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Developmental Changes in Presynaptic Calcium Channels Coupled to Glutamate Release in Cultured Rat Hippocampal Neurons

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Excitatory synaptic transmission in the hippocampus involves the participation of at least two types of presynaptic Ca^{2+} channels, N-type channels sensitive to ω-conotoxin GVIA (ω-CTx GVIA) and Q-type channels sensitive to ω-agatoxin IVA (ω-Aga IVA). Hippocampal pyramidal neurons in cell culture were used to examine the participation of these two classes of channels at different stages of synapse development. Specific Ca^{2+} channel toxins were used to block presynaptic Ca^{2+} channels while whole-cell voltage-clamp recordings were used to record evoked EPSCs in postsynaptic neurons. At immature synapses (cells in culture for 10–15 d), ω-CTx GVIA (1–5 μM) blocked transmission by more than 80% while ω-Aga IVA (1 μM) was less effective. In older cultures, however, ω-Aga IVA (1 μM) was more effective than ω-CTx GVIA (1–5 μM) in blocking synaptic transmission. The pharmacological properties of the ω-Aga IVA sensitive component of synaptic transmission were examined in more detail using ω-Aga IVA and ω-conotoxin MVIIC (ω-CTx MVIIC). The properties of this component of transmitter release indicated that a Q-type Ca^{2+} channel was involved in presynaptic Ca^{2+} entry. The results suggest that different classes of presynaptic Ca^{2+} channels begin to participate in transmitter release at different times during synapse development and maturation.

[Key words: ω-conotoxin GVIA, ω-conotoxin MVIIC, ω-agatoxin IVA, glutamate, EPSC, presynaptic calcium channel]

In the CNS of many lower vertebrates, presynaptic Ca^{2+} channels are blocked by ω-conotoxin GVIA (ω-CTx GVIA) (Rivier et al., 1987; Venema et al., 1992), a well-characterized peptide component of cone snail venom (Olivera et al., 1991). In mammals, however, ω-CTx GVIA blocks only a minor fraction of transmission at many central synapses (Reynolds et al., 1986; Cruz et al., 1988; Mintz et al., 1992a). The major fraction is blocked by another Ca^{2+} channel toxin, ω-agatoxin IVA (ω-Aga IVA) (Turner et al., 1992; Takahashi and Momiyama, 1993). For example, synaptic transmission in the rat hippocampus is partially inhibited by ω-CTx GVIA (Kamiya et al., 1988; Dutar et al., 1989; Horne and Kemp, 1991; Parfitt and Madison, 1993; Castillo et al., 1994; Wheeler et al., 1994), although ω-Aga IVA is a more effective blocker (Luebke et al., 1993; Takahashi and Momiyama, 1993; Castillo et al., 1994; Wheeler et al., 1994).

The hippocampus has been an important structure for the study of neurotransmitter action and synaptic plasticity, including facilitation and long-term potentiation (LTP). Specific forms of synaptic plasticity in the rat hippocampus have been shown to develop during a critical period around 1–2 weeks after birth (Harris and Teyler, 1984; Muller et al., 1989; Bekenstein and Lothman, 1991). Knowledge of changes in the molecular components of synapses during differentiation of hippocampal neurons is likely to provide key insights into the function of the synapse and mechanisms of plasticity. Because Ca^{2+} channels are critical for triggering the release of neurotransmitter, developmental changes in the properties or identity of the presynaptic Ca^{2+} channel will have significant effects on the properties of synaptic transmission and plasticity. This report describes the contribution of different Ca^{2+} channel subtypes to excitatory synaptic transmission between cultured hippocampal pyramidal neurons at different stages of synapse development.

