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Short Communication

Long-Term Potentiation is Associated With Changes in Synaptic Ultrastructure in the Rat Neocortex

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ABSTRACT Long-term potentiation (LTP) in the sensorimotor cortex of freely moving rats has been associated with changes in dendritic morphology and dendritic spine density. The current research examined changes in synaptic number and ultrastructure associated with LTP in this cortical region. LTP was induced over a 1 h period and the animals were sacrificed 2 h after the initial stimulation of the LTP group. Synapses within the terminal area of the apical dendrites from layer III pyramidal neurons were quantified by determining the total number of synapses per neuron, the number of excitatory and inhibitory contacts, number of synapses with different curvature subtypes, number of perforated synapses, and synaptic length. Several changes in synaptic morphology of excitatory synapses were revealed but no overall increase in the number of synapses per neuron was evident. Specifically, the induction of LTP was associated with an increased number of excitatory perforated and concave shaped synapses. Increased numbers of perforated concave synapses were also found to be significantly correlated with the degree of potentiation in the LTP animals. These and previous results suggest similar synaptic changes in both the cortex and hippocampus during the early phases of LTP maintenance and distinct synaptic changes during later phases of LTP maintenance. **Synapse 59:378–382, 2006.**

Discovering the neuronal mechanisms that underlie learning and memory continues to be an area of intense study. Changes in synaptic number or structure remain important candidates supporting lasting functional modifications. While the specific changes in synaptic efficacy that result from structural modifications are not fully understood, there is mounting evidence that these changes do affect fundamental synaptic activity by changing factors such as the probability of vesicular release (Ghaffari-Farazi et al., 1999) and receptor content (Ganeshina et al., 2004).

Previously, Ivanco et al. (2000) found increased spine density in the sensorimotor cortex following long-term potentiation (LTP) using Golgi techniques. Further, Kleim et al. (2004) found synatogenesis at the electron microscopic level only during the later phases of memory consolidation of a motor task. Thus, we hypothesized that the early phase of plasticity following the induction of LTP in the neocortex would be associated with synaptic change at the ultrastructural level.

Research examining synaptic structure following LTP has typically focused on the hippocampus, where morphological changes have been found (Geinisman et al., 1991; Weeks et al., 1999). Synaptogenesis or the formation of new synapses has not been a consistent

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finding following hippocampal LTP. The hippocampus is typically involved in processes that require rapid acquisition of information, whereas other structures may display more gradual changes (Ivanco and Racine, 2000; McClelland et al., 1995). The neocortex appears to be involved in more lengthy acquisition of memories for long term storage, and thus, may be modified differently. As the neural mechanisms for consolidation are not known, a central question addressed here is whether neocortical synapses are modified in a fashion similar to that for synapses in the hippocampus during the early phase of LTP.

All electrophysiological procedures were carried out at the University of Manitoba and all morphological procedures were conducted blind to group designation at Nipissing University. Fourteen adult male Long-Evans hooded rats were supplied by the University of Manitoba breeding colony, were housed individually, and were between 60 and 90 days old at the time of surgery.

Electrophysiological procedures followed the protocol described in Wawryko et al. (2004). Briefly, animals were deeply anesthetized with sodium pentobarbital (65 mg/kg, i.p.), placed in a stereotaxic apparatus, and body temperature maintained at 37 ± 1°C. Surgeries were ordered so that the LTP-stimulated and control animals were randomized. The coordinates used for the stimulating electrode were 0.5 mm anterior to Bregma, 3.0 mm lateral to the midline, and 3.0 mm ventral to the skull surface. The recording electrode was implanted in the homotopic site in the contralateral hemisphere (0.5 mm anterior to Bregma, 3.0 mm lateral to the midline, and 1.8 mm ventral to the surface). Final electrode depths were adjusted to maximize evoked response amplitude. Thus, the stimulating electrode was placed in the white matter directly below the cortex, and the bipolar recording electrode spanned the somatomotor cortex (see Chapman et al., 1998).

