Alterations in neurotransmitter-related enzyme activity in scrapie-infected PC12 cells

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Enzyme activities associated with the neurotransmitter pathways in nerve growth factor-treated, 139A scrapie strain-infected PC12 cells were examined. Since these cells show no morphological alterations during the time of agent replication, any scrapie-induced effects would have to be associated with non-vital cellular functions. When compared to controls, infection with the 139A scrapie strain resulted in decreased activity of the cholinergic pathway-related enzymes, choline acetyltransferase and acetylcholinesterase. However, the adrenergic pathway was unaffected by scrapie infection as evidenced by unaltered tyrosine hydroxylase activity, the putative rate-limiting enzyme in the synthesis of catecholamines. The effects of the 139A scrapie strain on the cholinergic system appeared to be dose-dependent and were first detected prior to the detection of scrapie agent replication in these cells. Furthermore, the altered enzymic activities observed were not the result of contaminating material in the scrapie brain homogenate because similar results were obtained when partially purified scrapie preparations were used as the inoculum. These scrapie agent-induced alterations in specific neuronal properties suggest a mechanism for the clinical manifestations observed in scrapie and perhaps other related central nervous system disorders.

Introduction

PC12 cells, a cloned cell line derived from a rat pheochromocytoma, have been used as an in vitro model to study developmental and functional aspects of neurons. This cell line, in the continuous presence of nerve growth factor (NGF), stops dividing and undergoes neuron-like morphological, electrophysiological and biochemical differentiation, which includes neurotransmitter metabolism (Greene & Tischler, 1976; Greene & Rein, 1977a, b, c; Melega & Howard, 1981). Previous reports have pointed to the usefulness of differentiated PC12 cells as an in vitro neuronal system for the study of the replication of scrapie agent, yielding consistently higher titres than any other tissue culture system (Rubenstein et al., 1984, 1990).

Scrapie is the prototype of the unconventional slow infectious diseases. The disease-specific markers of scrapie and related diseases are the scrapie-associated fibrils (SAF) (Merz et al., 1983, 1984), which are filamentous amyloid-like structures that co-purify with scrapie infectivity (Diringer et al., 1983; Prusiner et al., 1983). SAF are composed mainly of protease-resistant proteins (PrPs) (Bolton et al., 1982; Diringer et al., 1983), which are derived from a higher Mr, host-encoded glycoprotein (Chesebro et al., 1985; Oesch et al., 1985). In addition, these diseases are characterized histologically by spongiform encephalopathy, gliosis and, in some instances, amyloid plaque formation (Fraser, 1976, 1979; Bruce & Fraser, 1982). The events leading to these neuropathological changes are not known, and thus an in vitro model of scrapie replication would be extremely useful in that it would provide a system to examine the basis of the biochemical and molecular events associated with and/or responsible for these neurodegenerative diseases. Since it has been reported previously that the scrapie agent replicates in differentiated PC12 cells without causing any gross cytopathic changes (Rubenstein et al., 1984, 1990), it was assumed that any effects on these cells would be on their ‘luxury’ functions rather than on their housekeeping or vital functions. Therefore our studies centred upon the examination of possible changes in luxury functions in PC12 cells exposed to the scrapie agent.

Methods

Growth and maintenance of cells. PC12 cells were grown and subcultured as previously described (Rubenstein et al., 1990). In brief, cells were grown and passaged in RPMI 1640 supplemented with 10% heat-inactivated horse serum (Gibco), 5% foetal calf serum (Gibco), 200 mm-1-glutamine and antibiotics. Cells were maintained at 37 °C in
a 94% air, 6% CO₂ atmosphere and were refed and subcultured weekly. The induction of differentiation by NGF (Mobley et al., 1976) and the maintenance of these cells in the differentiated state on 60 mm² dishes coated with an air-dried extracellular matrix (Kleinman et al., 1986) were performed as previously described (Rubenstein et al., 1990).

**Infection of PC12 cells.** The cloned 139A scrapie strain (provided by Dr R. H. Kimberlin, SARDAS, Edinburgh, U.K.) was passaged and maintained in C57BL/6J mice as previously described (Rubenstein et al., 1990). Cells were exposed to diluted brain homogenate derived from mock-infected mice or mice clinically sick with the 139A scrapie strain 2 to 3 weeks after treatment with NGF. Cells were exposed for 5 h, washed, refed and maintained in the presence of NGF as previously described (Rubenstein et al., 1990). At different times after exposure to the brain homogenates, randomly chosen cultures of differentiated PC12 cells were harvested and prepared for enzyme analysis as described below.

