Glutamate and MPP+ specificity in Primary neuronal cultures

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ABSTRACT

Parkinson’s Disease (PD) is a movement disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). Although much research is currently devoted towards its cause, the etiology of PD remains unknown. In order to elucidate the molecular mechanisms, several compounds including MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine), glutamate, and 6-OHDA (6-Hydroxydopamine) have been used to induce neuronal death in vitro. However, to date, there has been no comparison on the toxicity of these different compounds on dopaminergic neurons. Therefore, in this study, the abilities of MPP+ (1-methyl-4-phenylpyridinium) and glutamate to specifically cause apoptosis in dopaminergic neurons from primary neuronal cultures were investigated. It was found that MPP+ preferentially induces apoptosis in dopaminergic neurons as compared to non-dopaminergic neurons, while glutamate induces apoptosis in both types of neurons. These findings have implications on in vitro models for studying the molecular mechanism of apoptosis in dopaminergic neurons and on development of drug screening assays for PD.

INTRODUCTION

Parkinson’s Disease (PD) is distinguished by the selective loss of dopaminergic neurons in the SNc. Several well-established animal models of PD have been developed. These are based on the pharmacological treatment of rats or mice with MPTP, glutamate, or 6-OHDA.

Of these, MPTP comes closest to replicating the motor symptoms of PD. In primary cultures, MPP+, the toxic metabolite of MPTP is well characterized in the pathogenesis of PD as it mimics the severe, irreversible motor symptoms of PD by mediating the loss of dopaminergic neurons (Langston et al., 1983).

A major neurotransmitter, glutamate has been known to cause excitotoxicity by over activating N-methyl-D-aspartate receptors. In PD, dopamine depletion leads to dysfunction of the basal ganglia circuitry, causing increased glutamatergic emission. The resulting overactivity mediates nigral degeneration and Parkinsonian symptoms (Vila et al., 1999). Though the toxicity of these two compounds in PD is well recognized, no studies have yet been done to distinguish between their selectivity for dopaminergic neurons.

Therefore, in this study, the goal was to investigate MPP+ and glutamate induced cell death of primary neuronal cultures. It was found that while neuronal death can be triggered by either MPP+ or glutamate in vitro, dopaminergic neurons are particularly vulnerable to MPP+ treatment.
MATERIALS AND METHODS

**Primary neuronal cultures**

The mesencephalon and olfactory bulb were dissected from embryonic day 16 (E16) mice and neuronal cultures prepared. The dissociated cells were plated at 2200 cells/mm² (mesencephalic cells) and 1100 cells/mm² (olfactory cells) and maintained in a humidified (37°C, 5% CO₂) incubator.

**Glutamate, MPP+ treatments**

Day 7 mesencephalic cultures were treated with 1.0 mM glutamate for 3, 6 and 9 hours. Treatment was halted and cells fixed by changing the drug-infused medium to a solution of 4% formaldehyde in PBS (Phosphate Buffer Saline). In a prior experiment, Day 7 olfactory bulb neurons which had been treated with 100 μM MPP+ for 1, 3, 5 and 7 hrs were similarly fixed. For both experiments, controls were prepared whereby no glutamate or MPP+ was administered.

**Apoptotic Assay**

Staining for apoptosis was performed with a TUNEL-Cy3 conjugate (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) according to the In situ cell death detection kit, TMR red (Roche).

**Immunocytochemistry**

Cells were then treated with Triton 100X in FCS (Fetal Calf Serum). Next, they were incubated for 1 hr with primary antibodies: mouse anti-TUJ1 (Neuronal Class III β-Tubulin, neuronal marker, Covance) and rabbit anti-TH (Tyrosine hydroxylase, Covance). The coverslips were then washed thrice with PBS. This was repeated with the secondary antibodies---anti-rabbit Alexa Fluor 488 (Invitrogen) and anti-mouse Alexa Fluor 633 (Invitrogen). The cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, nuclei marker). Images of the different time points were taken using a LSM 510 META Confocal Microscope (Zeiss).

