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Tetanus-induced LTD of Developing MNTB-LSO Synapses in Rat is Dependent on Postsynaptic Ca^{2+}

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Because synaptic refinement of medial nucleus of trapezoid body (MNTB) - lateral superior olive (LSO) synapses is most active during the first postnatal week and the long term depression (LTD) has been suggested as one of its mechanisms, LTD of MNTB-LSO synapses was investigated in neonatal rat brain stem slices with the whole cell voltage clamp technique. In Mg^{2+} free condition, tetanus (10 stimuli at 10 Hz for 2 min) in the current clamp mode induced a robust LTD of isolated D, L-APV-sensitive postsynaptic currents (PSCs) for more than 30 min (n=6, 2.4±0.4% of the control), while isolated CNQX-sensitive PSCs were not suppressed (n=6, 95.3±1.6%). Tetanus also elicited similar LTD in the isolated GABAergic/glycinergic PSCs (n=5, 3.6±0.5%) and mixed PSCs (GABAergic/glycinergic/glutamatergic) (n=4, 2.2±0.7%). However, such a strong LTD was not observed in the mixed PSCs when 10 mM EGTA was added in the internal solution (n=10), indicating that postsynaptic Ca^{2+} rise is needed for the strong LTD. This robust LTD might contribute to the active synaptic refinement occurring during the first postnatal week.

Key Words: MNTB-LSO, GABAergic, Glycinergic, Glutamatergic, Synaptic refinement, LTD, Rat

INTRODUCTION

The LSO, a binaural auditory brainstem nucleus, receives inhibitory GABAergic/glycinergic input from the contralateral ear via MNTB and excitatory glutamatergic input from the ipsilateral ear via the ventral cochlear nucleus (Boudreau & Tsuchitani, 1968; Oertel, 1999). However, the neurotransmission of the immature MNTB-LSO synapses before hearing onset is quite different from that of the mature MNTB-LSO synapses. While the mature MNTB-LSO synapses are purely glycinergic (Caspary & Finlayson, 1991), the MNTB-LSO synapses in neonates are both GABAergic and glycinergic (Kotak et al, 1998; Korada & Schwartz, 1999). Furthermore, the mature MNTB-LSO synapses are hyperpolarizing, whereas the immature MNTB-LSO synapses are depolarizing (Ehrlich et al, 1999). Immature MNTB-LSO synapses release even excitatory neurotransmitter glutamate during the first postnatal week, which acts on NMDA receptor (Gillespie et al, 2005).

MNTB-LSO synapses undergo synaptic refinement during the first postnatal week, characterized by active synaptic elimination of overproduced synapses at birth and strengthening of the maintained synapses (Kim & Kandler, 2003). LTD has been suggested as one of possible mechanisms for this synaptic refinement (Kotak & Sanes, 2000). Many conditions are favorable to LTD during the first postnatal week. First, MNTB-LSO synapses (GABAergic/glycinergic) are depolarizing due to high concentration of $[Cl^-]_{int}$ (Ehrlich et al, 1999), which would elicit Ca^{2+} increase (Kullmann et al, 2002). In other systems, this Ca^{2+} increase has been shown to elicit LTD or LTP at GABAergic or glycinergic synapses (Oda et al, 1995; Morishita & Sastry, 1996; Caillard et al, 1999; Kotak & Sanes, 2000). Second, glutamate is released from MNTB-LSO synapses during this period (Gillespie et al, 2005), which would act synergistically with depolarizing synapses for Ca^{2+} increase. Since NMDA- or mGluR-dependent synaptic plasticities have been reported in many systems, both depolarizing state and glutamate release from MNTB-LSO synapses may open the possibility of synaptic refinement by synaptic plasticity.

As described above, LTD has been reported to be a possible mechanism for the MNTB-LSO synaptic refinement in gerbil (Kotak & Sanes, 2000), however, the report shows the presence of LTD of MNTB-LSO synapses in gerbils aged over P7, therefore, not necessarily providing enough explanation for the early onset of synaptic refinement. Moreover, it does not show the LTD of each component of the post synaptic currents (GABAergic/glycinergic or glutamatergic), because the glutamatergic PSCs were not known in MNTB-LSO synapses at that time. In this study, a question of whether LTDs of each component of PSCs were induced in MNTB-LSO synapses of neonatal rats younger than P7 was examined with a help of specific blockers of neurotransmitters.

