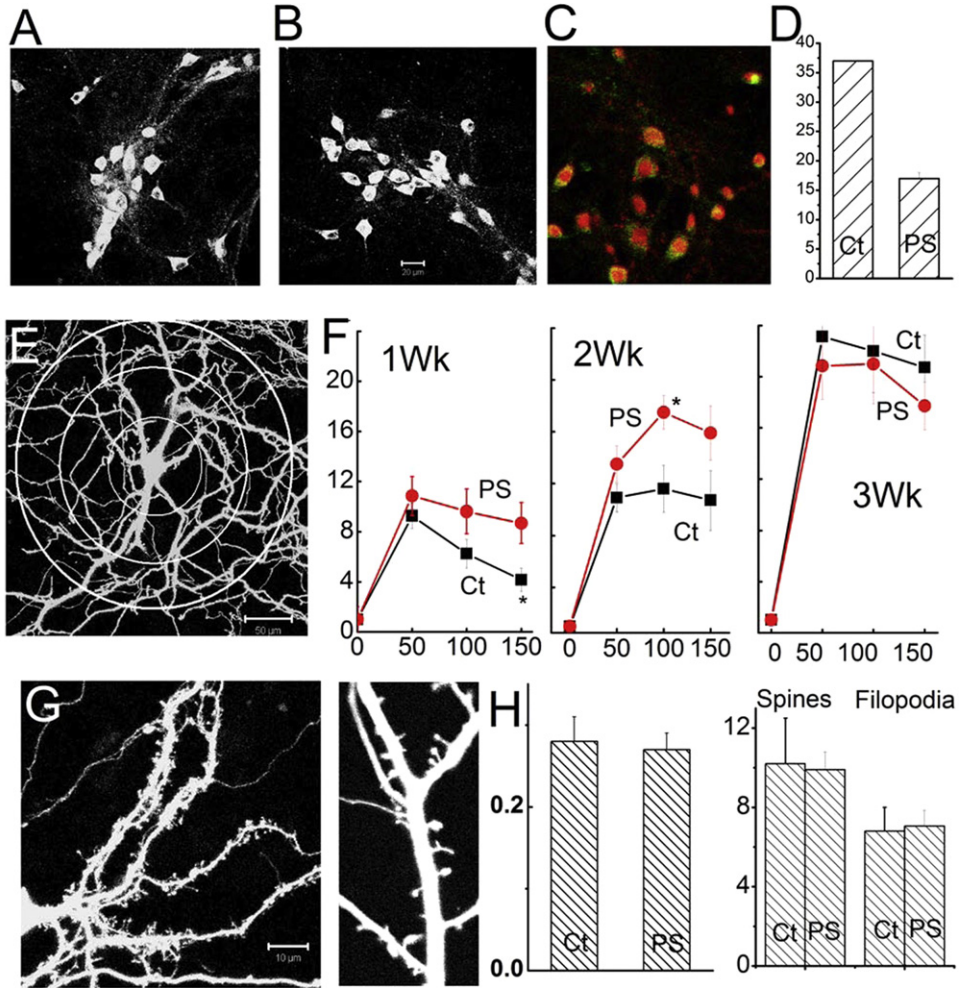

Figure 2. Morphological analysis of cultured hippocampal neurons derived from prenatally stressed (PS) pups. **(A, B)** NeuN immunostained neurons in control (Ct) **(A)** and PS cultures **(B)**. No difference in cell density between the two cultures types were found (see text for details). **(C)** Immunostained PS cultures for NeuN (red) and glutamic acid decarboxylase (green). **(D)** Summary of results, indicating a significantly larger proportion of glutamic acid decarboxylase-positive cells in the control cultures. **(E)** Sholl analysis of the dendritic arborization of the neurons that were transfected with green fluorescent protein to enhance visibility of their dendrites. Three spheres, 50 μm apart, are centered in the nucleus of the imaged neuron, and the number of dendritic crossings in each of the spheres is counted. **(F)** Averaged results of the Sholl analysis for 1-, 2-, and 3-week (Wk)-old cultures. Significant differences were found in the 150 μm sphere of the 1-week culture, and the 100 μm sphere of the 2-week cultures. The other points were of the same trend but did not reach significance. Ordinate, the number of crossings, abscissa, the distance from soma in μm. **(G, H)** Analysis of dendritic spine density in the green fluorescent protein transfected neurons, which were used also for the Sholl analysis. Three-dimensional high resolution images were used for the analysis. A dendritic length of 40 to 50 μm was used for the count, and two dendrites were analyzed for each neuron. Scale bar in **(G)** left = 10 μm. Spine sizes in **(G)** right, 1 to 1.5 μm. **(H)** No difference in dendritic spine density (ordinate, spines per μm) were found between the two groups, either in the total spine density or in the breakdown to spines and filopodia (right).