Biphasic square wave electrical pulses with 100-μs halfcyles were produced with a Grass S88 stimulator. Baseline-evoked field potentials were collected for 20 min at 0.1 Hz using a stimulus intensity of 500 μA, which produces a robust response amplitude and induces a response of ~60% of asymptotic value in all animals. To construct input/output curves (I/Os), stimulation pulses of increasing intensity were delivered to the stimulating electrode at a frequency of 0.1 Hz. Sweep durations of 50 ms were used to capture waveforms. Five responses were evoked and recorded for each of these intensities: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, and 1,200 μA.

Subsequently, LTP was induced in half of the animals ($n = 7$). One 100-ms train of pulses was delivered at a frequency of 300 Hz at an intensity of 1,000 μA via the stimulating electrode. Baseline-evoked responses were retested at 50 μA at 0.1 Hz for 20 min. This process continued until a total of four pulse trains had been delivered and sampling of an additional 480 evoked field-potentials was completed. Control animals ($n = 6$; one animal died prior to surgery due to the anesthetic) were not stimulated during these times, but retesting of the baseline responses occurred after similar delays as those of the LTP group to ensure that there was not any drift in the evoked responses over time, and to provide unstimulated, but implanted, controls for the anatomical portion of the experiment.

Tissue preparation and morphological procedures followed a protocol similar to that reported in Weeks et al. (2001). All animals, still anesthetized from the LTP procedure, were perfused intracardially 80 min after the initiation of LTP-inducing stimulation with 30 ml phosphate buffered saline followed by fixative (2% paraformaldehyde, 2.5% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.3). Two 1-mm^3 tissue blocks containing layer III cell bodies and apical dendrites were dissected from the sensorimotor cortex (contralateral and ipsilateral to the recording electrode) and embedded. Thick sections (1 μm) were taken from the block to quantify the number of neurons. Ultrathin sections (~80 nm) were then cut and placed on slot grids yielding ~40 ultrathin serial sections per series.

Four randomly selected subregions per ultrathin series from within the terminal area of the apical dendrites from layer III pyramidal neurons were examined in both the LTP and control animals. This was crucial, as Ivanco et al. (2000) found both dendritic and spine changes only in the apical dendrites. Estimates of the number of various subtypes of synapses were determined using an unbiased dissector technique (Kleim et al., 2004). This technique produces an estimate of the number of various types of synapses per neuron independent of tissue volume, assuming that the neuron number and volume do not change between the LTP and control animals. Briefly, neurons were counted by comparing adjacent thick sections, one a reference section and the other a look-up section immediately following it in the series. Neurons were distinguished from glial cells by the presence of a central nucleolus within a pale nucleus. Neurons were sampled (counted) if they were observed in a reference section micrograph within the area limited by an unbiased sampling frame (4,000 μm^2), but not observed in the corresponding look-up section (Q). The total volume for neuronal counting (V_{neur}) was derived as: $V_{neur} = A \times H$ where A is the area of the counting frame and H is the total thickness of the series. Neuron density (N_{neur}) was therefore derived as: $N_{neur} = Q/V_{neur}$.

Synapses were counted by comparing adjacent ultrathin sections. Synapses were identified by the presence of synaptic vesicles, dense material in a presynaptic axon terminal, and an accompanying postsynaptic density (PSD). Synapses were sampled (counted) if they were observed in a reference section micrograph

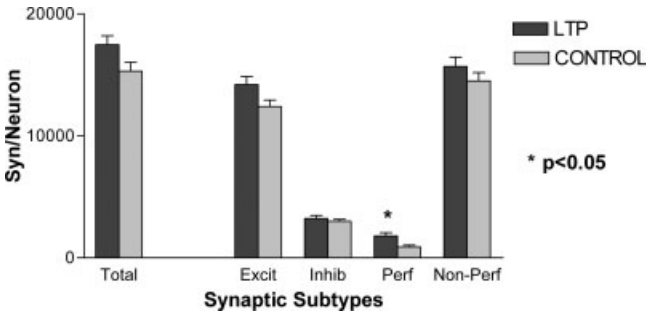

Fig. 1. The total number of synapses per neuron (mean ± SE) and various synaptic subtypes including excitatory, inhibitory, excitatory perforated, and excitatory nonperforated synapses are reported.

within the area limited by an unbiased sampling frame (50 μm^2), but not observed in the corresponding look-up section. Synaptic densities were determined by the equation $(N_{syn})(1/N_{neur})$.