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METHODS

Neonatal rats of both genders, aged between P1~P7 (Sprague-Dawley, Central Lab. Animal Inc, Seoul, Korea), were used. Experimental procedures were in accordance with NIH guidelines and approved by the Internal Review Board of Dankook University Hospital. After deep anesthesia with isoflurane, brains were removed, placed in ice-cold artificial cerebrospinal fluid (aCSF) with 1 mM kynurenic acid, and 300 μm thick coronal slices were cut with a vibratome (LEICA VT1000s, LEICA Microsystems, Heidelberger, Germany). Slices were allowed to recover for at least 30 min in an interface chamber before recording. Slices were transferred to a submersion-type chamber mounted on an upright microscope and were continuously perfused with aCSF, containing (in mM) NaCl (124), Mg_2SO_4 (1.3), KCl (5), KH_2PO_4 (1.25), glucose (10), $NaHCO_3$ (26), $CaCl_2$ (2). $Mg2SO_4$ was omitted in Mg^{2+}-free condition.

LSO inputs were stimulated by low resistance electrode (< 2 MΩ filled with aCSF) positioned at the lateral end of the MNTB (Master 8 and Isoflex, A.M.P.I., Israel). Whole cell patch clamp recordings were obtained from visualized principal type LSO neurons which were identified by their bipolar morphology. Electrodes (1~3 MΩ) were filled with solution containing (in mM) K-gluconate (42), EGTA (0.6), KCl (91), HEPES (20), Na_2GTP (0.3), Na_2ATP (1), KOH (12), and QX 314 (5 mM). When high concentration of EGTA was used, internal solution contained (in mM) KCl (91), EGTA (10), K-gluconate (22), HEPES (20), Na_2GTP (0.3), KATP (1), KOH (35), sucrose (5), and QX 314 (5 mM). All chemicals except QX 314, CNQX and bicuculline (Tocris) were purchased from Sigma Chemicals Co., unless otherwise noticed. Osmolarity was adjusted with sucrose (around 285 mOsm) (The Advanced^{TM} Micro Osmometer Model 3300, ADVANCED INSTRUMENTS, INC, USA). One mM $MgCl_2$ was added or omitted depending on the purpose of the experiment. Data were filtered at 5 kHz (EPC-8, HEKA, Germany), digitized at 10 kHz and stored on a computer using home-made program (R-clamp 1.23).

For a tetanic stimulation, MNTB was stimulated with trains of 10 stimuli at 10 Hz for 2 min. The intensity of stimulus was set to 3 times the threshold of the PSC. The same stimulus intensity was used for both control and tetanic stimulation. LSO cells were held in the voltage clamp mode (V_{hold}=−60 mV) during recording, except for the tetanic stimulation period, during which LSO cells were held in the current clamp mode (I=0). During control and post-tetanic periods, MNTB was stimulated at 0.05 Hz, and PSCs were recorded for 10 min for control and for at least 30 min for post-tetanic periods. Series resistance was not compensated, but monitored throughout experiments. If change of mean series resistance at 20~30 min post tetanus was greater than 25% of that of the first 10 min before tetanus, data were discarded.

Analysis of electrophysiological data and statistical tests were performed with Clampfit 9.2 (Molecular Devices), Origin 7.0 (OriginLab Corporation). Data are expressed throughout the text as mean±standard error of the mean.

RESULTS

LTD of glutamatergic PSCs

Whole cell voltage clamp recordings were made from LSO neurons in acute brain slices obtained from P1~P7 rats. To isolate the D, L-APV-sensitive PSC, strychnine (10 μM), a glycine receptor blocker, bicuculline (10 μM), a GABA receptor blocker, and CNQX (10 μM), an AMPA receptor blocker in Mg^{2+}-free solution were perfused from the beginning of experiments. The internal solution was also Mg^{2+} free. In this condition, electrical stimulation of MNTB produced inward currents in LSO neurons (V_{hold} = −60 mV) which was sensitively blocked by 10 μM D, L-APV (data not shown). After 10 min of recording of D, L-APV-sensitive PSCs which were elicited by stimulation of MNTB at 0.05 Hz, tetanic stimulation given in the current clamp mode (I=0) (10 stimuli at 10 Hz for 2 min) produced an almost complete suppression of the PSCs in 6 out of 7 cells tested (Fig. 1A, B). The mean PSC at 20~30 min post tetanus was 2.4±0.4% of the control, which was statistically significant (t-test, n=6).