In studies that involved exposure of NGF-treated PC12 cells to partially purified PrPs or similar preparations from mock-infected brain, the inoculum was prepared using a modified version of a previously published procedure (Hilmert & Diringer, 1984). A 20% brain homogenate was prepared in TBS (10 mM-Tris-HCl, 133 mM-NaCl pH 7-4) using a Tekmar tissumixer at setting 9. An equal volume of 20% Sarkosyl (prepared in TBS) was mixed with this homogenate and allowed to stand for 5 min at room temperature. The homogenate was microfuged for 5 min and the supernatant was mixed with a solution of TBS containing 0-1% sulphobetaine 3-14 (SB3-14) (4:1 v/v). Following centrifugation at 200000 g for 1.5 h at 20 °C in a Beckman TL-100 ultracentrifuge, the resulting pellet was resuspended by sonication in 400 µl TBS/10% NaCl/0-1% SB3-14 and layered over 100 µl of a TBS solution containing 20% sucrose/10% NaCl/0-1% SB3-14. After centrifugation at 200000 g for 1 h at 20 °C, each pellet was resuspended by sonication in 50 µl TBS/0-1% SB3-14 and treated with 50 µg/ml protease K for 1 h at 37 °C. After the addition of 0-05 mM-PMSF, the samples were centrifuged at 260000 g for 1 h at 20 °C and the final pellet was resuspended by sonication in 100 µl of water. This procedure was carried out in 1 day and bioassays of the final pellet and the original homogenate indicated that there was a recovery of greater than 90% of the starting infectivity in the final samples.

**Determination of enzymic activities.** At various times post-infection (p.i.), cells were harvested and washed in ice-cold phosphate-buffered saline pH 7-4. Cell lysates were prepared by homogenizing the cell pellets in ice-cold 100 mM-sodium phosphate buffer pH 7-4, containing 0-5% Triton X-100.

Choline acetyltransferase (CHAT) activity was assayed using the method of Fonnun (1975). In brief, aliquots of the extract were added to the incubation mixture, which contained (final concentration) 0-2 mM-[1-14C]acetyl-CoA (0-1 µCi/assay; 55 Ci/mmol), 300 mM-NaCl, 50 mM-sodium phosphate buffer pH 7-4, 8 mM-choline bromide, 20 mM-EDTA (pH 7-4) and 0-1 mM-physostigmine. The reaction was carried out for 60 min at 37 °C and terminated by the addition of 5 ml 10 mM-sodium phosphate buffer pH 7-4 followed by 2 ml acetonitrile containing 20 mg Kalignost and 10 ml of toluene-based scintillation fluid which contained 0-5% PPO and 0-03% POPP. The vials were gently shaken, resulting in the extraction of acetylcholine (ACh) into the toluene phase. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Acetylcholinesterase (AChE) activity was measured by the method of Eillman et al. (1961). Aliquots of a cell extract prepared as described above were added to an incubation buffer (final volume 3 ml) containing 0-1 mM-sodium phosphate buffer pH 8-0, 0-33 mM-5,5′-dithiobis(2-nitrobenzoic acid) (dissolved in 0-1 mM-sodium phosphate buffer pH 7-0, containing 15 mg sodium bicarbonate) and 0-5 mM-acetylthiocholine iodide. Changes in absorbance indicate the amount of acetylthiocholine hydrolysed. Absorbance readings were recorded at 412 nm over a 30 min period using a Beckman Model 34 spectrophotometer; the change in absorbance per min was calculated.

Tyrosine hydroxylase (TH) activity was measured according to Waymire et al. (1971) using aliquots of cell supernatant prepared as described above. This assay involves the conversion of 100 µM-[1-14C]-tyrosine to 14C-labelled L-dopamine (L-Dopa) by TH in the presence of 200 µM-2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropyrido[1,2,3-de]pyrimidine hydrochloride (Aldrich Chemical). L-Dopa is then decarboxylated in the presence of excess hog kidney L-amino acid decarboxylase, purified as described by Waymire et al. (1971). TH activity was determined from the 14CO₂ that was released and trapped.

All enzymic activities were measured in at least two individual experiments. In each experiment at least three cultures were used for every time point with triplicate assays on each culture.