Synaptic quantification included the total number of synapses per neuron, numbers of excitatory and inhibitory synapses, numbers of synapses with various synaptic curvatures, and the number of perforated synapses. Excitatory synapses were identified as having round vesicles and asymmetric pre- and postsynaptic dense material, whereas inhibitory synapses had oval vesicles and symmetric dense material. The synaptic curvature subtypes were identified as follows: concave, a protrusion of the presynaptic terminal into the postsynaptic bouton (spine); convex, a protrusion of the postsynaptic bouton (spine) into the presynaptic terminal; and flat, no noticeable curvature. Perforated synapses are defined here as completely partitioned segments of postsynaptic density, and the maximal length of each synapse was measured using the analySIS imaging system. In the LTP group, synaptic subtypes from individual animals were also correlated with the degree of potentiation that the particular animal expressed at the time of sacrifire. The degree of potentiation was derived from a composite measurement based on a standardized mean of the population-spike amplitudes evoked from the middle 12 intensities (as per Weeks et al., 1998), and the use of multiple correlations required the use of the Bonferoni correction.

Analyses of the electrophysiological data revealed a significant experimental group by intensity interaction for the population-spike amplitudes ($F(6,66) = 2.78$, $P < 0.05$) and for the EPSP amplitude ($F(6,66) = 2.78$, $P = 0.018$). The LTP group showed a significant increase in I/O measures after the delivery of trains ($P = 0.002$), indicating a significant level of LTP over time. Generally, the synaptic analyses yielded a mean of 74 (+13) synapses per animal counted through 34 (+4) dissector pairs and a mean volume of 168 (+7) μm^3. The neuronal sampling resulted in a mean of 122 (+12) neurons counted through 50 (+3) dissector pairs and a mean volume of 3.03×10^5 (2.95×10^4) μm^3. Importantly, the neuronal counts within the specified volume did not change between the LTP and control groups, which allows for the use of synapse per neuron measures. Analysis of the number of synapses per neuron revealed no difference in the total number of excitatory or inhibitory contacts between the LTP and control groups. No significant changes were observed between the ipsilateral and contralateral hemispheres on any of the neuronal or synaptic measures, resulting in the pooling of the data from the two hemispheres for all subsequent analyses.

MANOVA of various synaptic subtypes revealed a significant increase in excitatory perforated synapses in the LTP group ($F(1,12) = 8.23$, $P = 0.015$, see Fig. 1). A significant increase in concave-shaped excitatory synapses was also observed in the LTP animals ($F(1,12) = 6.75$, $P = 0.023$). A combined analysis revealed significantly more excitatory perforated concave synapses per neuron in the LTP group (303, +53) vs. controls (124, +32) ($F(1,12) = 7.53$, $P = 0.019$). In the excitatory perforated flat synapses, the LTP group averaged 1,804 (+333) and the control group averaged 862 (+157) ($F(1,12) = 5.83$, $P = 0.034$). No other significant differences were observed between synaptic subtypes. ANOVA of maximal synaptic length revealed that experimental condition did not interact with the various synaptic types. Thus, synaptic length was not included in the correlational analysis.

Analysis of the correlations between several morphological measures and the electrophysiological results revealed that the number of concave perforated synapses (see Fig. 2) was the only significant correlate of potentiation. Animals that expressed higher levels of potentiation also had more concave perforated synapses ($r = 0.78$, $P < 0.05$, see Fig. 3).