As CNQX-sensitive current has also been reported to exist in developing rat MNTB-LSO synapses (Gillespie et al, 2005), the possibility of LTD of the CNQX-sensitive current by tetanic stimulation was also tested. After perfusion with strychnine (10 μM), bicuculline (10 μM) and D, L-APV (10 μM) in Mg^{2+}-free solution, MNTB stimulation produced a fast, rapidly decaying inward current (V_{hold}=−60 mV) which was sensitive to 10 μM CNQX (data not shown). This CNQX-sensitive current did not show significant suppression (6 out of 6 cells tested) after tetanic stimulation given in the current clamp mode (I=0) (10 stimuli at 10 Hz for 2 min) (Fig. 1C, D). The mean PSC at 20~30 min post tetanus was 95.3±1.6% of the control, which was not statistically significant (t-test, n=6).

LTD of GABAergic/glycinergic PSCs

To isolate the GABAergic/glycinergic PSCs, D, L-APV (10 μM) and CNQX (10 μM) in Mg^{2+}-added aCSF were used for perfusion from the beginning of experiments. Mg^{2+} was also added to the internal solution. To rule out the possible contribution of mGluR in the generation of LTD, 1 mM (S)-alpha-methyl-4-carboxyphenylglycine (MCPG), a blocker of mGluR, was also added. In this condition, inward currents produced by MNTB stimulation were sensitively blocked by the mixture of strychnine and bicuculline (10 μM each) (data not shown). After 10 min of recording of strychnine, bicuculline-sensitive PSCs, tetanic stimulation given in the current clamp mode (I=0) (10 stimuli at 10 Hz for 2 min) produced an almost complete suppression of the PSCs in 5 out of 10 cells tested (Fig. 2). The mean PSC at 20~30 min post tetanus was 3.6±0.5% of the control (n=5).

LTD of mixed PSCs

To find out the presence of LTD of mixed PSCs (glutamatergic and GABAergic/glycinergic), electrical stimulation

