1-Methyl-4-phenylpyridinium induces synaptic dysfunction through a pathway involving caspase and PKCδ enzymatic activities

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration has been used, in various mammalian species, as an experimental model of Parkinson's disease. The pathogenesis for such pharmacologically induced Parkinson's disease involves 1-methyl-4-phenylpyridinium (MPP+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. This metabolite produces rapid degeneration of nigrostriatal dopaminergic neurons, which causes the parkinsonian syndrome. In this work, we show that injection of MPP+ into the presynaptic terminal of the squid giant synapse blocks synaptic transmission without affecting the presynaptic action potential or the presynaptic calcium currents. These effects of MPP+ were mimicked by the injection of an active form of caspase-3 and prevented by inhibitors of caspase-3 and protein kinase C δ. Ultrastructurally, MPP+-injected synapses showed a dramatic reduction in the number of neurotransmitter vesicles at the presynaptic active zone, as compared with control synapses. Otherwise, normal docking and clathrin-coated vesicles were observed, albeit at much reduced numbers. These results indicate that MPP+ acutely reduces presynaptic vesicular availability, not release, and that MPP+-induced pathogenesis results from presynaptic dysfunction that leads, secondarily, to dying-back neuropathy in affected neurons.

MPP+ | Parkinson's disease | presynaptic terminal | synaptic potentials | voltage clamp

Parkinson's disease (PD) is the most common movement disorder in the elder population and the second most common neurodegenerative disease (1). PD results primarily from the loss of function for nigrostriatal dopaminergic neurons. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been used extensively in various mammalian species to produce an experimental model of PD (2). In humans and monkeys, systemic injection of MPTP produces an irreversible and severe parkinsonian syndrome characterized by all of the features of PD, including tremor, rigidity, slowness of movement, postural instability, and freezing. After systemic administration, MPTP crosses the blood–brain barrier (3) and, once in the brain, it is eventually converted in glia and serotonergic neurons to 1-methyl-4-phenylpyridinium (MPP+), which is the active toxic molecule (1). MPP+ later is released into the extracellular space before entering dopaminergic neurons through the plasma membrane dopamine transporter (4, 5). MPTP administration results in pathological changes remarkably similar to those seen in PD, namely, degeneration of the nigrostriatal dopaminergic pathway (1). The MPTP animal model is regarded as one of the best experimental models for PD (6).

MPP+ is generally thought to induce cell death by targeting mitochondria (7). However, the mechanisms by which MPP+ induces degeneration of dopaminergic neurons through a dying-back neuropathy to produce Parkinsonian syndrome still are not well understood. We have found that direct presynaptic MPP+ injection into the squid giant synapse prevents the delivery of constitutive and neurotransmitter vesicles to the presynaptic terminal. These effects are reversed when MPP+ is coinjected in the presynaptic terminal with inhibitors of either caspase or protein kinase C δ (PKCδ). Based on these results, we report a previously undescribed pathway by which MPP+ affects neurotransmitter availability and synaptic function and propose a mechanism by which MPP+ induces a Parkinsonian syndrome. Alterations in presynaptic vesicular availability described here are addressed directly in an accompanying article by Morfini *et al.* (8) and suggests that Parkinson's and other CNS pathologies may result from deregulation of vesicle transport, which we have termed a "dysferopathy," from the Greek "fero": to carry or to transport. Dysregulation of vesicle transport would reduce the terminal availability both of synaptic vesicles and the constitutive vesicular commerce required for the survival of the telodendrion.

Results

Electrophysiological Results. After MPP+ injection into the squid giant presynaptic terminal, trains of presynaptic action potentials were elicited and the postsynaptic response was used as a test for the integrity of synaptic transmission (Fig. 1). MPP+ injection produced no direct effect on the amplitude, duration, or threshold for presynaptic spike generation or conduction, nor did it affect synaptic release acutely, indicating that no direct damage to presynaptic machinery or membrane function was triggered by the injection (Fig. 1*B*). It became clear, nevertheless, that over a period of tens of minutes after injection, there was a gradual reduction in postsynaptic potential amplitude (Fig. 1*B*). To determine whether this reduction was due to a deficit in synaptic availability or synaptic release, presynaptic stimulus trains were delivered at a frequency of 200 Hz for 1 sec at 5-sec intervals until all postsynaptic responses were below the threshold for spike initiation (Fig. 1 *C* and *D*). At that point, presynaptic stimulus trains were discontinued for 5 min. This stimulation paradigm was continued until all postsynaptic potentials were subthreshold for spike generation (Fig. 1 *Ciii* and *Diii*) (9).