Materials and Methods

Cell culture. The techniques used for the preparation of cell cultures and characterization of the cultures have been described previously (Bartlett and Banker, 1984; Scholz et al., 1988; Scholz and Miller, 1991). The only difference in the culture technique was that the poly-L-lysine used as a substrate for cell adhesion to the glass coverslips was conjugated to the glass in the present study (Aplin and Hughes, 1981). This was accomplished by evaporation of 3-aminopropyltriethoxysilane onto the coverslips in a vacuum chamber overnight. This was followed by submersion of coverslips in 5% glutaraldehyde for 2–5 min and four washes in distilled water. Coverslips were then autoclaved and placed in sterile petri dishes. Fresh poly-L-lysine (0.05–0.1% in borate buffer) was then applied and allowed to react overnight. Criteria used for identification of pyramidal neurons have been described previously (Scholz and Miller, 1991). After the cells had grown for 15 d in culture, the media was supplemented with 1 mM kynurenic acid and 10 mM $MgCl_2$. These conditions provided optimal survival of neurons beyond this time (Furshpan and Potter, 1989).

Some of the experiments were performed on coverslips that had a growth-supporting substrate applied in a pattern to isolate single cells or pairs of cells. Patterns were constructed by a method derived from previously described techniques (Kleinfeld et al., 1988; Rohr et al., 1991). KTFR negative photoresist (PC and E, Frazer, PA; 1 part resist:2 parts thinner) was applied to the coverslips and allowed to flow to a relatively thin layer. The use of negative photoresist obviated the need to create a microscopically thin layer. The photoresist was allowed to dry and then exposed to UV light through a film mask made from Kodak LPD4 precision-line film. The coverslips were developed in KTFR developer, which removes unexposed resist. Coverslips were then exposed to 3-aminopropyltriethoxysilane as described above. The resist pattern was then removed by sonicating in xylene. From this point, the coverslips were treated with glutaraldehyde and poly-L-lysine as described above. The next day, the coverslips were washed three times in

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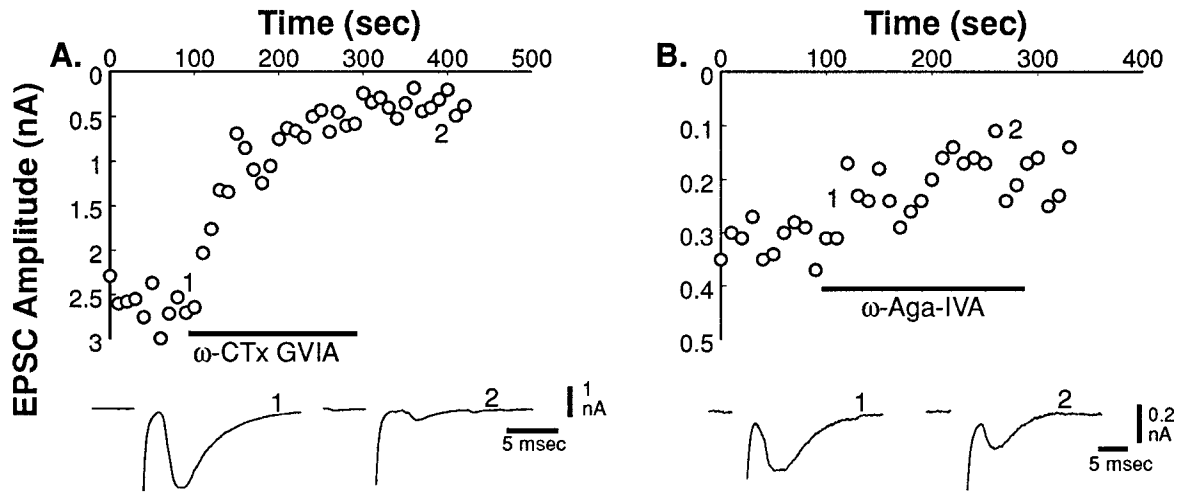

Figure 1. Block of EPSCs by Ca^{2+} channel toxins in immature neurons. *A*, Time course of block of the EPSC by ω-CTx GVIA (2.5 μM) recorded from a cell in culture for 10 d. (*Inset*) EPSCs recorded at the times indicated. (*B*) Effect of ω-Aga IVA (1 μM) on the EPSC recorded from a cell in culture for 14 d.