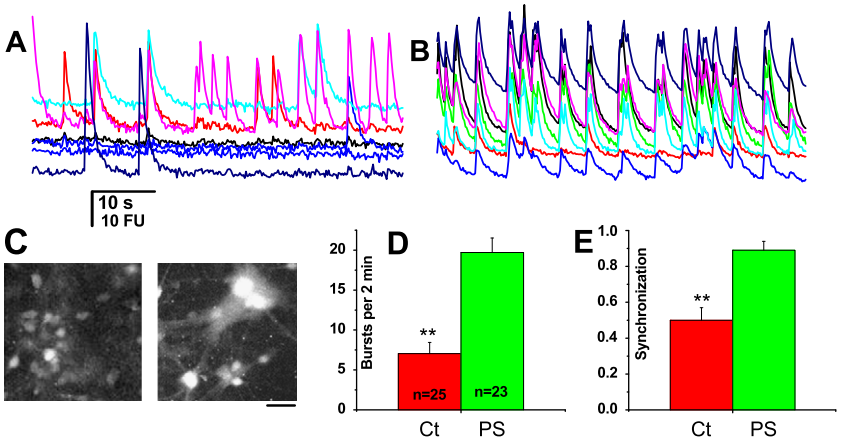

Figure 3. Spontaneous network activity in control (Ct) and prenatal stress (PS) cultures. **(A, B)** Traces of $[Ca^{2+}]_i$ variations in individual neuron somata in a field of view (e.g., sample fields in **[C]**). Some, but not all, of the neurons in the control culture **(A)** fire in synchrony, in contrast to the PS derived cultures **(B). (C)** Sample illustrations of images of Fluo-4 filled neurons. **(D)** Number of bursts per 2-minute sampling interval, in control and PS cultures, showing a significantly higher rate of activity in the latter group. Likewise, **(E)** shows a higher synchronization value in the PS group. $^{**}p < .01$. FU, fluorescence units; s, seconds.

conditions. These two possibilities were examined in separate sets of experiments.

Intrinsic Properties. Activity of single neurons in 14 DIV cultures was recorded with patch pipettes in whole cell voltage or current clamp configuration, under standard conditions. Resting membrane potential and input resistance were measured, as well as properties of action potentials discharged to depolarizing current pulses. There were no differences in either passive properties of the neurons (Figure 4B,C) or in active properties, expressed as the action potential threshold, overshoot, duration, or after-potentials (eight and six cells in control and PS cells, respectively; Figure 4D). These data demonstrate that intrinsic properties of the neurons are not affected by PS.

Synaptic Currents. Miniature excitatory postsynaptic currents were recorded in the presence of TTX and bicuculline from 10 control and 10 PS 14 DIV neurons, in a total of three experiments.

