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14. ABSTRACT

A lysate of an immortalized monoclonal cell line derived from the striatum (X61) contains two types of chemically distinct factors which are capable of increasing the dopamine content of an immortalized, dopaminergic mouse mesencephalic cell line (MN9D). One type of factor could be extracted from the cell lysate by isoamyl alcohol/chloroform and was identified to be composed primarily of oleic acid. The other type of factor was water-soluble and consisted of ethanolamine and phosphoethanolamine. Our experimental results indicate that both types of dopaminergic stimulatory factors elevate the dopamine content of mesencephalic-derived MN9D cells through an increase in storage capacity, possibly via a membrane fusion mechanism, rather than by enhancing neurotransmitter synthesis. These findings suggest a novel mechanism of action by which such factors regulate the dopaminergic phenotype. Although developing, fetal primary dopaminergic neurons in reaggregate culture were not responsive to the dopaminergic stimulatory effect of ethanolamine, there remains the possibility that phospholipid precursor therapy may be of some utility in neurodegenerative disorders, such as Parkinson's disease, given the need of neurons to repair damaged cell membranes.

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Dopamine, MN9D, nigrostriatal, Parkinson's Disease, trophic factor

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INTRODUCTION

This report covers the research conducted under Award Number DAMD17-01-1-0819 during the period September 15, 2001 to November 30, 2006. Parkinson's disease is a debilitating disorder resulting in severe motor dysfunction including muscle rigidity, bradykinesia and resting tremor. The motor disabilities are a result of degeneration of a dopaminergic projection arising in the mesencephalon and providing an extensive innervation to the corpus striatum a subdivision of brain subserving extrapyramidal motor function. The disease is widespread affecting approximately a million individuals in North America. The prevalence, morbidity and mortality associated with Parkinson's disease has stimulated an intense investigation of a variety of strategies for the treatment of this disorder (for a detailed review see Alexi et al., 2000). Given that it is, as yet, unclear as to what specific molecules will be useful therapeutic agents, investigation of the factors which are involved in the development and maintenance of the nigrostriatal projection remains an active and enlarging field of research and the development of new approaches to the investigation of dopaminergic differentiation, development and cell survival is an essential element in this research endeavor.

Among the treatment modalities currently under investigation is the use of trophic agents capable of increasing dopaminergic cell survival which could halt or delay the degenerative process. As an approach to the discovery of unique factors capable of increasing dopaminergic function, we have, during the period of Army support under Award Number DAMD17-01-1-0819, succeeded in purifying two types of chemically distinct dopaminergic stimulatory factors from the lysate of the X61 cell, an immortalized hybrid monoclonal cell derived from the corpus striatum (Wainwright et al.., 1995). The stimulatory activities derived from the X61 lysate were routinely assayed during purification by use of an immortalized, dopaminergic mouse mesencephalic cell line (MN9D)(Choi et al., 1991; Heller et al., 2000). One stimulatory factor is low molecular weight and highly hydrophilic which we have recently identified as ethanolamine and phosphoethanolamine. The second, in contrast, could be extracted by organic solvents from a concentrate of the X61 lysate and was found after purification to contain primarily the long-chain, unsaturated fatty acid cis-9-octadecenoic acid (oleic acid). Studies have been conducted on the mechanism of action and possible utility of these compounds as potential pharmacological approaches to the investigation of the Parkinson's disease and its treatment.

BODY

The primary objective of the research supported by Grant DAMD17-01-1-0819 focused on the purification, identification and mechanism of action of dopaminergic stimulatory factors obtained from the immortalized monoclonal hybrid cell, X61, derived from the corpus striatum (Heller et al., 2000; Wainwright et al., 1995).

During the period of this report (September 15, 2001 to November 30, 2006), research has been conducted on the following specific tasks outlined in the currently approved Statement of Work. These include: 1) purification and identification of low molecular weight dopaminergic stimulatory factors from the lysate of X61 cells; 2) examination of the mechanism by which these factors elevate MN9D dopamine; and 3) examination of the effect of the dopaminergic

stimulatory factors on primary dopaminergic neurons maintained in three-dimensional reaggregate culture.

Purification of Low Molecular Weight Dopaminergic Stimulatory Factors from an Immortalized Striatal Cell Line (X61)

We have purified and identified multiple low molecular weight biomolecules from X61 cells (a monoclonal line derived from the corpus striatum) that are responsible for markedly increasing the dopamine content of MN9D cells (a dopaminergic cell line derived from the mesencephalon). They are ethanolamine, phosphoethanolamine, and oleic acid (cis-9-octadecenoic acid).