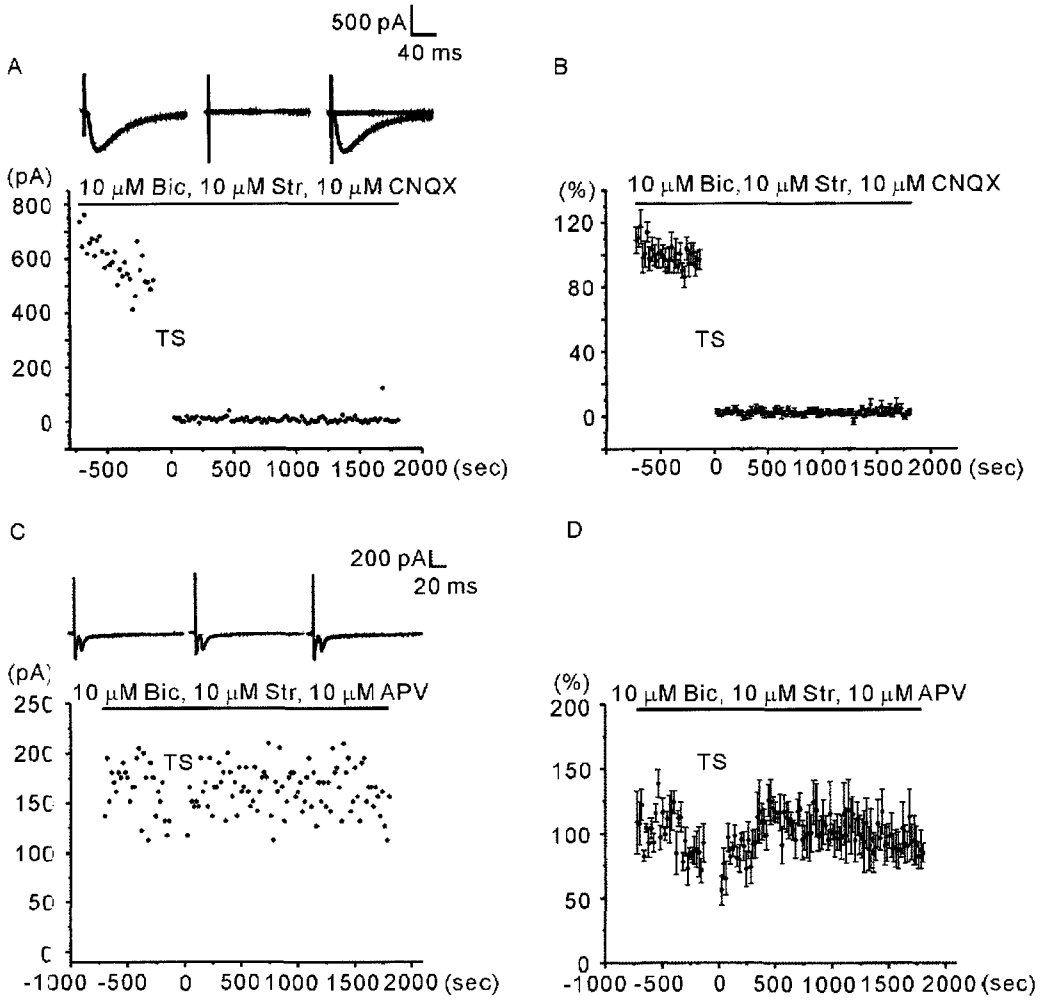

Fig. 1. The peak amplitudes of the D, L-APV-sensitive PSCs are plotted against time (A). Tetanic stimulation (TS) for 2 min (10 stimuli at 10 Hz) suppressed D, L-APV sensitive PSCs almost completely for 30 min after 10 min of recording of PSC (A). Tetanic stimulation was given while the postsynaptic cell was in the current clamp condition (I=0). Bicuculline (Bic, 10 μM), strychnine (Str, 10 μM) and CNQX (10 μM) were present in the bath. Mg^{2+} was absent in both intracellular and extracellular sides. The upper left inset over A is an average PSCs at 10 min before tetanus, and the middle one is average PSCs at 20~30 min post tetanus. The right figure is the overlap of two figures. The vertical bars in the insets are the stimulus artifacts. The percent changes of the peak amplitudes against time are shown in B (n=6). The effects of tetanus on CNQX-sensitive PSCs are shown in (C), and (D). Tetanic stimulation (TS) was given in the presence of bicuculline (Bic, 10 μM), strychnine (Str, 10 μM) and D, L-APV (APV, 10 μM), which did not produce any significant changes of PSCs (C, D). The upper left inset over C is an average PSCs at 10 min before tetanus, and the middle one is an average of PSCs at 20~30 min post tetanus. The right one is the overlap of two figures. The percent changes of the peak amplitudes against time are shown in D (n=6).

of MNTB was given without any blockers added in the bath. Both internal and external solutions were Mg^{2+} free. After 10 min of recording of mixed PSCs, tetanic stimulation given in the current clamp mode (I=0) (10 stimuli at 10 Hz for 2 min) produced also an almost complete suppression of the PSCs in 4 out of 9 cells tested (Fig. 3A, B), and the mean PSC at 20~30 min post tetanus was 2.2±0.7% of the control (n=4).

As tetanic stimulation provided a favorable condition for intracellular Ca^{2+} increase, involvement of Ca^{2+} in LTD induction was studied. To elucidate the dependency of LTD on Ca^{2+}, 10 mM EGTA was included in the internal solution of recording electrode. In that condition, tetanic stimulation did not induce LTD in 10 out of 10 cells tested (Fig. 3C, D, n=10), and the mean PSC at 20~30 min post tetanus was 116.0±3.0% of the control.