The synapse then was left unstimulated for 15 min to allow the replenishment of transmitter vesicles at the terminal (9) and then stimulated again. In uninjected synapses, a return of transmitter release was observed after the rest period (Fig. 1*Civ*). However, when the terminal was injected with MPP+ (50 μM estimated final concentration) ($n = 4$), a return of postsynaptic response was not observed after the 15-min rest period (Fig. 1*Div*). These results suggested that MPP+ could have reduced transmitter

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Abbreviations: MPP+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; PKCδS, PKCδ peptide substrate.

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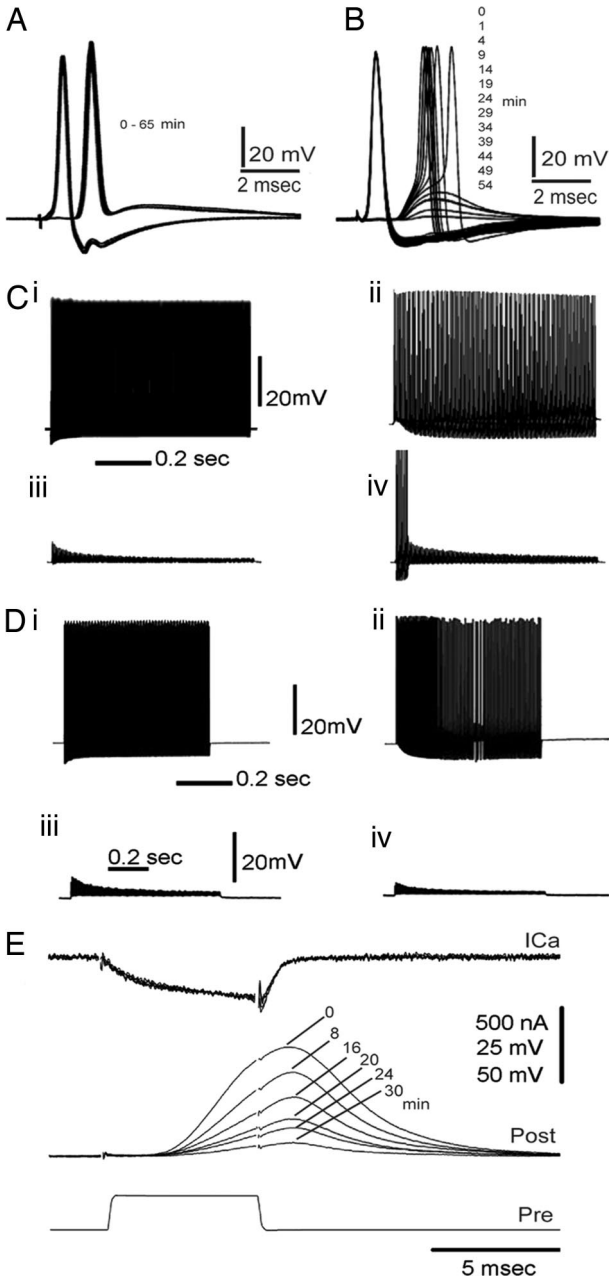

Fig. 1. Effect of MPP+ on transmitter availability. (*A* and *B*) Pre- and postsynaptic recordings in control conditions (no injection) (*A*) and after MPP+ injection (*B*). (*A*) No reduction of the postsynaptic potential in control conditions. (*B*) Reduction of postsynaptic potential without affecting the amplitude or duration of the presynaptic spike after MPP+ injection. Time after injection is indicated on the right. (*C*) Simultaneous presynaptic (*Ci*) and postsynaptic (*Cii*) recordings after repetitive presynaptic stimulation at 200 Hz in the control. (*Ciii*) The stimulus trains are repeated until all postsynaptic spikes fail. (*Civ*) After 15 min, stimulus train demonstrates recovery from transmitter depletion. (*D*) Same as in *C*, but after MPP+ injection, showing lack of recovery from transmitter depletion. (*E*) MPP+ injection reduces transmitter release without modifying the inward Ca^{2+} current (Ica). Reduction of transmitter release (*Middle*) after a presynaptic voltage clamp (*Bottom*) at different times after MPP+ injection. Note the lack of change of Ica (*Top*).

availability by reducing synaptic vesicle replenishment. Indeed, no modification of the presynaptic spike characteristics was observed in any of the experiments, and although the postsynaptic responses were very small, their latency was unchanged.