This experiment provides novel evidence for early synaptic change following neocortical LTP. Changes in synaptic ultrastructure occurred in the apical field of layer III sensorimotor neurons within hours of LTP induction. Although overall synaptogenesis was not observed, more excitatory synapses were found to be perforated and to have a concave shape in the LTP animals. Further, the degree of LTP was significantly and positively associated with these concave perforated synapses. It is important to note that the current experimental design did not include a stimulated/no-LTP group. We have previously investigated a stimulated/no-LTP group and found synaptic changes observed following LTP induction are LTP specific and do not result from tetanization alone (Weeks et al., 2003). Thus, inclusion of a stimulated/no-LTP group may not be necessary as the current findings are similar to the LTP-specific synaptic modifications observed in the hippocampus.

Current and past research strongly suggests that synaptic remodeling associated with LTP is dynamic

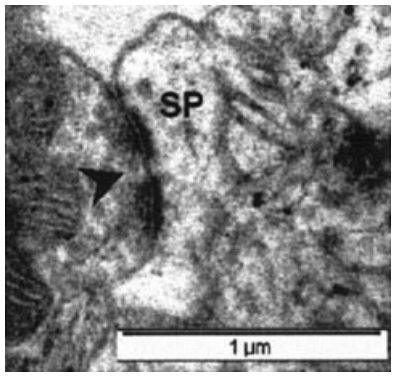

Fig. 2. Electron micrograph of a concave perforated synapse. "Sp" identifies the synaptic spine and the arrow located in the presynaptic terminal shows the concave shape (×20,000).

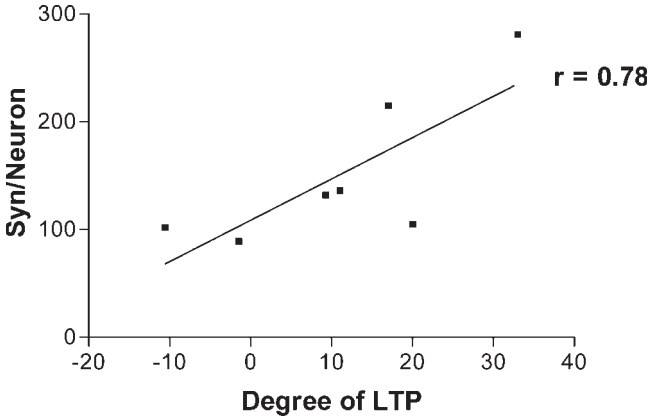

Fig. 3. This plot shows the relationship between the degree of LTP expressed by individual animals and the number of concave perforated synapses per neuron.

and complex, involving alterations in morphological characteristics that are both region- and time-dependent. The current results suggest that the early phase of synaptic plasticity may be common to both the neocortex and hippocampus, since increases in perforated and concave-shaped synapses has been a consistent finding in the hippocampus shortly after the induction of LTP (Geinisman et al., 1991; Weeks et al., 2000). Importantly, computer modeling has suggested that concave perforated synapses may sequester calcium in a way that increases the probability of transmitter release (Ghaffari-Farazi et al., 1999). Further, perforated synapses, in general, are thought to represent large, mature, and highly efficacious synapses that could affect the amount of potentiation observed (Calverly and Jones, 1990; Jones and Harris, 1995). These researchers have theorized that the perforations may allow for a greater proximity of the presynaptic calcium channels to the vesicular release sites, and therefore, a greater probability of transmitter release. Ganeshina et al. (2004) examined perforated and nonperforated synapses using immunogold labeling of AMPA and NMDA receptors. They found that perforated synapses always expressed AMPA receptors, whereas only 64% of nonperforated synapses were AMPA positive. Finally, perforated synapses were found to express more AMPA label than nonperforated AMPA-positive synapses. Ganeshina et al. argue that as a result of these differences in AMPA expression, perforated synapses may evoke larger postsynaptic responses than nonperforated synapses.

Although caution should be taken when comparing synaptic change following LTP with changes observed during a learning task, the current findings appear to fit with those of Kleim et al. (2004), who did not find synatogenesis during the early stages of acquiring a motor task. Thus, results from the neocortex suggest a logical time-sensitive cascade of plastic synaptic change following neural stimulation or learning that begins with morphological alterations and ends with more synapses.

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