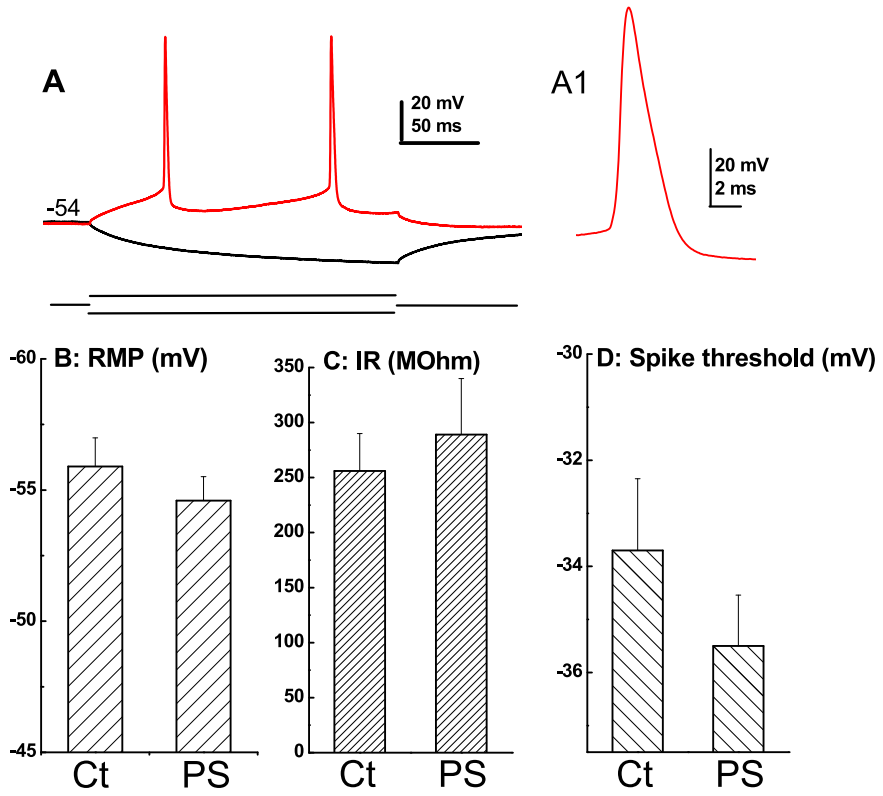

Figure 4. Analysis of active and passive properties of whole cell patch clamped neurons recorded in control (Ct) and prenatal stress (PS) cultures. Action potentials were evoked by a series of depolarizing current commands in current clamped neurons **(A)**. The spike threshold, height, duration, and after potentials were measured from these records. Input resistance was measured from responses to hyperpolarizing current pulses in the same neurons. **(A1)** Sample illustration of an action potential to illustrate the measured parameters. **(B–D)** Averages of resting membrane potential (RMP) **(B)** (mV), input resistance (IR) **(C)** (in MΩ), and spike threshold **(D)** (mV) in the two groups of neurons. There was no difference in the three measured parameters, although the spike threshold had a tendency to be lower in the PS group compared with the Ct.

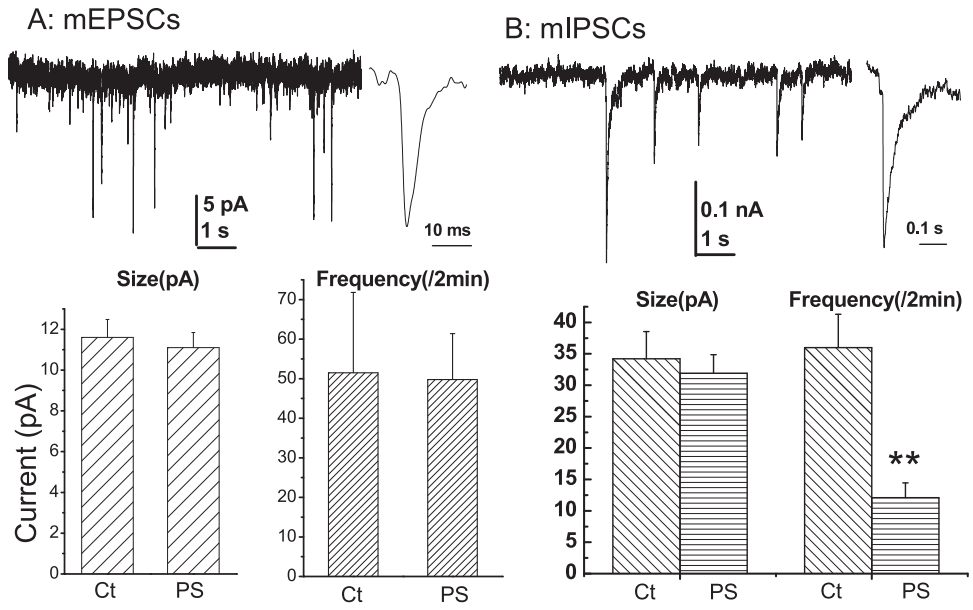

Figure 5. Synaptic activity in control (Ct) and prenatal stress (PS) neurons. Miniature excitatory postsynaptic currents (mEPSCs) **(A)** were recorded in 10 control and 10 PS neurons in presence of TTX and bicuculline. Top, sample trace of mEPSCs taken from a control neuron. Bottom, there was no difference in either amplitudes or frequencies of mEPSCs between the two groups. Cells were voltage clamped at −60 mV. **(B)** Miniature inhibitory postsynaptic currents (mIPSCs) recorded from control ($n = 21$) and PS ($n = 20$) neurons in presence of TTX and DNQX, to eliminate excitatory synaptic activities. Note that the mIPSCs are much larger than the mEPSCs in the same group of cells. There were no differences between the Ct and PS in amplitudes of the mIPSCs, but there were significantly fewer mIPSCs in the PS group. $^{**}p < .01$.