An essential initial step in the purification was to allow preparative amounts of X61 cell lysate to "autodigest" at room temperature over a 3-day period. This procedure results in the progressive conversion of the stimulatory activity from a high molecular weight form, that is associated with the particulate fraction and cannot be readily fractionated, to a lower molecular weight, soluble form. The latter was then separated from the crude cell lysate material by pressure filtration through a 5 kDa (Amicon, YM-5) cut-off membrane, and it had a stimulatory activity (yield) equal to, or greater than, that of the initial crude X61 cell lysate, before autodigestion. We found, however and to our surprise, that the autodigested cell lysate material on the concentrated side of the YM-5 membrane had even more stimulatory activity than the ultrafiltrate, demonstrating that the autodigestion had resulted in a multi-fold activation of the X61 cell lysate stimulatory activity. Purification and chemical analysis, described below, has now permitted us to identify the stimulatory activity in the YM-5 ultrafiltrate as ethanolamine and phosphoethanolamine, and that in the YM-5 concentrated, X61 cell lysate material as oleic acid.

When the YM-5 ultrafiltrate activity was pressure-filtered through a YC-05 membrane (Amicon; 500 Da nominal cut-off), somewhat more that one-half of the stimulatory activity underwent concentration (termed "YC-05 concentrate"), and the remainder passed through this membrane (termed "YC-05 ultrafiltrate"). The YC-05 ultrafiltrate fraction was further purified by adsorption to charcoal. The charcoal extraction reduced the UV absorbance to about 1% of the initial (at 280 and 260 nm) and to 2% of the initial (at 230 nm), whereas no significant activity was removed by charcoal. The charcoal-adsorbed fraction was then lyophilized and re-dissolved in water at one-fifteenth the initial volume. Multiple, one-ml aliquots were then gel-filtered on a Superdex-peptide column, equilibrated and run in 5 mM sodium phosphate, pH 7.4, 10 mM NaCl, at 0.5 ml/min, and 1.0 ml fractions were collected. The stimulatory activity eluted in fractions 18-20 with the peak in fraction 19 (representing a relatively low molecular weight position within this column's sizing range). The Superdex-peptide fractions 18-20 were re-extracted with charcoal which further reduced the UV absorbance at 280 and 260 nm to about one-half the initial, and that at 230 nm to about two-thirds the initial, again without significant loss of activity onto charcoal.

The charcoal-adsorbed, Superdex-peptide YC-05 ultrafiltrate stimulatory activity was then lyophilized, re-dissolved in about one-third the initial volume of 0.05% trifluoroacetic acid in water (0.05% TFA) and multiple, 0.50 ml aliquots were chromatographed on a C18 reverse phase column, equilibrated and eluted in 0.05% TFA at 0.2 ml/min. The activity associated

transiently with the resin and eluted separate from most of the A215-absorbing material and residual salt. Following purification on the C18 column, the activity was chromatographed in similar fashion (100 ul injected per run) on a C1 (trimethyl, SAS hypersil) column, resulting in considerable additional purification, based upon a reduction of A215 UV-absorbing material.

Nuclear magnetic resonance (NMR) spectral analysis of this highly purified, YC-05 ultrafiltrate product by Josh Kurutz (Technical Director of the Biomolecular NMR Facility at The University of Chicago) confirmed a very high degree of purity. He found it to contain several amino acids (especially abundant is lysine, which we have found to be inactive) and an unknown with the simple structure R-CH2-CH2-R', where R could be –OH or –COOH, and R' could be –NH2. With this information, we have tested and found that ethanolamine produces the same dose-dependent and saturating stimulation of dopamine content in MN9D cells as the activity purified from the YC-05 ultrafiltrate. When ethanolamine was subjected to NMR analysis by Josh Kurutz, it was found to produce the same spectrum as that produced by the R-CH2-CH2-R' unknown (Fraction #15) in the purified preparation (Figure 1). In addition, amino acid analysis (by Giri Reddy, Technical Director of the Amino Acid and Polypeptide Core Laboratory of The University of Chicago) of two different purified preparations of the stimulatory activity from the YC-05 ultrafiltrate demonstrated that 60-70% of each preparation is composed of ethanolamine, about 20% is lysine, and the remainder is trace amounts of threonine, serine, glycine, and alanine. These results provide compelling evidence that the active metabolite purified from the YC-05 ultrafiltrate fraction derived from the X61 cell lysate is ethanolamine.