1 M NaCl, pH 7.5 to remove residual polylysine that was not conjugated to the surface.

Electrophysiology. Whole-cell voltage-clamp recordings were obtained from a postsynaptic neuron while a bipolar tungsten electrode was used to stimulate a presynaptic neuron. For autaptic connections, the extracellular electrode stimulated the same cell from which the whole-cell recording was obtained. For monosynaptic connections between cell pairs, the extracellular electrode stimulated a presynaptic cell from which no recording was obtained. Because in most cases there was only one presynaptic neuron, there was no ambiguity regarding the efficacy of the presynaptic stimulus. In these cases, the postsynaptic neuron was voltage clamped at a depolarized holding potential (−30 mV) to inactivate Na^+ channels and prevent the postsynaptic neuron from firing.

For recordings of EPSCs the bath contained (in mM) NaCl, 143; KCl, 3; $CaCl_2$, 1; $MgCl_2$, 2; HEPES, 10; glucose 10 at pH 7.35 and the pipette contained (in mM) K-fluoride, 140; $MgCl_2$, 1; EGTA, 10; HEPES, 10 at a pH of 7.15. Intracellular fluoride was chosen as the major intracellular anion since it has been shown to reduce activation of postsynaptic voltage-gated currents, thereby allowing for a more faithful recording of EPSCs (Kay et al., 1986). Pipettes had resistances ranging from 2.0 to 3.5 MΩ. Series resistance was <10 MΩ and was corrected by 60%, typically. Currents were recorded by a List EPC7 patch-clamp amplifier, filtered at 3 kHZ and digitized at 10 kHZ. Data collection and analysis were performed by custom written programs based on Indec C-LAB subroutines (Scholz and Miller, 1991). Experiments with unstable baseline responses or obvious contamination from polysynaptic responses were discarded. EPSC amplitudes were measured from the peak of the EPSC to the baseline before the stimulus. In each case, the amplitude of 10 EPSCs was averaged for each condition. ω-CTx GVIA was obtained from Peninsula Labs (Belmont, CA) and Bachem Bioscience (Philadelphia, PA). ω-Aga IVA was obtained from Peptides International (Louisville, KY) and from Pfizer (Groton, CT) through Bachem Biosciences. ω-CTx MVIIC was a gift from Neurex Corp. All experiments were performed at room temperature (21–24°C) and in the continuous presence of 0.5 mg/ml chicken egg albumin (Sigma grade V; a normal component of the cell culture media) in order to block low-affinity binding sites. All toxins were applied by bath superfusion.

Results

The experiments were performed on hippocampal pyramidal neurons cultured from embryonic rats at 17 d gestation. Cultures prepared at this point in development are highly enriched in pyramidal neurons (Scholz and Miller, 1991). Some of the experiments were performed on coverslips that had a growth-supporting substrate applied in a pattern to isolate single cells or pairs of cells (see Materials and Methods). This procedure permitted experiments to be performed on cells that received synaptic input from only one presynaptic neuron. In other cases, cells were plated very sparsely (approximately 4 cells/mm²). Autaptic connections (cf. Bekkers and Stevens, 1991; Segal, 1991; Pan et al., 1993), as well as monosynaptic connections between cell pairs, were used for these studies; the results were indistinguishable. From previous work on this preparation (Scholz and Miller, 1991, 1992), it is known that synapses can be detected by electrophysiological methods beginning around day 7 in culture (corresponding roughly to postnatal day 2–3). By 9–10 d in culture, excitatory postsynaptic currents (EPSCs) can be observed readily and increase in prevalence and amplitude for several more days.

ω-CTx GVIA is a peptide first isolated from the venom of the cone snail *Conus geographus* (Olivera et al., 1991). It is well characterized as a selective and high affinity blocker of N-type Ca^{2+} channels (Williams et al., 1992; Dubel et al., 1992; Fujita et al., 1993). In the rat hippocampus, ω-CTx GVIA partially blocks synaptic transmission at Schaffer collateral synapses (Kamiya et al., 1988; Dutar et al., 1989; Horne and Kemp, 1991; Parfitt and Madison, 1993; Castillo et al., 1994; Wheeler et al., 1994). When they have been compared, ω-CTx GVIA was found to be a less effective blocker than ω-Aga IVA at Schaffer collateral synapses (Luebke et al., 1993; Wheeler et al., 1994). In contrast, in this study ω-CTx GVIA (1–5 μM) was found to be a very effective blocker of synaptic transmission between immature cultured hippocampal pyramidal neurons (Fig. 1*A*). In cells that were in culture for 10–15 d inclusive, ω-CTx GVIA blocked EPSCs by 86 ± 5% (n = 7; mean ± SEM).