There were no significant differences in either the average size or frequency of mEPSCs between the two groups (Figure 5A).

Miniature inhibitory postsynaptic currents were recorded from 14 DIV neurons in the presence of TTX and 6,7-dinitroquinoxaline-2,3-dione (21 control and 20 PS neurons), in a total of five different experiments (Figure 5B). While the mean amplitudes of the mIPSCs was the same in both groups, the PS neurons generated significantly fewer mIPSCs than the control neurons in fixed measuring intervals of 2 minutes each. The rising and decaying kinetics of the mIPSCs were not different between the groups (data not shown).

To examine if this difference in mIPSC frequency seen in 14 DIV neurons can already be recorded in younger cultures, we compared 14 control neurons and 14 PS neurons at 7 DIV, in three separate experiments. There were no differences in the size of the mIPSCs in the two groups (30.3 ± 2.6 compared with 29.7 ± 1.6 pA in the control and PS neurons, respectively). However, there were fewer events in the PS group compared with the control group (6.14 ± 1.22 vs. 10.2 ± 1.7 events per 2 minutes in PS and control groups, respectively, $t = 1.92$, $p < .05$). Once again, there were no differences in rise or decay time of the mIPSCs in the two groups. Interestingly, in both groups, the number of events was significantly lower than those in the 14 DIV cells.

These data indicate that prenatal stress retards the expression of inhibitory synaptic connections in cultured neurons. This action can either result from a reduction in the number of inhibitory neurons, an actual reduction in the number of synapses, or a reduction in release probability of the inhibitory synapses. The reduction in number of GABAergic neurons in the culture indicates that the reduction in mIPSCs is likely to be of presynaptic origin and reflect a reduction in GABAergic terminals. A reduction in inhibitory tone may also contribute to the enhanced synchronized network activity, which we observed above (Figure 3).

Slice Physiology

To quantify the strength of GABA-mediated inhibition on pyramidal neuron, the paired-pulse stimulation protocol was used in hippocampal slices; two successive stimuli of equal intensity were delivered at 10- and 20-millisecond interpulse intervals (IPIs) to Schaffer collaterals in stratum radiatum. Stimulation of the conditioning pulse was set to evoke 75% of the maximal population spike. The activation of inhibition with the conditioning stimulus produced a reduction in the second PopS, the strength of which was quantitated as a PopS2/PopS1 ratio. The inhibition of PopS in the group of control animals was observed at both IPIs (PopS2/PopS1 ratio .47 ± .16 at IPI of 10 msec and .79 ± .16 at IPI of 20 msec, $n = 6$ control and $n = 8$ PS slices). On the other hand, PS rats showed no inhibition of the test PopS compared with the conditioning one (PopS2/PopS1 ratio 1.03 ± .04 [$p < .05$, compared with control animals] at IPI of 10 msec and .99 ± .09 at IPI of 20 msec) (Figure 6). These results confirm the reduction in inhibitory tone seen in the cultured neurons.

Discussion

The present experiments demonstrate that cultures taken from postnatal day 1 pups that were born to pregnant stressed dams are different from those of control nonstressed mothers in several morphological and functional parameters. The most striking difference was the higher frequency and synchronicity of network activity recorded from populations of 14 DIV PS cultures. This can be linked to a higher density of excitatory connections among the neurons or the lower inhibitory tone in these cultures. The present results favor the second possibility, as we found a reduction in the density of GABAergic neurons, as well as a reduction in the frequency of mIPSCs in these cultures. The more elaborate dendritic tree seen in the PS cultures can also