The effects of MPP+ injection on postsynaptic response could be due to either a reduction in transmitter availability, or a modification of the presynaptic Ca^{2+} current (ICa), which would affect transmitter release. To test this possibility, a set of presynaptic voltage-clamp experiments (Fig. 1*E*, $n = 3$) was implemented. After MPP+ injection, the presynaptic terminal was voltage clamped and a set of 6 test presynaptic voltage steps capable of producing a large postsynaptic response were delivered at 0.5 Hz every 5 min. The amplitude of ICa and of the postsynaptic potential was determined for each set of release events. As seen on Fig. 1*E*, the postsynaptic responses decreased with each voltage clamp trial (Fig. 1*E Middle*). However, there was no change in the amplitude, time course, or onset in the presynaptic ICa (Fig. 1*E Top*). It thus was concluded that MPP+ injection does not affect the presynaptic ICa.

Apoptotic effects of MPP+ on dopaminergic neurons have been shown to involve the activation of caspase-3, an aspartate-specific cysteinyl protease (7, 10, 11). To determine whether endogenous squid caspases mediate the effects of MPP+ on transmitter availability at the squid giant synapse, we injected a recombinant, active form of caspase-3 (0.5 nM) (Fig. 2). Injections of caspase-3 produced a lack of recovery of synaptic transmission after repetitive stimulation similar to that found in MPP+-injected synapses (Fig. 2*A*, $n = 3$). To find whether this result was also independent of any effect on the presynaptic ICa, we implemented a set of voltage-clamp experiments similar to those in MPP+-injected synapses (Fig. 2*B*). As shown in Fig. 2*B*, although the activated caspase-3 injection had no effects on ICa 30 min after injection ($n = 3$), it did reduce the postsynaptic response with a similar time course as that after MPP+ injection (Fig. 2*B*). As a further control for this experiment, we injected an inactive form of caspase-3 (Fig. 2*C*). This could allow us to confirm that the effects of this protein were in fact due to its enzymatic activity. Injection of inactive caspase-3 (0.5 nM) did not affect the recovery of transmitter release (data not shown), nor did it decrease the postsynaptic response (Fig. 2*C*) ($n = 2$), indicating that caspase-3 effects were due to its enzymatic activity.

The next set of experiments examined the possibility that coinjection of both MPP+ and a specific inhibitor of caspase-3 into the presynaptic terminal would prevent MPP+ effects on transmitter availability. To this end, we coinjected presynaptic terminals with MPP+ and Z-DEVD-fmk (Fig. 3*A*). Z-DEVD-fmk is a potent, irreversible peptide inhibitor of several caspases (caspases 3, 6, 7, 8, and 10) (12, 13). Indeed, contrary to the results illustrated in Fig. 1*D*, coperfusion of Z-DEVD-fmk peptide prevented MPP+ effects on transmitter availability (Fig. 3*Aiv*) ($n = 4$). We concluded, therefore, that MPP+ decreases transmitter availability by activating endogenous axonal caspases.

Finally, the mechanism by which caspase-3 mediates the effects of MPP+ was addressed. It has been shown that MPP+-induced caspase-3 activation results in proteolytic cleavage of protein kinase C δ (PKCδ). Proteolytic cleavage of PKCδ results in increased PKCδ activity (14). To determine whether the effects of caspase-3 on transmitter availability depend on PKCδ activity, active caspase-3 was coinjected with PKCδ peptide substrate (PKCδS) (Fig. 3*B*), a highly specific peptide substrate for PKCδ corresponding to residues 422–443 of murine elongation factor eEF-1 α (15). PKCδS is a selective substrate for PKCδ, and the structural basis of PKCδ selectivity toward PKCδS has been established (15). As can be seen on Fig. 3*B*, after coinjection of caspase-3 and PKCδS (500 μM) ($n = 3$), there was a complete recovery of transmitter release (Fig. 3*Biv*), confirming that PKCδ activity mediates caspase-3-induced decrease in transmitter availability. Finally, coinjection of PKCδS (500 μM) with MPP+ also blocked the effects of MPP+ on transmitter availability ($n = 4$) (data not shown),