**Determination of choline and ACh levels.** Choline and ACh levels in infected PC12 cells were determined by thin-layer chromatography as described by Rebois et al. (1980) utilizing the method of Freeman et al. (1975). At various times p.i., cells were preincubated for 15 min in a low K⁺ incubation buffer (60 mM-sucrose, 10 mM-glucose, 130 mM-NaCl, 48 mM-KCl, 1-3 mM-CaCl₂, 1-2 mM-MgCl₂, 1-2 mM-KH₂PO₄, 25 mM-HEPES pH 7-3). The preincubation buffer was replaced with fresh buffer containing 1 µCi/ml medium of [3H]choline chloride (84 Ci/mm; New England Nuclear, NEN). The cells were labelled for 3 h, and washed with ice-cold incubation buffer containing 40 µM-eserine and then with 41 µM-sucrose. The cells were harvested and resuspended in ice-cold incubation buffer containing 32 µM-sucrose and 40 µM-eserine. An aliquot was first extracted with 1 M-formic acid-acetone (15:85) followed by dichloromethane with dipicrylamine. The sample was dried under nitrogen, redissolved in acetone and spotted on cellulose thin-layer plates. The plates were developed in ethylacetate-formic acid-H₂O (70:20:10). The spots were visualised with iodine and identified by comparison with the migration of standards. The radioactive samples were scraped into scintillation vials containing 0-5 ml 0-1 M-HCl, 10 ml NEN scintillation cocktail was added and the radioactivity was determined.

**Results**

**Effects of scrapie agent replication on neurotransmitter-related enzyme activity.**

CHAT activity in cultures exposed to a 1:500 dilution (an approximate m.o.i. of 1) of 139A scrapie strain brain homogenate began to decrease between 2 and 3 weeks p.i. (Fig. 1a). In contrast, CHAT activity in cultures exposed to a 1:500 dilution of normal brain homogenate did not show any appreciable change at any point throughout the 42 days following exposure (Fig. 1a). In cells exposed to scrapie enzyme activity continued to decrease until the end of the experiment (42 days p.i.), such that the infected cells retained only 18% of the enzyme activity present immediately after the 5 h exposure (0 days p.i.) to the normal brain homogenate. No change in enzyme activity was detected in NGF-treated cultures immediately following the 5 h exposure to either normal or scrapie brain homogenate when compared to cultures prior to exposure (data not shown).
Fig. 1. Neurotransmitter-related enzyme activity in mock- (dashed line) and 139A scrapie-infected (solid line), NGF-treated PC12 cells. For each experiment the specific enzyme activity for ChAT (a), AChE (b) and TH (c) for each sample was calculated and the mean specific activity ± S.E.M. was determined for the samples at each time point. Each of these values was compared to the absolute activity from cultures exposed to normal mouse brain homogenate 5 h p.i. (0 days p.i.) and plotted as the percentage activity remaining.

The initial and continued decrease in ChAT activity closely paralleled the kinetics of 139A scrapie agent replication in infected NGF-treated PC12 cells, which was initially seen at approximately 3 weeks p.i. and thereafter increased throughout the experiment (Table 1; Rubenstein et al., 1990).

Another enzyme involved in Ach metabolism, AChE, was also examined in differentiated PC12 cells following exposure to normal and scrapie brain homogenate (Fig. 1b). Decreases in AChE activity following scrapie infection were more dramatic than those in ChAT activity, beginning within 7 days p.i. Approximately 60% of the AChE activity was lost by day 14 p.i. and an additional 20% drop occurred between 14 and 35 days p.i. In contrast, no change in AChE activity was detected in cells exposed to normal brain homogenate throughout the experiment. The change in AChE activity was detected prior to the onset of scrapie agent replication in these cells (Table 1; Rubenstein et al., 1990).

The decrease in the enzyme activities indicated that the cholinergic functions of these cells were markedly disrupted. To address the question of whether contaminating material in the scrapie brain homogenate caused the change in enzyme activities, we performed studies using partially purified PrP preparations as the inoculum, using an approximate m.o.i. of 1 (Table 2). Cultures

### Table 1. Relationship of scrapie infectivity and enzyme activity in NGF-treated, 139A scrapie strain-infected PC12 cells

<table>
<thead>
<tr>
<th>Time p.i. (days)</th>
<th>Infectivity titre (LD50/cell)*</th>
<th>ChAT (nmol/min/mg protein)</th>
<th>AChE (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.008</td>
<td>16 ± 3</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>7</td>
<td>0.004</td>
<td>15 ± 2</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>14</td>
<td>0.003</td>
<td>16 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>21</td>
<td>0.002</td>
<td>13 ± 1</td>
<td>3 ± 0.4</td>
</tr>
<tr>
<td>28</td>
<td>0.01</td>
<td>7 ± 1</td>
<td>3 ± 0.3</td>
</tr>
<tr>
<td>35</td>
<td>0.06</td>
<td>ND†</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>42</td>
<td>0.50</td>
<td>3 ± 0.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Infectivity data are taken from Rubenstein et al. (1990).
†ND, not done.