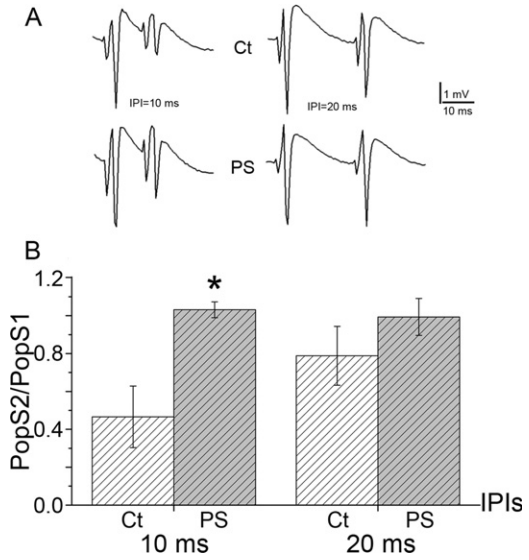

Figure 6. Reduced inhibition in hippocampal slices taken from prenatal stress (PS) rats. Population spikes were recorded from stratum pyramidale of region cornu ammonis 1 in transverse slices from control (Ct) and PS rats. **(A)** Sample illustrations of paired pulse stimulation at 10-msec interpulse interval (IPI) (left) and 20 msec-interval (right). Top row, control, bottom, PS. **(B)** Group data taken at 10-msec and 20-msec interpulse interval. Ordinate, ratio of the population spike (PopS) responses to the test/conditioning stimulation. A significant difference was found at 10 msec interpulse interval, indicating that inhibition is markedly suppressed in the PS rats. *$p < .05$.

be related to the reduced inhibitory tone and ultimately result from enhanced activity of the cultured neuronal network. As we have not conducted a time series study of the emergence of network activity relative to morphological maturation, we cannot attest to what leads, morphology or activity. Within the 3 weeks of observation, network activity, as well as the morphological complexity, grew extensively. In this respect, it is interesting to note that the excitatory tone, expressed in the frequency and amplitudes of mEPSCs, was not different between the two groups of neurons. Likewise, there was no difference in the density of dendritic spines, the locus of excitatory synaptic junctions, or the proportion of spines/filopodia, an indicator of maturity state of the dendrites.

The morphological consequences of prenatal stress are interesting indeed. Previous reports indicate that PS extends dendritic complexity in some brain regions (5) or that it has a complex effect to expand dendritic morphology in male dentate gyrus, while causing shrinkage of dendrites in both CA3 and CA1 areas of the hippocampus (4). These results indicate that the effects of PS on morphology is not universal but is region, and probably stimulus and gender, specific. It may also indicate that these effects result from other, more primary factors that enhance activation of some networks related to behavior. Interestingly, the effects of PS on brain biochemistry and behavior are reported to be gender-specific (4,8), indicating that stress hormones, like sex hormones, may have region and gender-selective actions in the brain. For this reason and to maximize the chances to detect some effects of stress in cultured neurons and to minimize variability, we restricted our studies to male-originated cultures.

The results that we obtained with cultured neurons are complemented by our studies both in the intact animals and in brain slices taken from them. In both test systems, there is an apparent reduced inhibition, expressed as a reduction in paired pulse inhibition in the slice (Figure 6) as well as an enhanced motility in the behaving PS rats. The fact that these rats are more explorative and less afraid of open arenas compounds an attempt to study cognitive effects of PS but clearly indicates that the changes that we observed in the cultured neurons are not due to a sheer artifact related to the preparation of cultured neurons but are found also in hippocampal networks in vivo, as expressed in the results obtained in the slice. Needless to say, the behavioral results obtained by us and others are not entirely due to intrinsic changes in the hippocampus but may be affected by neuromodulatory actions, extrinsic to the hippocampus, and by postnatal interactions between the pups and with their mothers. Thus, the results obtained in culture express net changes that take place in the hippocampus of the prenatally stressed rats and thus allow the study of the affected mechanisms and their functional implications in a rather clean environment.