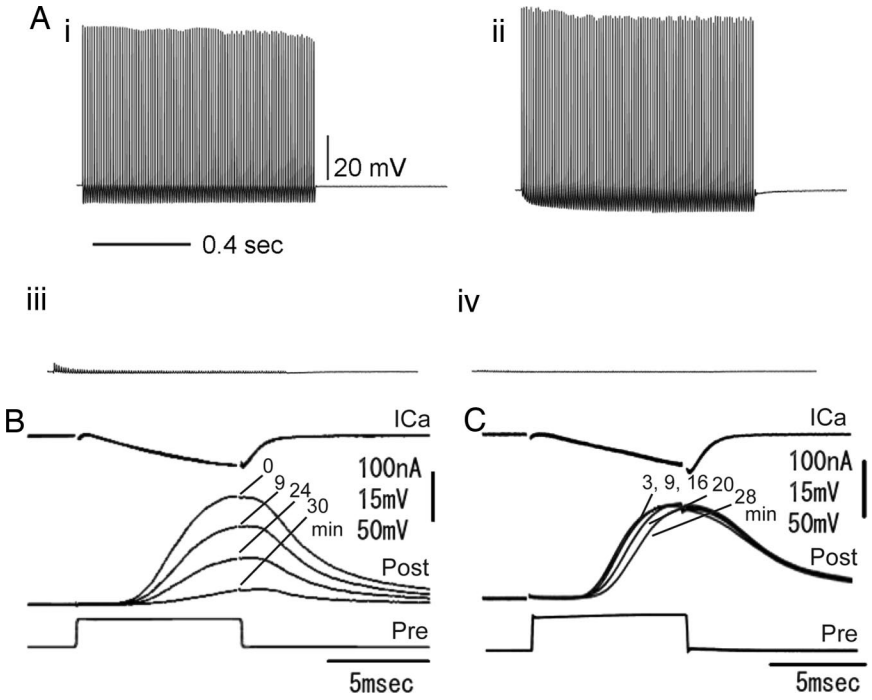

Fig. 2. Injection of active caspase-3 in the presynaptic terminal of squid mimics the effect of MPP+ on transmitter availability. (*A*) Simultaneous presynaptic (*Ai*) and postsynaptic (*Aii*) recordings after repetitive presynaptic stimulation at 200 Hz. (*Aiii*) The stimulus trains are repeated until all postsynaptic spikes fail. (*Aiv*) After 15 min, traces show lack of recovery from transmitter depletion. (*B*) Active caspase-3 reduces transmitter availability without affecting presynaptic Ca^{2+} (ICa). Traces show reduction of transmitter release (*Middle*) after a presynaptic voltage clamp (*Bottom*) at different times after caspase-3 injection. Note the lack of change in ICa (*Top*). (*C*) Same as in *B*, but presynaptic terminal was injected with inactive caspase-3. No significant change of transmitter release was observed.

Ultrastructural Results. To address the possibility that a reduction in synaptic vesicle availability was responsible for our electrophysiological findings, quantitative electron microscopy was implemented on each of the synapses studied electrophysiologically, as in previous studies (8, 16). The initial quantification compared synapses that were tetanically stimulated after the experimental paradigm shown in Fig. 1 but that were not injected (stimulated control) with synapses that were not stimulated or injected (unstimulated control). The mean number of vesicles in an active zone of the stimulated control was 52.41 ± 3.48 (n = 78 active zones), almost half that in the unstimulated control, 89.28 ± 6.27 (n = 34 active zones) ($P < 0.001$) (Fig. 4 *A* and *B* and Table 1). This is in agreement with previous reports using a similar stimulus paradigm (8, 16). When MPP+ was injected into the presynaptic terminal, the mean number of vesicles in the active zone dramatically decreased to 26.08 ± 1.71 (n = 97 active zones) ($P < 0.001$ compared with stimulated controls) (Fig. 4*C* and Table 1). These results suggest that MPP+ decreases transmitter availability by decreasing the number of synaptic vesicles present at the presynaptic terminal.