### Table 2. Effects of inoculum on neurotransmitter-related enzyme activity in 139A-infected, NGF-treated PC12 cells*

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>ChAT activity† (pmol/min/mg protein)</th>
<th>TH activity† (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-purified‡</td>
<td>Normal 19 ± 0.3</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>139A 3 ± 0.4</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Purified§</td>
<td>Normal 16 ± 0.5</td>
<td>2.3 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>139A 3 ± 0.5</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Uninfected cells</td>
<td>18 ± 5</td>
<td>2.3 ± 0.2</td>
</tr>
</tbody>
</table>

*All cultures were harvested and assayed 42 days p.i.
†Values are expressed as mean specific activity ± S.E.M.
‡Non-purified inoculum refers to total brain homogenate.
§Purified inoculum refers to PrP preparations.
were harvested 42 days p.i. and assayed for ChAT and TH activity as described in Methods. As shown in the table, similar decreases in ChAT activity were detected in cultures infected at an m.o.i. of 1 with either brain homogenate or partially purified PrP preparations.

Since these cells also contain the enzymes necessary for the synthesis and degradation of catecholamines (Greene & Tischler, 1976), TH activity following scrapie infection was assessed. TH is the first enzyme and the putative rate-limiting step in the synthesis of catecholamines. As shown in Fig. 1(c), TH activity remained essentially constant throughout the experiment in cells exposed to either normal or scrapie brain homogenate. Similar results were obtained when partially purified PrP preparations were used as the scrapie inoculum (Table 2). In addition, dopa decarboxylase activity was not altered in scrapie-infected PC12 cells throughout the experiment (data not shown).

Alterations in choline and ACh levels in scrapie-infected PC12 cells

PC12 cells take up choline and rapidly convert the accumulated choline to ACh (Greene & Rein, 1977c; Melega & Howard, 1981). ACh is compartmentalized into specific storage granules and thereby protected from degradation by the normally high levels of AChE activity in these cells (Rebois et al., 1980; Melega & Howard, 1981). Therefore the levels of choline and ACh should be affected by the changes in the ChAT and AChE activities. Our studies showed that choline is converted into and stored as ACh in control cells and in cells tested early after infection with scrapie (Fig. 2). By day 28 p.i., however, the amount of ACh in scrapie agent-exposed cultures was reduced and relatively large amounts of choline were detectable (Fig. 2). These results are not surprising and support our findings on the scrapie-induced altered enzyme activities.
Extent of scrapie-induced alterations in enzyme activity is proportional to the amount of inoculum

To test the dose–response relationship of inoculum concentration and altered enzymic activities, NGF-treated PC12 cells were infected with different dilutions of 139A scrapie brain homogenate using normal mouse brain homogenate as the diluent and enzymic activities were determined 28 days p.i. (Fig. 3). A 1 : 5000 dilution did not alter the specific activity of any of the enzymes (ChAT, AChE or TH) when compared to cultures exposed only to normal brain homogenate; however, a 1 : 1000 dilution caused a dramatic change in ChAT and AChE activities. These changes were more pronounced when higher concentrations (lower dilutions) of brain homogenate were used as inoculum. As expected, TH activity was virtually unchanged regardless of the concentration of scrapie brain inoculum used.

Discussion

Scrapie infection of NGF-treated PC12 cells and subsequent replication of the 139A scrapie strain causes alterations in specific neurotransmitter-related enzymes. The early changes in neurotransmitter-synthesizing enzymes observed in the scrapie-infected, NGF-treated PC12 cells were remarkable in that they occurred prior to the earliest detection of infectivity in the PC12 cells. The dramatic changes seen in both ChAT and AChE activities at relatively early times p.i. suggested that at the time of infection most, if not all, of the cells were both infected and affected, at least with regard to specific, non-vital or luxury functions. Whether the decreased enzyme activities were due to diminution in the specific activities of the enzymes or to inhibition of new enzyme synthesis is under investigation. Extensive studies to determine whether the alterations in these neurotransmitter enzymes are specific for the 139A scrapie strain or are a general scrapie-related phenomenon are also being pursued by infecting NGF-treated PC12 cells with other scrapie strains.