The effects of stress are believed to be mediated by peripheral release of corticosterone, which penetrates the brain of the embryo to produce long-term effects on gene expression and protein synthesis that result eventually in cognitive deficits (17). Interestingly, these effects can be ameliorated by antidepressants (17). Antidepressants may have several loci of action, one of which involves regulation of BDNF. BDNF is associated with many developmental and plasticity-related molecular cascades, among them the regulation of GABAergic neuronal maturation (11). It has been already suggested that PS reduces the expression of mature BDNF in the hippocampus, which may underlie the reduction in ability to express long-term potentiation and the enhanced long-term depression in these prenatally stressed rats (9). Prenatal stress reduces the expression of a subpopulation of GABAergic neurons in the hippocampus that contain nitric oxide (13), which is similar to the results presented here, and may underlie the reduction in mIPSCs and the increase in network activity that we observed in the current study. Another recent observation indicates that glutamic acid decarboxylase 67, the enzyme that produces GABA, is downregulated in PS mice, which also show hyperactivity (18), similar to our combined observations.

Another suggested mechanism for the effects of PS on synaptic properties involves the downregulation of mineralocorticoid receptors (19). Interestingly, mineralocorticoid receptors are associated with regulation of inhibitory postsynaptic currents in the hippocampus (20). Thus, this may be another link between PS and regulation of GABAergic inhibition that should be further explored.

Other long-term alterations in electrophysiological properties of the PS rodents involve a reduction in field excitatory postsynaptic potentials (7), as well as a reduction in ability to express long-term potentiation (9,21,22). The effects on synaptic plasticity are attributed to a massive reduction in *N*-methyl-D-aspartate receptors in the hippocampus (21), but this is not likely to explain the reduction in alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid mediated field excitatory postsynaptic potentials. These and other possibilities can be further explored in future studies with cultured neurons.

Recent studies on the effects of PS on behavior of the juvenile/adult rodent report an enhanced anxiety and impaired spatial learning, as well as fear conditioning (18). Our results indicate that the PS rats express enhanced locomotion, also in the elevated plus maze, but exhibit faster learning in the MWM, which are not likely to reflect anxiety or hippocampal dysfunction. The reasons for this discrepancy need to be further explored. One possible difference is the age of the tested

animals, and it is likely that the more mature rats will express different behavioral deficits. Another compounding factor is the intensity of the PS protocol. In our case, the maternal stress is rather mild, compared with extensive restraint stress used elsewhere [e.g., (23)]. Mild prenatal stress has been shown to facilitate learning in rodents (24). Undoubtedly, the magnitude of the stress can have differential effects on the newborn, as it may also have on the mothers. This possibility has to be explored in further studies. Regardless of the behavioral consequences of PS in the adult, the observations made in our tissue cultures, which are consistent with previous in vivo studies, indicate that the changes in GABAergic functions are genuine and can be studied under these rather isolated conditions.

This work was supported by a grant from the Israeli Science Foundation and the German Israeli Project Cooperation.

We thank Mr. Shimshon Chalaf for the handling and treatment of the pregnant rats and Ms. Efrat Biton for the preparation of the cultures.

The authors report no biomedical financial interests or potential conflicts of interest.

Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.biopsych.2013.02.003.