ω-Aga IVA is another peptide Ca^{2+} channel toxin first isolated from the venom of the funnel web spider *Agelenopsis aperta* (Mintz et al., 1992a). This toxin was initially characterized as a blocker of P-type channels and transmitter release in the mammalian CNS with an affinity for P-type channels on the order of 2 nM (Mintz et al., 1992a,b). ω-Aga IVA shows no blocking activity at N-type channels (or non-Ca^{2+} channels) at concentrations of 1 μM or below (Mintz and Bean, 1993; Wheeler et al., 1994). Recent work has concluded that ω-Aga IVA may also block another class of channels, termed Q-type Ca^{2+} channels, with somewhat lower affinity (IC_{50} of 100–1000 nM; Sather et

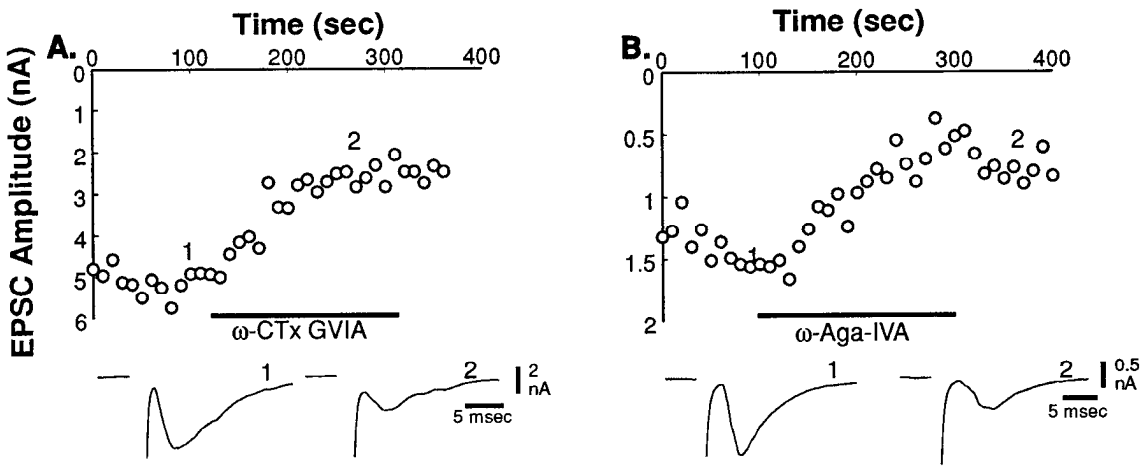

Figure 2. Block of EPSCs by Ca^{2+} channel toxins at a more advanced stage of synapse development. *A*, Effect of ω-CTx GVIA (2.5 μM) on the EPSC recorded from a cell in culture for 23 d. *B*, Effect of ω-Aga IVA (1 μM) on the EPSC recorded from a cell in culture for 21 d.

al., 1993). Such Q-type Ca^{2+} channels have been proposed to be responsible for the majority of Ca^{2+} influx that triggers transmitter release at Schaffer collateral synapses, since the kinetics of block by ω-Aga IVA at this synapse are slower than for block of P-type channels (Wheeler et al., 1994;, see also Wu and Saggau, 1994). Figure 1*B* shows that ω-Aga IVA (1 μM) was a much less effective blocker of synaptic transmission in immature neurons as compared to previous reports using adult synapses. The efficacy of ω-Aga IVA (1 μM) in blocking transmission between pyramidal neurons grown in culture for 10–15 d, inclusive, averaged 32 ± 9% (n = 8). The source of variability in the effects of ω-Aga IVA observed at this age is not clear.