DISCUSSION

In the present study, MNTB-LSO synapses were shown to undergo strong LTD by the tetanic stimulation. The LTD presented in this study was quite strong in the extent of depression, and this kind of strong LTD has rarely been

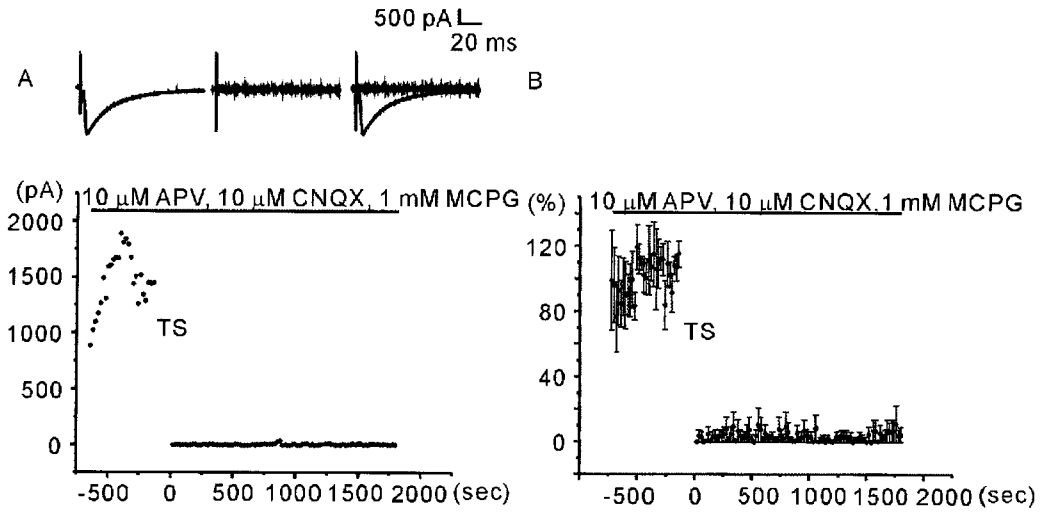

Fig. 2. The peak amplitudes of the GABAergic/glycinergic PSCs are plotted against time (A). Tetanic stimulation (TS) for 2 min (10 stimuli at 10 Hz) suppressed GABAergic/glycinergic PSCs almost completely for 30 min after 10 min of recording of PSC (A). Tetanic stimulation was given while the postsynaptic cell was in the current clamp condition (I=0). D, L-APV (APV, 10 μM), CNQX (10 μM), and MCPG (1 mM) were present in the bath. Mg^{2+} was absent in both intracellular and extracellular sides. The upper left inset is the average PSCs at 10 min before tetanus and the middle one is the average PSCs at 20~30 min post tetanus. The right one is the overlap of two figures. The vertical bars in the insets are stimulus artifacts. The percent changes of the peak amplitudes against time are shown in B (n=5).

reported so far. One of the possible explanations for this strong LTD might be presynaptic axonal damage by tetanic stimulation. However, this possibility could be ruled out, because it should occur regardless of the presence of EGTA in the recording pipette if LTD presented in this paper were the result of axonal damage. However, it was not the case. There might be another possibility of postsynaptic neuronal damage, which might have been caused by postsynaptic Ca^{2+} increase by tetanic stimulation. However, even though very rare, recovery of the postsynaptic currents was observed in some cells after a long period of strong LTD (data not shown), thus, indicating that this strong LTD was not due to pathologic changes of pre- or postsynaptic neurons.

The mechanism of LTD induction may lie at the postsynaptic site. The fact that LTD induction was not observed in the presence of 10 mM EGTA in the internal solution supports this idea. Some studies to support the role of postsynaptic Ca^{2+} in LTD have been reported: 10 Hz-induced LTD in rat deep cerebellar nuclei was reduced by phosphatase inhibitors, such as microcystin LR or okadaic acid (Morishita & Sastry, 1996), calcineurin, a Ca^{2+}-dependent phosphatase, was needed in LTD-induction in mice CA1 inhibitory synapse (Wang et al, 2003), and Ca^{2+} dependent process in LTD was also reported in the auditory system. According to previous reports on gerbil MNTB-LSO, suggesting a postsynaptic site of LTD expression, MNTB-LSO LTD required postsynaptic activation of $GABA_B$, tyrosine kinase receptors, tyrosine kinases, protein kinases, and also an increase of intracellular [Ca^{2+}] (Kotak & Sanes, 2000; Kotak et al, 2001; Kotak & Sanes, 2002).