1. Weinstock M (2008): The long-term behavioural consequences of prenatal stress. *Neurosci Biobehav Rev* 32:1073–1086.
2. Afadlal S, Polaboon N, Surakul P, Govitrapong P, Jutapakdeegul N (2010): Prenatal stress alters presynaptic marker proteins in the hippocampus of rat pups. *Neurosci Lett* 470:24–27.
3. Bogoch Y, Biala YN, Linial M, Weinstock M (2007): Anxiety induced by prenatal stress is associated with suppression of hippocampal genes involved in synaptic function. *J Neurochem* 101:1018–1030.
4. Bock J, Murmu MS, Biala Y, Weinstock M, Braun K (2011): Prenatal stress and neonatal handling induce sex-specific changes in dendritic complexity and dendritic spine density in hippocampal subregions of prepubertal rats. *Neuroscience* 193:34–43.
5. Muhammad A, Carroll C, Kolb B (2012): Stress during development alters dendritic morphology in the nucleus accumbens and prefrontal cortex. *Neuroscience* 216:103–109.
6. Mychasiuk R, Gibb R, Kolb B (2012): Prenatal stress alters dendritic morphology and synaptic connectivity in the prefrontal cortex and hippocampus of developing offspring. *Synapse* 66:308–314.
7. Saboory E, Ahmadzadeh R, Roshan-Milani S (2011): Prenatal exposure to restraint or predator stresses attenuates field excitatory postsynaptic potentials in infant rats. *Int J Dev Neurosci* 29:827–831.
8. Biala YN, Bogoch Y, Bejar C, Linial M, Weinstock M (2011): Prenatal stress diminishes gender differences in behavior and in expression of hippocampal synaptic genes and proteins in rats. *Hippocampus* 21:1114–1125.
9. Yeh CM, Huang CC, Hsu KS (2012): Prenatal stress alters hippocampal synaptic plasticity in young rat offspring through preventing the proteolytic conversion of pro-brain-derived neurotrophic factor (BDNF) to mature BDNF. *J Physiol* 590:991–1010.
10. Neeley EW, Berger R, Koenig JI, Leonard S (2011): Prenatal stress differentially alters brain-derived neurotrophic factor expression and signaling across rat strains. *Neuroscience* 187:24–35.
11. Subburaju S, Benes FM (2012): Induction of the GABA cell phenotype: An in vitro model for studying neurodevelopmental disorders. *PLoS One* 7:e33352.
12. Valtschanoff JG, Weinberg RJ, Kharazia VN, Nakane M, Schmidt HH (1993): Neurons in rat hippocampus that synthesize nitric oxide. *J Comp Neurol* 331:111–121.
13. Vaid RR, Yee BK, Shalev U, Rawlins JN, Weiner I, Feldon J, Totterdell S (1997): Neonatal nonhandling and in utero prenatal stress reduce the density of NADPH-diaphorase-reactive neurons in the fascia dentata and Ammon's horn of rats. *J Neurosci* 17:5599–5609.
14. Diz-Chaves Y, Baquedano E, Frago LM, Chowen JA, Garcia-Segura LM, Arevalo MA (2013): Maternal stress alters the developmental program of embryonic hippocampal neurons growing in vitro. *Psychoneuroendocrinology* 38:455–459.
15. Ivenshitz M, Segal M (2006): Simultaneous NMDA-dependent long-term potentiation of EPSCs and long-term depression of IPSCs in cultured rat hippocampal neurons. *J Neurosci* 26:1199–1210.
16. Cohen E, Ivenshitz M, Amor-Baroukh V, Greenberger V, Segal M (2008): Determinants of spontaneous activity in networks of cultured hippocampus. *Brain Res* 1235:21–30.
17. Abdul Aziz NH, Kendall DA, Pardon MC (2012): Prenatal exposure to chronic mild stress increases corticosterone levels in the amniotic fluid and induces cognitive deficits in female offspring, improved by treatment with the antidepressant drug amitriptyline. *Behav Brain Res* 231:29–39.
18. Matrisciano F, Tueting P, Dalal I, Kadriu B, Grayson DR, Davis JM, *et al.* (2012): Epigenetic modifications of GABAergic interneurons are associated with the schizophrenia-like phenotype induced by prenatal stress in mice [published online ahead of print April 28]. *Neuropharmacology*.
19. Tamura M, Sajo M, Kakita A, Matsuki N, Koyama R (2011): Prenatal stress inhibits neuronal maturation through downregulation of mineralocorticoid receptors. *J Neurosci* 31:11505–11514.
20. Maggio N, Segal M (2009): Differential corticosteroid modulation of inhibitory synaptic currents in the dorsal and ventral hippocampus. *J Neurosci* 29:2857–2866.
21. Son GH, Geum D, Chung S, Kim EJ, Jo JH, Kim CM, *et al.* (2006): Maternal stress produces learning deficits associated with impairment of NMDA receptor-mediated synaptic plasticity. *J Neurosci* 26: 3309–3318.
22. Yaka R, Salomon S, Matzner H, Weinstock M (2007): Effect of varied gestational stress on acquisition of spatial memory, hippocampal LTP and synaptic proteins in juvenile male rats. *Behav Brain Res* 179:126–132.
23. Marrocco J, Mairesse J, Ngomba RT, Silletti V, Van Camp G, Bouwalerh H, *et al.* (2012): Anxiety-like behavior of prenatally stressed rats is associated with a selective reduction of glutamate release in the ventral hippocampus. *J Neurosci* 32:17143–17154.
24. Fujioka T, Fujioka A, Tan N, Chowdhury GM, Mouri H, Sakata Y, Nakamura S (2001): Mild prenatal stress enhances learning performance in the non-adopted rat offspring. *Neuroscience* 103: 301–307.