Based on this conclusion, we hypothesized that the active metabolite present in the YC-05 concentrate fraction derived from the X61 cell lysate could be phosphoethanolamine. Supporting this hypothesis is the technical information from Amicon Corp. that orthophosphate undergoes concentration when subjected to ultrafiltration against a YC-05 membrane, despite its nominal molecular weight cut-off of 500 Da. Therefore, we tested and found that phosphoethanolamine produces a similar dose-dependent and maximal stimulation of the dopamine content of MN9D cells as compared to ethanolamine, strongly suggesting that the stimulatory activity purified from the YC-05 concentrate fraction is phosphoethanolamine (see Figure 4 of this report). To try to confirm this possibility, we subjected the purified fraction and standards to thin layer chromatography on flexible cellulose sheets (20 x 20 cm; Selecto-Scientific; cellulose 300; cat. No. 10089) with the following chromatography fluid: 3 vol. n-butanol; 1 vol. isopropanol; 1 vol. formic acid; and 1 vol. water. Chromatography lanes were then either stained with ninhydrin or cut at 0.50 cm intervals followed by elution with PBS and assay of eluates on MN9D cells. The results indicated that the purified, YC-05 concentrate activity migrated identically to phosphoethanolamine (Rf=0.09) and different from ethanolamine, lysine, and CDP-ethanolamine (the last of which we found to be as active as ethanolamine and phosphoethanolamine). Finally, when the activity purified from the YC-05 concentrate was subjected to amino acid analysis, we found that phosphoethanolamine was the most abundant species, and the quantity of phosphoethanolamine contained within the YC-05 concentrate accounts, fully, for its stimulatory activity when compared to a phosphoethanolamine standard. The analysis also demonstrated a number of amino acids (aspartate,glutamate/glutamine, threonine, glycine, and ornithine), that were about one-half (or less) as abundant as phosphoethanolamine, and none of which have stimulatory activity (see

page 11 of this report). These results, taken together, demonstrate that the stimulatory activity purified from the YC-05 concentrate fraction is phosphoethanolamine.

Figure 1

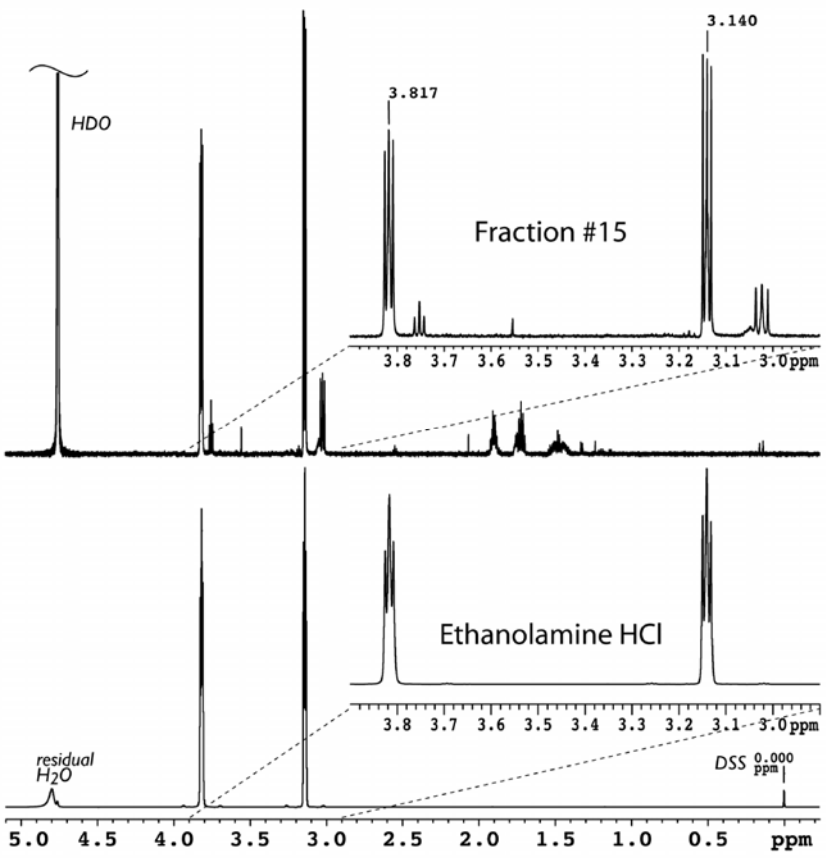


Figure 1. ^{1}H NMR spectra of Fraction #15, dissolved in 99.996% D_2O (Isotec), and commercial ethanolamine HCl, dissolved in 90% H_2O/10% D_2O. The H_2O signal in the ethanolamine sample was suppressed using presaturation. DSS-d6 (3-trimethylsilyl-1-propanesulfonic acid d6 sodium salt) was added to the ethanolamine sample as a chemical shift reference. Comparison of the spectra clearly shows that the most intense signals in Fraction #15 correspond to ethanolamine's methyl and methylene signals, both in terms of their resonance frequencies and splitting patterns.