Table 1. Quantitation of total, docked, and clathrin-coated vesicles in the active zones of control and injected presynaptic terminals

Terminals	Total	Clathrin-coated	Docked
Control	89.28 ± 6.27	1.24 ± 0.21	8.9 ± 0.36
Stimulated control	52.41 ± 3.46	4.37 ± 0.35	9.2 ± 0.41
MPP+	26.08 ± 1.71**	2.06 ± 0.21**	3.49 ± 0.27**
MPP+ + Z-DEVD-fmk	52.5 ± 2.5	1.1 ± 0.16**	7.13 ± 0.53
Caspase-3	37.84 ± 3.23*	2.46 ± 0.26**	2.26 ± 0.25**
Inactive caspase-3	48.58 ± 6.32	1.35 ± 0.27**	3.26 ± 0.78
Caspase-3 + PKCdS	113.74 ± 8.97	2.41 ± 0.72	6.37 ± 0.95

*, $P < 0.005$; **, $P < 0.001$.

Given that the electrophysiological results indicated that the effects of MPP+ were mediated by caspase-3 and that an inhibitor of this enzyme prevented the effects of MPP+ in the presynaptic terminal, a second set of ultrastructural determination was implemented to examine such terminals. In agreement with the first set of ultrastructural findings, coinjection of caspase-3 inhibitor with MPP+ prevented the MPP+-induced decrease of synaptic vesicles in the active zone, bringing the number of vesicles to 52.5 ± 2.5 (n = 52 active zones) (Fig. 4*D* and Table 1), which is similar to the number of vesicles in the stimulated control. Also in support of our electrophysiological findings, caspase-3 injection decreased the number of vesicles to 37.84 ± 3.23 (n = 60 active zones) (Fig. 4*E* and Table 1), which is significantly less than the stimulated control ($P < 0.005$). On the other hand, inactive caspase-3 did not significantly affect the number of vesicles in the active zone (48.58 ± 6.32) (n = 13 active zones) (P = 0.67 compared with stimulated controls) (Table 1). The effect of caspase-3 injection was prevented completely by coinjection of caspase-3 with the PKCδ inhibitor PKCδS (113.74 ± 8.97) (n = 9 active zones) (Fig. 4*F* and Table 1). Interestingly, when PKCδS was coinjected with MPP+, it not only prevented the effects of caspase-3, but also increased the number of vesicles when compared with the stimulated control by almost 2-fold ($P < 0.001$) (Table 1).

The decrease in the number of vesicles induced by MPP+ and caspase injection could be due to a defect in vesicle endocytosis. We examined this possibility by counting the number of clathrin-coated vesicles (CCV) present in the active zone. Consistent with previous reports (9), the stimulus paradigm used in our exper-

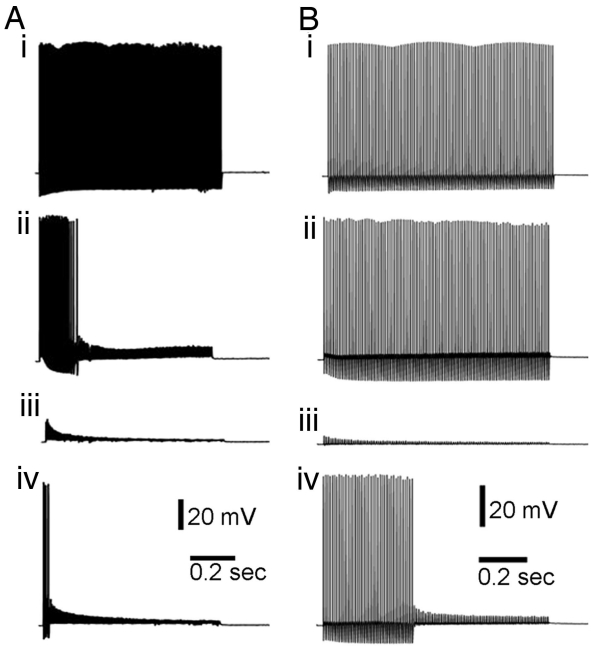

Fig. 3. The effects of MPP+ on transmitter availability are mediated by caspase activity and PKCδ. (*A*) Coinjection of MPP+ with the caspase inhibitor Z-DEVD-fmk. The experiment is similar to the ones shown in Figs. 1 *C* and *D* and 2 *A*. Stimulus train shows recovery from transmitter depletion (*iv*) when MPP+ is coinjected with Z-DEVD-fmk. (*B*) Coinjection of caspase-3 with the PKCδ inhibitor PKCδS. Contrary to injection of caspase-3 alone, note the recovery from transmitter depletion (*iv*).