**Plotting Kill Curves**

Each neuron was assessed on the basis of being dopaminergic by TH immunoreactivity (THir). Each neuron showing apoptosis (TUNEL+) was scored as apoptotic. For each treatment condition, about 20-30 dopaminergic and 200 non-dopaminergic neurons were assessed.

Kill curves of percent of survival by TUNEL versus post treatment time (Hrs) were plotted for both THir and non THir neurons. Each point is represented by its Mean ± Standard error of Measurement (SEM), with SEM calculated by assuming binomial distribution, using the formula, √(x ( 1-x)/ (n-1)), where n = Total no. of cells and x = No. of TUNEL- cells/ n.

RESULTS

**Immunocytochemical Results**

Neurons are shown (in figure 1) in white indicated by immunoreactivity with TUJ1. Dopaminergic neurons are TH immunoreactive (THir), shown in green. From the confocal images, we can clearly identify dopaminergic neurons and apoptotic cells. The latter are purple, because of the co-localisation of blue staining for nucleus and red for apoptosis. Apoptotic
neurons clearly exhibit a different morphology from non-apoptotic ones, having much retracted neurites and smaller, fragmented nuclei.

**Figure 1: MPP+ and glutamate treatment of primary neurons**

**MPP+ and glutamate specificity**

The mean ± SEM of the number of surviving dopaminergic and non-dopaminergic neurons was plotted against time for glutamate and MPP+. As shown in Fig 3, the glutamate exhibit little effects as treated cells still have 80-90% of survival rate. In contrast, MPP+ treated dopaminergic neurons have a much lower rate of survival than the non-dopaminergic ones. This clearly shows that MPP+ is selective for dopaminergic neurons while glutamate is not.

**DISCUSSION**

It was found that MPP+ selectively induces apoptosis in dopaminergic neurons, while glutamate does not. Our findings indicate that MPP+ could provide a better model system *in vitro*, to study cellular mechanisms and to develop drug screening assays.

Although 100 μM of MPP+ was able to cause about 40-50% neuronal death, 1.0 mM of glutamate caused only 10-20% of neuronal death, even at 9hr. The low cell loss for glutamate is unlikely to be due to the dose being too low since we used is one of the highest in literature. It is more likely because the cells were fixed immediately after treatment whereas recovery periods (24-48hr) were given in typical drug screening studies involving MPP+ and glutamate.
This shows that the dosage and incubation time are very important in determining the rate of death of the neurons. For the dose, too low a concentration would only cause little apoptosis in dopaminergic and non-dopaminergic neurons, so differences in the rate of death between the two would not be statistically significant. Conversely, too high a concentration would cause rapid apoptosis and make the kill curve difficult to interpret. Thus, the optimal dose should be obtained by a titration of different doses as shown in other studies.

The higher plating density of the glutamate-treated cultures (2200 cells /mm²) could have been a factor in determining the rate of apoptosis. Watanabe et al. (1998) showed that increasing plating density prolonged cell viability in cortical neurons. Glutamine was found to be highly up-regulated in the medium of high density plates (2000 cells /mm²) and it could have been released from astrocytes, which convert glutamate to glutamine (Farinelli and Nicklas, 1992). We also observed a higher proportion of astrocytes in mesencephalic than cortical cultures (data not shown). Since mesencephalic cultures were used in this study and plated at a high density, there would have been a substantial number of astrocytes in the culture, which could have converted the exogenous glutamate to glutamine. Thus, addition of 1mM of exogenous glutamate was not toxic to the neurons.

FUTURE WORK

Given that 100 μM of MPP+ administered for 3 to 6 hrs causes the greatest difference in the rate of cell loss between dopaminergic and non-dopaminergic neurons, different compounds can be tested for their ability to shift the kill curve for the dopaminergic neurons. Compounds shown to reduce the gap between the two types of neurons would be said to exert a therapeutic effect specific to dopaminergic neurons. In this respect, glutamate antagonist, MK-180 has been widely investigated for its defense against MPP+, with mixed results. Novel substances like CDP-choline and Kavain have also been postulated to have a therapeutic effect.

Thus, knowing that MPP+ and not glutamate possesses specificity towards dopaminergic neurons paves the way towards testing the effect of therapeutics and elucidation of pathways in future work.

REFERENCES