As indicated above and as previously reported (see Annual Report of December 2004), we have found that the autodigested X61 cell lysate material on the concentrated side of the YM-5 membrane has even more stimulatory activity than the YM-5 ultrafiltrate. Consequently, we subjected the YM-5 concentrate to fractionation and purification so that we might identify the active ingredient. When the YM-5 concentrate was extracted with 2M NaCl, only 10% of its activity was solubilized, but when it was subsequently extracted with a 1:1 mixture of isoamyl alcohol:choloform, the remaining 90% was totally solubilized in the organic mixture. The isoamyl alcohol/chloroform soluble stimulatory activity is soluble in 70% acetonitrile/30% (0.05% TFA in water) and taken up by a C18 reverse phase column. The activity can be eluted from this column by linear gradient elution from 70-100% acetonitrile. All of the eluted fractions show absorbance at 215 nm. While some absorbance is present in the fractions containing dopaminergic stimulatory activity, the vast bulk of absorbance is present in fractions devoid of such activity, suggesting that the reverse phase separation resulted in considerable purification of the activity. Subsequently, the activity from reverse phase chromatography was subjected to gel filtration on a Phenogel, 5 micron, 50 Angstrom, 300 x 7.8 mm column (Phenomenex) in acetonitrile, and the activity eluted in a distinct low molecular weight peak, coincident with a major absorbance (215 nm) peak (Phenogel peak #49). When evaporated to dryness, this material was a liquid. Mass spectrographic analysis showed a mass of 283. NMR analysis revealed a 98% pure compound with a spectrum identical to that of 9-octadecenoic acid (Figure 2). Standard ^{1}H and ^{13}C one-dimensional NMR spectra were taken of the purified unknown, dissolved in deuterated chloroform. Two-dimensional correlation NMR spectra (DQCOSY and TOCSY) have been acquired to help assign peaks to individual hydrogen and carbon atoms. Initial comparison of these spectra with those in the Aldrich Library strongly suggested that the substance was oleic acid, but that it could potentially also be elaidic acid, in which the carbon-carbon double bond of oleic acid is in the trans form rather than cis. Detailed analysis of the ^{1}H and ^{13}C spectra of the unknown compound and those of pure oleic and elaidic acids shows conclusively that the unknown is primarily oleic acid (cis 9-octadecenoic acid). Both spectral analysis and the liquid state of the purified material indicate that it is cis-9-octadecenoic acid (oleic acid), which has a melting point of 10 ^{o}C while the trans form (elaidic acid) has a melting point of 45 ^{o}C and is a solid at room temperature.

To verify that the increase in dopamine levels of MN9D cells by the purified product, derived from X61 cell lysate retained by a 5 kDa filter, is indeed due to oleic acid, the dose response to known cis and trans forms of 9-octadecenoic acid was determined (Figure 3). The cis form of 9-octadecenoic acid, oleic acid, produced a marked maximal increase in MN9D dopamine with an EC_{50} of approximately 50 μM. The trans form of 9-octadecenoic acid (elaidic acid) was clearly without significant effect on MN9D dopamine. Finally, when the dose-respose (MN9D cell dopamine) produced by the Phenogel-purifed unknown was compared to that produced by commercial oleic acid (normalized by absorbance at 215 nm), they were found to be identical. These results clearly demonstrate that the factor purified from the isoamyl/chloroform extract of striatal-derived X61 cells, based upon its stimulation of MN9D cell dopamine, is oleic acid.