iments resulted in an increase of CCV number, from 1.24 ± 0.21 (n = 34) in the unstimulated control to 4.37 ± 0.35 (n = 78) in the stimulated control ($P < 0.001$) (Fig. 4*B* and Table 1). When MPP+ was injected, the CCVs decreased to approximately half of the stimulated control 2.06 ± 0.21 (n = 97; $P < 0.001$) (Table 1). However, the number of CCVs with MPP+ injection was almost two times higher than in the unstimulated control ($P < 0.05$) (Table 1), suggesting that the machinery required for endocytosis still was functioning well after MPP+ injection. In fact, the 2-fold decrease of total vesicles in MPP+-injected synapses compared with the stimulated control may explain the similar decrease of CCVs on this group of synapses. These results suggest that the reduction of vesicles in the terminal is not caused by a defect on vesicle endocytosis.

Finally, to rule out any defect in vesicle docking, we counted the number of docked vesicles in the active zone. The mean number of docked vesicles in active zones of MPP+-injected terminals was 2.5 times smaller (3.49 ± 0.27, n = 97) than in the stimulated control (8.9 ± 0.36, n = 77) ($P < 0.001$) (Table 1). However, this decrease could reflect a reduced number of total vesicles present at the active zone. To assess the capability of docking on each experimental condition, we calculated the probability of vesicle docking for each experimental group by dividing the mean number of docked vesicles by the mean number of vesicles in each active zone. There was no difference in the probability of vesicle docking between the stimulated control and the terminal with MPP+ injection (0.20 ± 0.01 vs. 0.26 ± 0.07) (n = 78 and n = 97, respectively) (P = 0.54). We conclude that MPP+ injection does not affect vesicle docking.

Discussion

The results presented here indicated that MPP+ has a direct effect on vesicular availability that results in a decrease and cessation of transmitter release within tens of minutes to hours after intracellular presynaptic injection. These effects of MPP+ are mediated by a caspase-dependent activation of PKCδ.

As reported in the accompanying article by Morfini *et al.* (8), studies of axonal transport in squid axoplasm indicate that MPP+ treatment results in abnormal vesicular transport, and this effect is mediated by an axonal caspase and PKCδ activity. Dysregulation of vesicular mobility results in an increased retrograde transport and a concurrent decrease in anterograde movement of vesicles, leading to a net movement of vesicles away form the axonal terminal arbor and a reduction of vesicular availability at the presynaptic terminal. This centripetal organelle movement we propose is the basis for the reduction in the number of vesicles at the presynaptic terminal and the failure of transmission, in addition to the overall reduction in all aspects of preterminal activity resulting in the terminal dying back observed in many degenerative diseases of the CNS. Moreover, as expressed in Morfini *et al.* (8), we have named such dying-back phenomena due to alterations in vesicular trafficking a dysferopathy, from the Greek fero: to carry or to transport. These mechanisms may be operant not only in pathological conditions but also during development, where axonal branch retraction is a common finding during the period of synaptic competition before final synaptic consolidation (17). Furthermore, the pos-

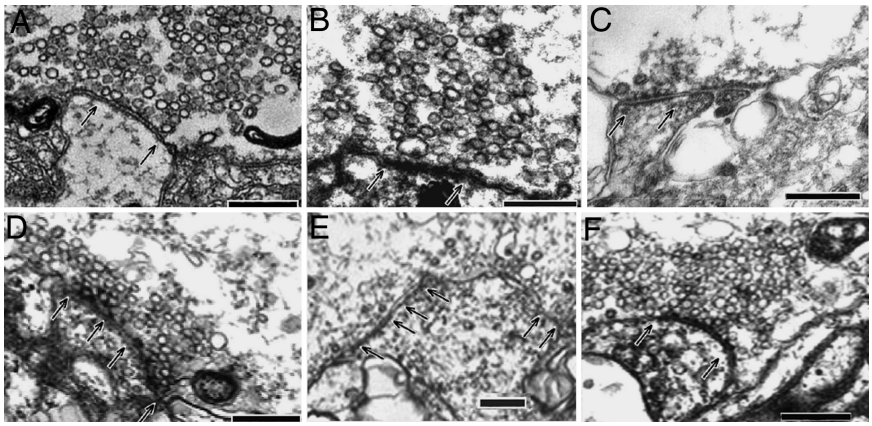

Fig. 4. Ultrastructure of the giant squid synapse showing reduced number of vesicles in the active zone after MPP+ and caspase-3 injection. (*A*) Unstimulated control. (*B*) Stimulated control; note the decrease number of vesicles in the active zone compared with the unstimulated terminal. (*C*) MPP+; note the reduced number of vesicles in the active zone compared with stimulated control. (*D*) MPP+ with the caspase-3 inhibitor Z-DEVDfmk showing block of the effects of MPP+ in vesicle number. (*E*) Caspase-3; note the reduced number of vesicles compared with stimulated control. (*F*) Caspase-3 with the PKCδ inhibitor PKCδS showing block of the effects of caspase-3 in vesicle number. Arrows denote active zones at the synaptic junctions. (Scale bars: 300 nm.)