The scrapie-specific changes reported in this paper may be due to a direct effect of the agent or its products on the molecular and biochemical mechanisms responsible for neurotransmitter synthesis. It is also possible, however, that the neurotransmitter-related alterations could be the result of an indirect action on these neuron-like cells. One possibility is that the scrapie agent may block the effects of NGF on PC12 cells; treatment of PC12 cells with NGF causes an increase in neurotransmitter-related enzyme activity (Greene & Tischler, 1976). Since the effects of NGF on PC12 cells are reversible upon its removal, it is likely that selectively blocking some of the effects of NGF, possibly by binding to NGF receptors or interfering with second messengers, could cause altered enzyme activity; alternatively, NGF may be exerting its effects by regulating the expression of specific genes. It has been reported recently (Mobley et al., 1988) that NGF increases the levels of mRNA for the precursor proteins of both the scrapie-specific PrPs and the β-amyloid precursor protein, or A4, in developing hamster brain. PrPs are a component of the amyloid plaques present in scrapie and other unconventional slow infections, whereas A4 is found in neuritic plaques in human brains affected with Alzheimer’s disease (AD). Following the injection of NGF, the increase in mRNAs for PrP and A4 coincided with the increase in ChAT activity in brain regions containing cholinergic neurons. The authors therefore suggest that NGF increases the mRNA levels for PrP and A4 specifically in cholinergic neurons. It is tempting to speculate that the altered enzyme activities detected in the scrapie-infected PC12 cells are regulated by PrPs. Studies by Wion et al. (1988) have shown that NGF can cause an increase in the PrP mRNA levels in PC12 cells.

Scrapie and AD share a number of common features. Both diseases display a slowly progressive and unremitting course, and pathological changes in each disease are confined to the central nervous system and are degenerative, with little or no evidence of inflammatory reaction. In addition, using certain scrapie strain–host strain combinations, it is possible to reproduce a major pathological feature of AD, the occurrence of amyloid-containing plaques (Bruce et al., 1976).

It is well established that AD is associated with a pronounced loss of cholinergic neurons. The degeneration of cholinergic neurons is evidenced by decreased ChAT and AChE activities, a decline in choline uptake, reduced ACh synthesis and a loss of cholinergic cell bodies, which are located mainly in the nucleus basalis of Meynert (Bowen et al., 1976; Davies & Maloney, 1976; Davies, 1979; Marchbanks, 1982; Coyle et al., 1983). Furthermore, it has been suggested that the degenerative changes in cholinergic neurons which occur in the nucleus basalis in AD may be caused by a lack of trophic support by NGF (Hefti, 1983). As suggested above, it is possible that a similar mechanism involving NGF could be responsible for scrapie-induced alterations in neurotransmitter enzymes in differentiated PC12 cells. Infected PC12 cells may not only be a useful model to study scrapie-like diseases but may also prove to be valuable for the examination of the mechanism of neuropathogenesis associated with AD.

The ability of a virus to alter the luxury functions of a cell without disturbing its vital functions, i.e. the cell’s ability to survive, was noted in murine neuroblastoma cells persistently infected with lymphocytic choriomeningitis virus (LCMV) (Oldstone et al., 1977). In this
system, LCMV infection of murine neuroblastoma cells did not cause cytopathic change or affect cell growth or protein or RNA synthesis, but did reduce ACh levels by causing a decrease in both ChAT and AChE activities. These alterations occurred in cells infected in vitro and in mice inoculated at birth with LCMV. As demonstrated by cloning experiments, ACh metabolism was affected in most of the neuroblastoma cells and not just a subpopulation. These studies indicated that viruses can alter cellular functions without causing cell pathology; the results agree with our findings that non-cytopathic agents can alter specialized functions of cells without affecting their vital functions.

The dramatic clinical changes seen in scrapie-affected animals appear to be accompanied by only minimal neuronal damage. There have been numerous studies attempting to relate scrapie-induced histopathology and neuronal dysfunction to specific alterations in luxury functions, i.e. neurotransmitter-related mechanisms (McDermott et al., 1978; Masullo et al., 1984; Quinn & Somerville, 1984; Durand-Gorde et al., 1985; Cross et al., 1985; Quinn et al., 1988). Taken together these studies have yielded confusing and conflicting results. Among other changes, decreases in both ChAT activity and the number of muscarinic cholinergic receptors were reported by some investigators but not others. The variations in findings may have been due to many factors including the different scrapie strain–host strain combinations used, the dose of agent used as inoculum, the brain regions examined and the time after infection at which the animals were killed. It is also possible, however, that the changes may occur only in specific cells or cell populations and that these alterations may be partially masked by enzyme levels in unaffected cells. As shown by the present study, the use of a tissue culture model can alleviate many of these problems and generate a well defined, easily controlled system to study these changes. Assessing scrapie-induced changes in luxury functions of homogeneous cell populations might provide insight into the fundamental mechanisms of disease induction in unconventional slow infections.

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