Figure 2

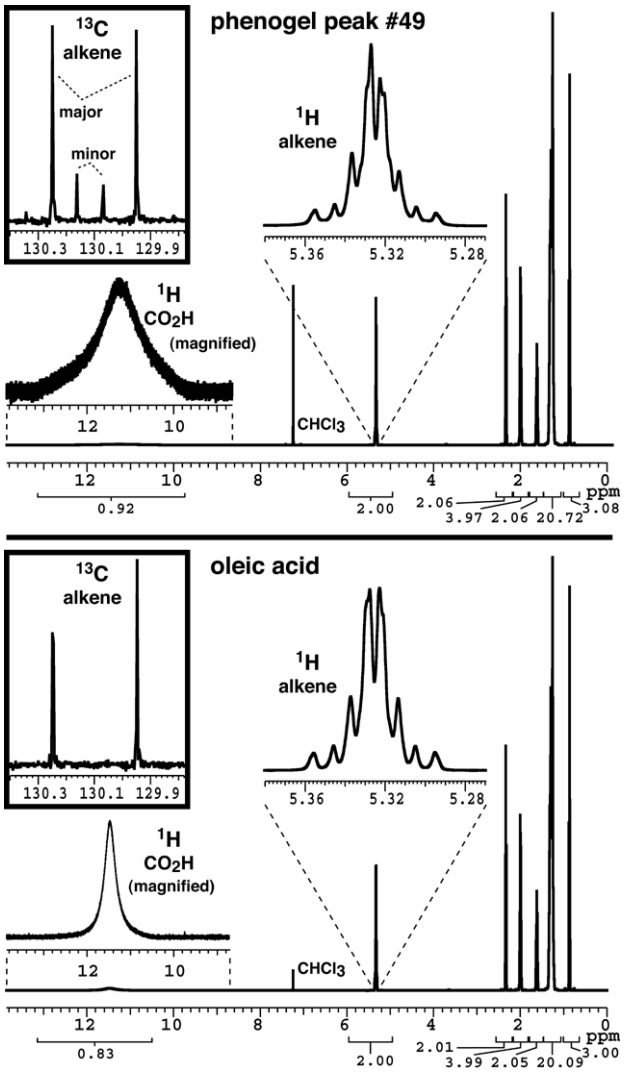
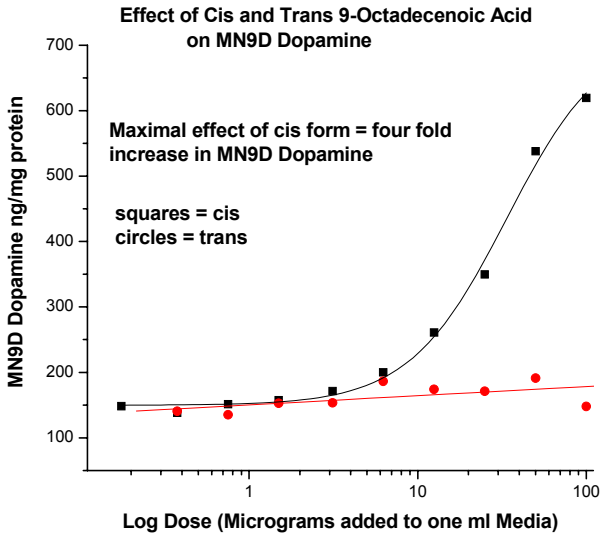


Fig. 2 The NMR spectra of peak #49 from the Phenogel column purified material (top) as compared to commercial oleic acid (bottom). The ^{1}H spectrum is shown at the bottom of each panel, and the peak integral values of the intensities are indicated below the axes. The carboxylic acid and alkene regions of the ^{1}H spectra are magnified and expanded. The alkene regions of the ^{13}C spectra are shown as insets. It is apparent that the Phenogel #49 sample contains one major and one minor species. The minor species was identified by NMR analysis to be cis-vaccenic acid.

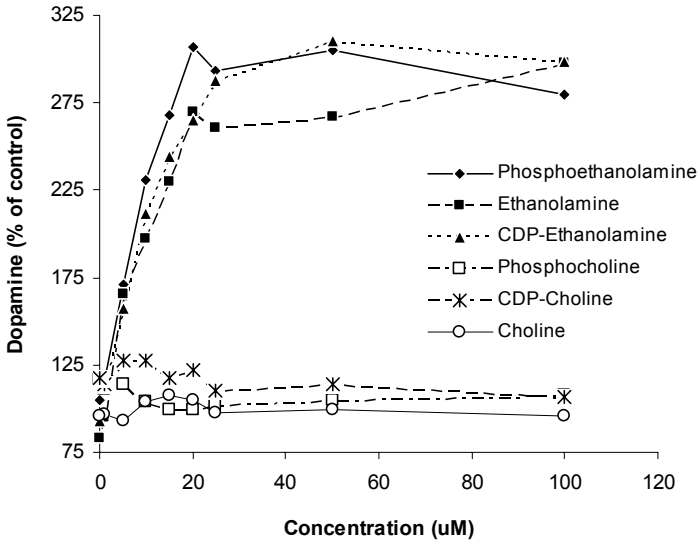
Figure 3



Evaluation of Low Molecular Weight Compounds for Dopaminergic Stimulatory Activity

NMR and amino acid analysis identified ethanolamine and phosphoethanolamine as major components of the YC-05 ultrafiltrate (>500 Da) and YC-05 concentrate (>500 Da) of the X61 cell lysate, respectively. Concentration-effect studies were conducted with ethanolamine and phosphoethanolamine as well as other known substrates for phospholipid synthesis to determine whether these compounds possess stimulatory activity with respect to elevating MN9D dopamine content.