However, presynaptic mechanisms can not be completely ruled out. The retrograde messenger, such as endocannabinoid, released from postsynaptic site can also produce the similar LTD. In this study, moderate frequency stimulation (10 stimuli at 10 Hz for 2 min) was used to induce LTD. The fact that endocannabinoid generated by moderate frequency stimulation induces LTD in rat striatum (Ronesi & Lovinger, 2005) raises the possibility of involvement of endocannabinoid in LTD of MNTB-LSO synapses. As the endocannabinoid related LTD has been reported in many systems such as striatum (Gerdeman et al, 2002), nucleus accumbens (Hoffman et al, 2003), neocortical layer 5 pyramidal neuron pairs (Sjostrom et al, 2003), basolateral amygdala (Marsicano et al, 2002), and hippocampus (Chevaleyre & Castillo, 2003), the possible involvement of the retrograde messenger should be investigated in future.

Postsynaptic [Ca^{2+}] might be increased during tetanic stimulation, because postsynaptic membrane potential changed freely during tetanus because LSO cells were held in the current clamp mode. The possible routes of Ca^{2+} are VOCC (voltage operated Ca^{2+} channel) (Christie et al, 1995; N'Gouemo & Rittenhouse, 2000; Normann et al, 2000; Kullmann et al, 2002), ionotropic glutamate receptors (Waters & Allen, 1998; Fischer et al, 2002), and increase of intracellular Ca^{2+} secondary to mGluR activation (Fagni et al, 2000; Ene et al, 2003; Rae & Irving, 2004). Among them, AMPA receptor might not be a proper Ca^{2+} route to induce LTD in developing rat MNTB-LSO synapses as CNQX-sensitive PSCs did not show LTD. Although the percentage of the AMPA PSCs in the whole glutamatergic PSCs was not measured in this study, it might be small compared to that of NMDA PSCs, because it has been reported that PSCs through NMDA receptor contributes over 80% of the whole glutamatergic synaptic charges (Gillespie et al, 2005). Thus, the failure of LTD induction of CNQX-sensitive PSCs might be due to not enough increase of intracellular Ca^{2+} through the AMPA receptors. Except AMPA receptor, the extent of contribution of each Ca^{2+} routes in the induction of LTD was not determined in this study. Some cells show LTD when GABA/glycine/AMPA