As the neurons developed in culture, the efficacy by which ω-CTx GVIA blocked synaptic transmission decreased (Fig. 2*A*), while that of ω-Aga IVA increased (Fig. 2*B*). After the cells were in culture for 21 d or more, ω-CTx GVIA blocked EPSCs by 30 ± 5% (n = 9). This effect was significantly less than at 15 d or earlier (two-tailed Mann-Whitney test, $p < 0.001$). In contrast, during this same time period ω-Aga IVA blocked EPSCs by 62 ± 4% (n = 10). This effect was significantly greater than the effect of ω-Aga IVA at 15 d or earlier (two-tailed Mann-Whitney test, $p < 0.05$).

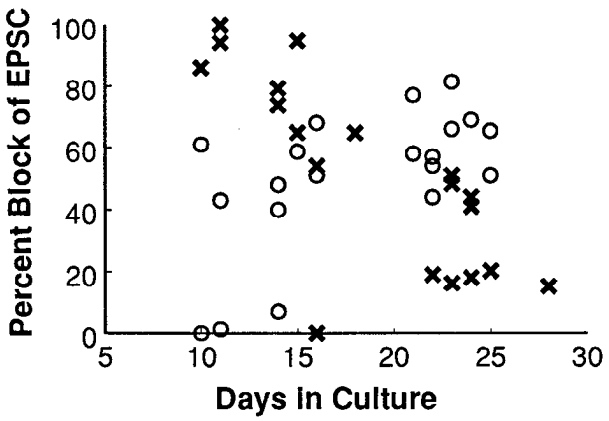

Figure 3. Summary of results showing block of EPSCs by Ca^{2+} channel toxins at different times in development. *X*, Percent block of the EPSC by ω-CTx GVIA (1–5 μM) for each cell plotted as a function of the time spent growing in culture. *O*, Percent block of the EPSC by ω-Aga IVA (1 μM) for each cell. Each symbol represents a different cell. The extrapolated day of birth corresponds roughly to day 4–5 in culture.

Figure 3 summarizes the effects of the two toxins in cultured neurons of different ages. Notice that in immature neurons (e.g., before 15 d in culture), ω-CTx GVIA was a more effective blocker of synaptic transmission than ω-Aga IVA. In contrast, later in development (e.g., after 21 d) ω-Aga IVA was the more effective blocker. These results suggest that N-type Ca^{2+} channels are important for transmitter release at an early stage of synapse development. As the cells mature, a second type of Ca^{2+} channel is incorporated into the transmitter release pathway and eventually plays a dominant role there. This second type of channel is blocked by 1 μM ω-Aga IVA.

The change in presynaptic Ca^{2+} channel is unlikely to be due to treatment of cells with kynurenic acid or 10 mM Mg^{2+} because it was already underway by day 15 (see Fig. 3) and because it could be observed in cells that survived up to day 18 without such treatment (data not shown). Furthermore, in three cells that were not treated with kynurenic acid or elevated Mg^{2+} and from which recordings were obtained at 23 d in culture, ω-CTx GVIA reduced the EPSC by 24 ± 2%. This value is within the range of values observed in cells grown in kynurenic acid and elevated Mg^{2+} (see Discussion).

Figure 4*A* demonstrates that the combination of ω-Aga IVA (1 μM) and ω-CTx GVIA (2.5 μM) blocked EPSCs almost completely. In three cells, all in culture more than 21 d, the successive application of these two toxins blocked the EPSC by 83 ± 5%. As mentioned earlier, ω-Aga IVA is thought to recognize at least two classes of channels, P-type and Q-type (Sather et al., 1993), which may be related. The ω-Aga IVA sensitive channel that played a dominant role in transmitter release late in development was characterized further by the use of ω-Aga IVA and another cone snail toxin ω-CTx MVIIC (Hillyard et al., 1992). ω-CTx MVIIC blocks a number of Ca^{2+} channel subtypes including N-type channels (Hillyard et al., 1992; Grantham et al., 1994). This toxin also blocks P- and Q-type channels, and is thought to block Q-type channels with higher affinity, although the precise dose dependence is not well established (Hillyard et al., 1992; Sather et al., 1993).