Figure 4

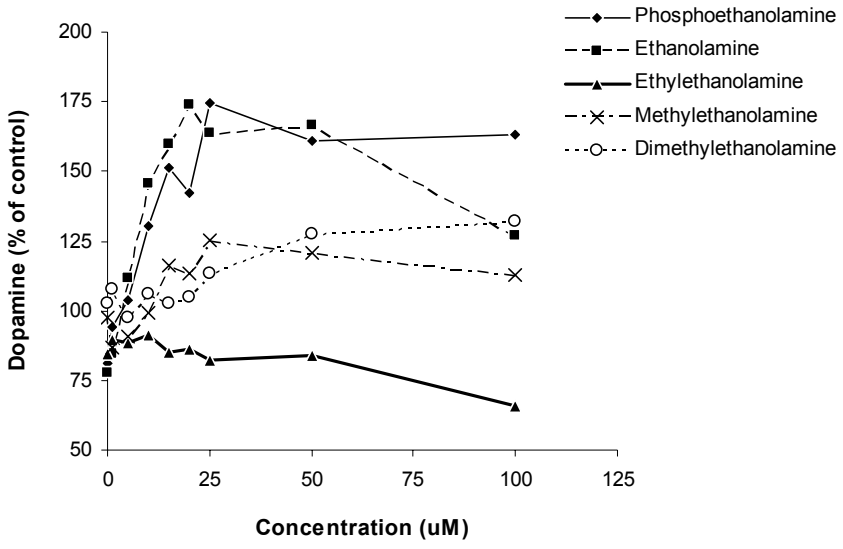


Ethanolamine, phosphoethanolamine and CDP-ethanolamine significantly elevated MN9D dopamine content and had similar dose-effect curves, with EC50's of approximately 6-8 μM, and saturation at 20-25 μM (Figure 4). Neither choline, phosphocholine or CDP-choline had any dopaminergic stimulatory activity over the concentration range tested (0.01-100 μM) Other phospholipid precursors, myo-inositol and glycerol, examined over a similar concentration range, were without any effect on MN9D dopamine (data not shown).

Other small biomolecule compounds with a chemical structure similar to ethanolamine, as well as amino acids identified as components of the YC-05 concentrate, were evaluated over a varied concentration range for ability to increase MN9D dopamine level. These included L-citrulline (0.05-5 mM), arginosuccinic acid (0.05-2 mM), N,N-dimethylglycine (0.1-100 μM), glycolic acid (0.1-50 μM), glyoxylic acid (0.1-50 μM), glycoaldehyde (0.1-50 μM), sarcosine (0.1-50 μM), L-aspartate (1-10 mM), L-cysteine (0.05-5 mM), L-glutamine (0.1-4 mM), L- and D-glutamate (2.5-10 mM), L-ornithine (0.01-2 mM), and, L- and D-serine (1-10 mM). None of these compounds produced an appreciable elevation in MN9D dopamine content (data not shown).

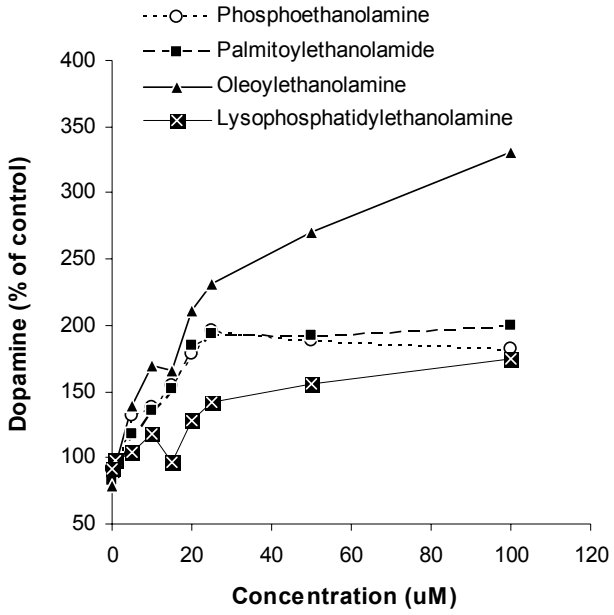
It appeared that dopaminergic stimulatory activity resided in compounds containing ethanolamine. Structure-activity studies were carried out in MN9D cells on various ethanolamine analogs (Figure 5). Methylethanolamine and dimethylethanolamine produced a similar stimulation of dopamine content that was somewhat less than one-half the effect of ethanolamine or phosphoethanolamine. Ethylethanolamine had an inhibitory effect.

Figure 5



Different classes of lipids, with potential intracellular signaling function and containing ethanolamine within their structure, were evaluated for dopaminergic stimulatory activity over a concentration range of 0.1-100 μM (Figure 6). N-acyl-ethanolamines (palmitoylethanolamide, oleoylethanolamine) are products of phosphatidylethanolamine metabolism and have been shown to exhibit neuroprotective activity (Hansen et al., 2002). Lysophosphatidylethanolamine, a product of phospholipase digestion of phosphatidylethanolamine, has recently been shown to have neurotrophic effects (Nishina et al., 2006).