sibility that the dying-back phenomenon due to a dysferopathy is not a *sui generis* case for the MPTP Parkinson's, but rather a possible mechanism for other thalamocortical dysrhythmias (18), has wide implications both in neurological as well as psychiatric conditions.

Materials and Methods

Reagents. MPP+ (D048) was from Sigma (St. Louis, MO). PKCδ peptide substrate (539563) and caspase inhibitor II (Z-DEVD-fmk, 264155) were both obtained from Calbiochem (San Diego, CA). Active recombinant caspase-3 (14-264) was from Upstate Biologicals (Lake Placid, NY).

Electrophysiology. The isolation of the squid stellate ganglion, electrophysiological techniques, and protocol, as well as the composition of the continuously superfused artificial seawater, were the same as in our previous experiments (6). The database consists of 34 preparations that were successfully studied. The controls (4) reproduced the experimental protocol without presynaptic injection. Electrophysiological recordings were obtained with microelectrodes filled with a mixture of cesium chloride and tetraethylammonium chloride for voltage-clamp studies or with potassium citrate for other studies. The presynaptic and postsynaptic terminals were impaled with recording and current-injecting microelectrodes. Electrical-stimulus trains were delivered directly to the preterminal axon at 200 Hz for 1 sec at 5-sec intervals until all postsynaptic responses during the train were subthreshold for spike generation. This occurred such that the initial stimuli in the train were the most resistant to depression because they were delivered immediately after the 5-sec stimulus silence.

Voltage-Clamp Experiments. Voltage-clamp experiments ($n = 8$) involved double presynaptic implement of the presynaptic terminal (19). The ionic currents responsible for spike generation (e.g., voltage-gated sodium and potassium conductances) were blocked by tetrodotoxin and tetraethylammonium, respectively. Ca^{2+} currents were activated by using voltage steps from a -70 mV holding potential. The Ca^{2+} currents were leakage subtracted and monitored for the duration of the experiment.

Presynaptic Terminal Microinjection. Injection fluid containing 500 mM potassium acetate, 100 mM Hepes (pH 7.2), and MPP+ or other effectors as appropriate was pressure-injected into the presynaptic terminal in conjunction with a fluorophore, which allowed fluorescence imaging of the injected site and progression of the bolus along the presynaptic terminal length. The progression was monitored at 10-min intervals for the duration of the experiment by using fluorescent microscopy and was recorded in a video frame grabber (Argus 100; Hamamatsu Photonics, Bridgewater, NJ). This procedure allowed bolus progression to be correlated with changes in postsynaptic response properties.

Ultrastructure. After electrophysiological recording, the ganglia were removed from the recording chamber and fixed by immersion in 6% glutaraldehyde in Ca^{2+}-free sea water, postfixed in osmium tetroxide, and in-block stained with uranyl acetate (20). They were dehydrated in ethanol, substituted with propylene oxide, and embedded in Araldite resin (CY212) or Embed 812 (EM Sciences). Ultrathin sections were collected on Pyoloform (Ted Pella, Redding, CA) and carbon-coated single-sloth grids, contrasted with uranyl acetate and lead citrate, observed, and digitally photographed in a JEOL 200CX transmission electron microscope adapted with an AMT digital camera. Electron micrographs were taken at initial magnifications of 10,000–30,000. Morphometry and quantitative analysis of the synaptic vesicles were performed with in-house program designed in Labview for vesicle counting and density determination. Vesicle density was determined as the number of vesicles per micrometer squared. Clathrin-coated vesicles were identified by size and the characteristic electron-dense coats. Docked vesicles were identified as those vesicles in contact with the presynaptic membrane of each cluster. Statistical analysis of the data were performed by using GraphPad (San Diego, CA) InStat software. Significant values were calculated by using a Kruskal–Wallis test (nonparametric ANOVA).

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