For these experiments, cells in culture for more than 21 d were used. At the onset, ω-CTx GVIA was applied at a saturating concentration to block N-type channels. This was followed

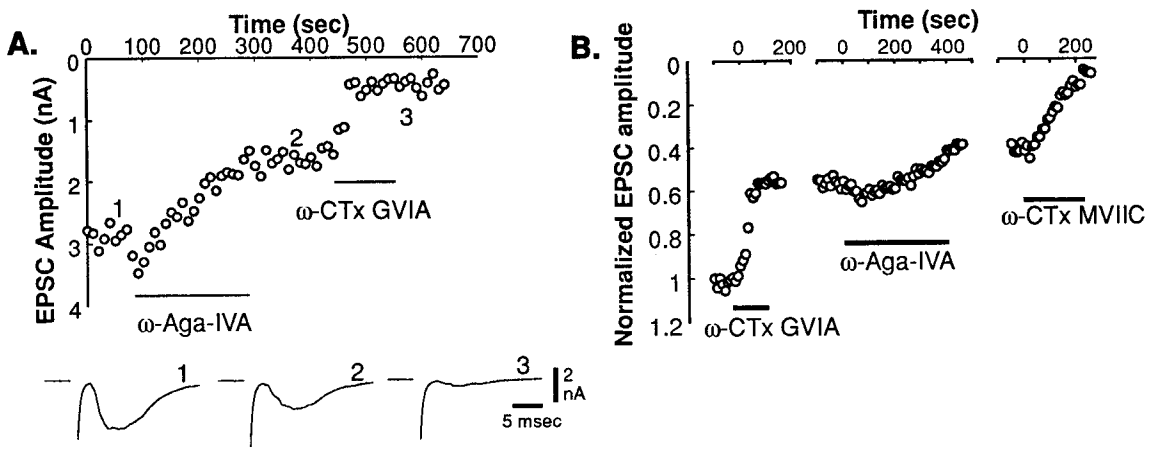

Figure 4. Pharmacological properties of the ω-Aga IVA sensitive presynaptic Ca^{2+} channel. *A*, Application of ω-CTx GVIA (2.5 μM) after block of ω-Aga IVA sensitive channels (cell in culture for 22 d). *B*, Ensemble average of results from three cells. After block of the ω-CTx GVIA sensitive component of the EPSC, a low concentration of ω-Aga IVA (50 nM) produced block at a very slow rate. Subsequent application of ω-CTx MVIIC (3 μM) produced a rapid and nearly complete block of the remaining synaptic current (cell in culture for 24 d).

by the application of 50 nM ω-Aga IVA, a concentration sufficient to block P-type channels within a few minutes but that will block Q-type channels very slowly (Mintz et al., 1992b; Mintz and Bean, 1993; Sather et al., 1993; Wheeler et al., 1994). Finally, ω-CTx MVIIC was applied to the remaining EPSC at a concentration of 3 μM. The results (Fig. 4*B*) indicate that the presynaptic Ca^{2+} channel that was resistant to block by ω-CTx GVIA was blocked by 50 nM ω-Aga IVA at a very slow rate, consistent with the properties of a Q-type channel (see Discussion). Furthermore, 3 μM ω-CTx MVIIC blocked the remaining EPSC with a time course that also suggests the participation of a Q-type channel at the presynaptic terminal. Thus, the Q-type presynaptic Ca^{2+} channel, previously shown to trigger transmitter release in the adult rat hippocampus, is likely to account for the presynaptic Ca^{2+} channel that couples to glutamate release late in development of hippocampal synapses in culture.

Discussion

The results of these studies suggest that immature excitatory hippocampal synapses utilize predominantly N-type channels for the regulation of transmitter release. During the first 1–3 weeks of postnatal life, the synapses change so as to utilize a mixture of N- and Q-type channels, with Q-type channels predominating at more mature synapses. The evidence for the participation of a Q-type channel, as opposed to a P-type channel, was obtained from an analysis of the blocking actions of ω-Aga IVA and ω-CTx MVIIC. It has been estimated that synthetic ω-Aga IVA blocks P-type channels with a K_d of 1.4 nM and an on-rate of 2.4×10^5 M^{-1} sec^{-1} (Mintz and Bean, 1993). From these values, it has been calculated that 30 nM toxin would provide 98% block of P-type channels within 120 sec (Wheeler et al., 1994). In contrast, Figure 4*B* shows that 50 nM toxin produced block of the EPSC at a much slower rate. A higher concentration of ω-Aga IVA (1 μM) blocked nearly all of the ω-CTx GVIA insensitive transmitter release (Fig. 4*A*).