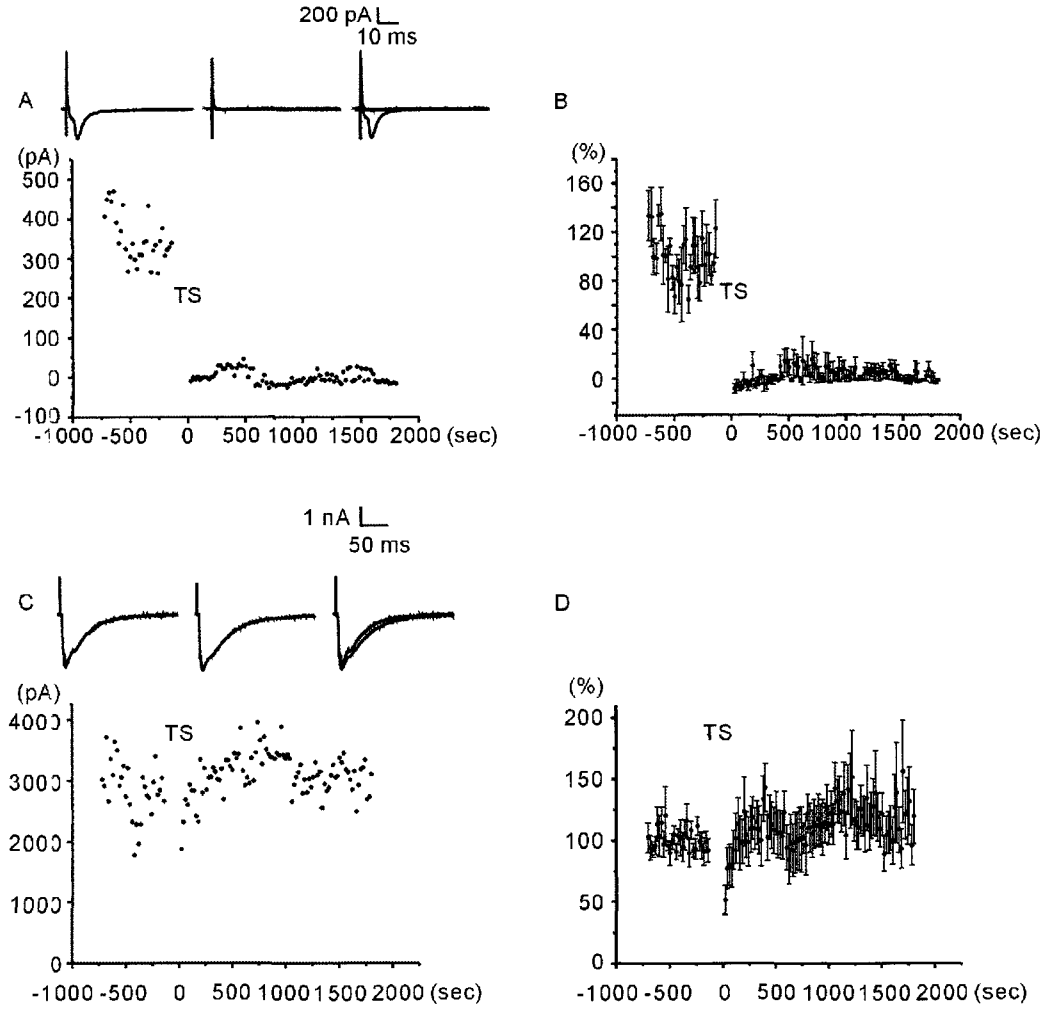

Fig. 3. The peak amplitudes of mixed (GABAergic/glycinergic/glutamatergic) PSCs were plotted against time (A). Tetanic stimulation (TS) for 2 min (10 stimuli at 10 Hz) suppressed mixed PSCs almost completely for 30 min after 10 min of PSC recording (A). Tetanic stimulation was given while the postsynaptic cell was in the current clamp condition (I=0). The upper left inset over A is an average PSCs at 10 min before tetanus and the middle one is the average of PSCs at 20~30 min post tetanus. The right one is the overlap of two figures. The vertical bars in the insets are stimulus artifacts. The percent changes of the peak amplitudes against time are shown in B (n=4). However, the strong LTD of the mixed PSCs was not observed, when 10 mM EGTA was added to an internal solution (C, D).

receptors are blocked, whereas other cells show LTD when NMDA/AMPA/mGlu receptors are blocked. The contribution of each Ca^{2+} route in the induction of LTD might be dependent on its specific developmental stage of MNTB-LSO synapses.

The functional mapping study of developing MNTB-LSO synapses revealed a fourfold decrease in the MNTB-LSO convergence ratio and a 12-fold increase in the synaptic conductance of individual maintained MNTB inputs (Kim & Kandler, 2003). According to the study just described, LTD presented in the present study might not be enough to explain the whole synaptic refinement process during the first postnatal week, because this LTD can explain only synapse elimination. The other mechanism, such as LTP, seems to exist to explain the synaptic strengthening of MNTB-LSO synapses.

Spontaneous synaptic activity has been suggested to be responsible for the synaptic refinement (Kotak & Sanes, 1996). Unfortunately, however, the pattern of spontaneous synaptic activity is not yet known in developing rat MNTB-LSO synapses. Investigation of spontaneous synaptic activity might help us understand the significance of this robust LTD, because results imply that functionally active synapse would fall in LTD and be eliminated more easily.

With identical experimental conditions, I was able to observe similar strong LTD in rats older than P8. However, a question of whether similar LTD really occurs in vivo after P8 needs to be further investigated, because the environmental conditions are not favorable for LTD induction after P8: MNTB-LSO synapses are hyperpolarizing and glutamate co-release decreases, suggesting that the strong

LTD presented in this study might not play a crucial role in synaptic refinement in hyperpolarizing period.

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