Figure 6

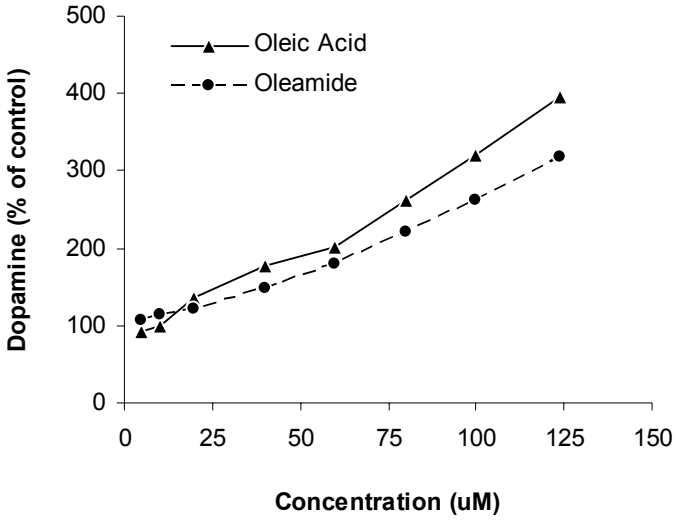


All of these compounds were capable of increasing MN9D dopamine. The most striking effect was observed with oleoylethanolamine which produced over a 3-fold elevation in neurotransmitter compared to vehicle control. Phosphatidylethanolamine was also examined over a lower concentration range (0.05-50 μM) due to poor solubility, but it did not enhance MN9D dopamine content (data not shown).

Evaluation of Compounds Structurally Related to Oleic Acid for Dopaminergic Stimulatory Activity

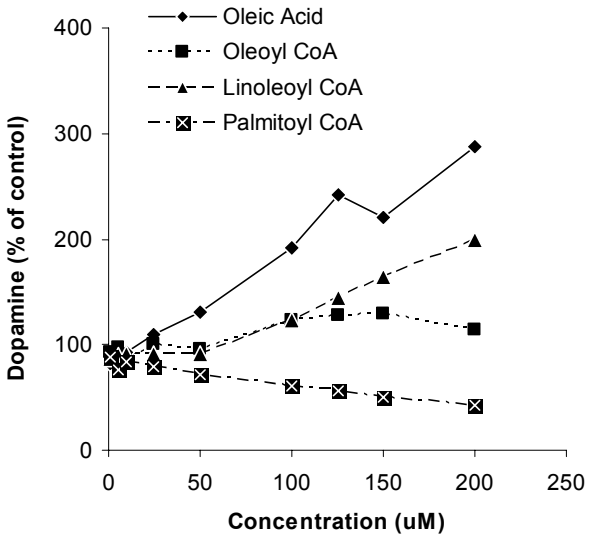
We previously reported that a number of unsaturated, long-chain fatty acids, in addition to oleic acid, are capable of increasing the dopamine content of MN9D cells (see Annual Report of December 2004 and Heller et al., 2005). These studies have now been extended to other structurally related compounds. One such agent is oleamide, a naturally occurring fatty acid amide in brain, which has been shown to have sleep-inducing properties in mammals (Cravatt et al., 1995). The concentration-response relationship of oleamide is similar to that of oleic acid (Figure 7).

Figure 7



Long-chain fatty acids are important metabolites for generating energy for the cell as well as lipid biosynthesis. Prior to being metabolized, the fatty acids must be esterified to coenzyme A (CoA). A concentration-effect study was carried out to evaluate whether several long-chain fatty acid CoA derivatives were capable of increasing the dopamine content of MN9D cells similar to that seen with oleic acid. As seen in Figure 8, none of the fatty acid CoA compounds tested were as effective as oleic acid with respect to elevating MN9D dopamine.

Figure 8



Neurochemical Mechanisms by Which Factors Derived from X61 Cell Lysate Increase Dopamine in the MN9D Cell

As previously reported, oleic acid, an active component purified from X61 cell lysate, increased the cellular dopamine content of MN9D cells by a mechanism that did not appear to involve an increase in neurotransmitter synthesis (see Annual report of December 2005). Preliminary results in that report suggested that the enhanced level of cellular dopamine in MN9D was due to increased neurotransmitter storage or uptake. In addition, we demonstrated that the vesicular compartment contributes markedly to neurotransmitter storage in MN9D cells since the oleic acid-induced elevation in dopamine was drastically reduced by agents which interfere with vesicular storage function by either inhibiting the vesicular monoamine transporter 2 (reserpine) or by disrupting the proton electrochemical gradient (bafilomycin A1). We have conducted additional studies to further define the neurochemical mechanism of action of oleic acid on regulating the dopaminergic phenotype of these cells.