Although the cells were treated with 1 mM kynurenic acid and 10 mM Mg^{2+} to enhance survival beyond day 15, it is unlikely that such treatment was responsible for the developmental changes observed, since untreated cells underwent a similar change. Nevertheless, it will be important to examine the role of activity in the control of the presynaptic Ca^{2+} channel identity. The possibility that activity participates in the control of the presynaptic Ca^{2+} channel that couples to release has not been ruled out. Furthermore, since the majority of plated cells survive when treated with kynurenic acid, the results are unlikely to arise from a change in the neuronal population resulting from selective death of specific cell populations.

Other workers have reported nonadditive effects of ω-CTx GVIA and ω-Aga IVA on synaptic transmission (Takahashi and Momiyama, 1993; Wheeler et al., 1994) and attributed this to the nonlinear relationship between Ca^{2+} entry and transmitter release (Dodge and Rahamimoff, 1967; Augustine et al., 1987). While we also see some evidence for this effect it is not as dramatic as these earlier reports. However, this issue cannot be addressed properly without adequate voltage control of the subsynaptic membrane in the postsynaptic neuron.

Developmental changes in the expression or localization of different somatic Ca^{2+} channels has been described in hippocampal and other neurons (Yaari et al., 1987; McCobb et al., 1989; Jones et al., 1989; Thompson and Wong, 1991; Gruol et al., 1992; Desarmenien et al., 1993). In addition, pharmacological studies in the chick have detected a developmental change in which dihydropyridine-sensitive channels contribute to transmitter release early in development but not later (Gray et al., 1992). Excitatory amino acid receptors also undergo changes during development in the CNS (cf. Williams et al., 1993, for a recent study). Indeed, changes in the expression patterns of ion channels is likely to be an extremely important feature of neuronal development.

The results raise the question whether changes in the ability of hippocampal synapses to undergo synaptic plasticity during development can be explained by developmental changes in the presynaptic Ca^{2+} channel. For the case of LTP, such a scenario seems unlikely, even though LTP does undergo dramatic changes during the same period of development (Harris and Teyler, 1984; Muller et al., 1989; Bekenstein and Lothman, 1991). Indeed, there is good evidence that the type of presynaptic Ca^{2+} channel is not important for the generation or expression of LTP, at least during the first hours (Muller and Lynch, 1989; Castillo et al., 1994; Wheeler et al., 1994). For other forms of synaptic plasticity, however, the type of presynaptic Ca^{2+} channel is likely to be very important. This is particularly true for G protein–

coupled presynaptic inhibition, which is likely to involve modulation of the presynaptic Ca^{2+} channel. It will be interesting to examine developmental changes in presynaptic inhibition at these synapses. In addition, paired-pulse facilitation changes at the same developmental period during which the presynaptic Ca^{2+} channel changes (Muller et al., 1989). Thus, the utilization of multiple Ca^{2+} channels for transmitter release is likely to be involved in the control of synaptic plasticity and neuromodulatory actions.

Other important issues raised by the results reported here involve the nature of the developmental change in the presynaptic Ca^{2+} channel. For instance, does each release site have a single type of Ca^{2+} channel or does each release site incorporate more than one type of channel? A related question is whether each newly formed release site incorporates N-type channels first and Q-type channels later. The alternative is that the release sites formed in early postnatal life utilize N-type channels indefinitely whereas release sites that develop later utilize Q-type channels. In this regard, ω-CTx GVIA sensitive channels and ω-Aga IVA sensitive channels can be detected in the soma of cultured hippocampal neurons by the earliest times included in this study (Scholz and Miller, unpublished observations). It is also not clear whether Q-type channels are present in the presynaptic terminal of immature cells but do not contribute to release. Finally, it will be important to determine whether release sites formed anew in the adult nervous system incorporate N-type channels first, thereby following the same developmental sequence as release sites developing in the neonatal brain.

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