It is known that high concentrations of fatty acids such as oleic acid can have detergent-like properties (Horwitz and Davis, 1993). Thus in our previous studies examining dopamine uptake (see Annual report of December 2005), exposure of MN9D cells to high concentrations of oleic acid may have resulted in a more permeable membrane permitting more exogenous dopamine in the culture medium to enter the cell. If this is the case, then treatment of other cell types with oleic acid should result in increased accumulation of exogenous dopamine. For this purpose, we measured the uptake of exogenous dopamine in control and oleic acid-treated MN9D cells as compared to N18TG2 cells, the parental neuroblastoma from which the MN9D cell line is derived which does not synthesize catecholamine.

The experiment was conducted the same as that previously described (see Annual report of December 2005). Dopamine was measured in MN9D cells incubated with exogenous dopamine following depletion of endogenous neurotransmitter stores by alpha-methy-p-tyrosine (AMT), an inhibitor of tyrosine hydroxylase. MN9D and N18TG2 cells were treated for 48 hrs with DMSO or 124 μM oleic acid. Some of the cultures were exposed to 1 mM AMT for the last 24 hrs to block further dopamine synthesis. At the end of 48 hrs, the cultures were incubated in 100 μM dopamine for 20 min.

Exposure of DMSO or oleic acid-treated MN9D cells to 1 mM AMT for 24 hrs resulted in significant reductions in cellular dopamine (DMSO, -81.4%; oleic acid, -78.4%), as compared to non-AMT treated cells, confirming the inhibition of dopamine synthesis (Table 1). In the presence of AMT, MN9D cells treated with oleic acid and exposed to exogenous dopamine exhibited a significant 2.2-fold increase in net accumulation of neurotransmitter (142.08 ± 10.92 ng/mg) as compared to DMSO vehicle control (65.85 ± 4.19 ng/mg) suggestive of an increase in MN9D dopamine uptake or storage capacity. In the absence of AMT, there was less absolute net accumulation of dopamine, however, accumulation of dopamine in MN9D cells exposed to oleic acid was still 7.4-fold greater as compared to cells treated with DMSO (48.43 ± 9.90 versus 6.51 ± 4.15 ng/mg). AMT depletion of endogenous dopamine content significantly enhanced the amount of exogenous dopamine accumulated by both DMSO-treated (10.1-fold) or oleic acid-treated (2.9-fold) cells.

Table 1

Effect of Oleic Acid on Dopamine (DA) Storage Capacity of MN9D versus N18TG2 Cells

	Endogenous DA ng/mg Pr	+ AMT Endogenous DA ng/mg Pr	Net DA Accumulated ng/mg Pr	+ AMT Net DA Accumulated ng/mg Pr
MN9D				
DMSO	101.57 ± 1.64	18.89 ± 0.48[b]	6.51 ± 4.15	65.85 ± 4.19[#]
Oleic	446.38 ± 4.75[a]	96.55 ± 3.33[b]	48.43 ± 9.90*	142.08 ± 10.92*,[#]
N18TG2				
DMSO	0.00 ± 0.00	0.00 ± 0.00	24.33 ± 2.06	21.92 ± 2.68
Oleic	0.00 ± 0.00	0.00 ± 0.00	32.69 ± 5.24	23.81 ± 3.00

The values represent the mean ± SEM, n = 6 cultures. Net accumulated dopamine was calculated by subtracting the amount of dopamine in MN9D or N18TG2 cells not incubated with exogenous dopamine from corresponding cultures incubated with 100 μM dopamine.
[a]Significantly different from DMSO vehicle control, $p<0.001$, two-tailed t-test.
[b]Significantly different from corresponding non-AMT treated MN9D or N18TG2 cells (i.e., DMSO or oleic acid), $p<0.001$, two-tailed t-test.
*Significantly different from DMSO vehicle control (in the absence or presence of AMT) incubated with dopamine, $p<0.01$, two-tailed t-test.
[#]Significantly different from corresponding DMSO- or oleic acid-treated MN9D cells exposed to exogenous dopamine, but not to AMT, $p<0.001$, two-tailed t-test.

In comparison to MN9D cells, N18TG2 cells accumulated exogenous dopamine, however, neither oleic acid treatment or AMT significantly affected transmitter accumulation (Table 1). It is interesting that in the absence of oleic acid and AMT treatment, N18TG2 cells accumulated more exogenous dopamine than the MN9D cells (N18TG2, 24.33 ng/mg versus MN9D, 6.51 ng/mg). It is possible that under basal conditions, neurotransmitter storage capacity in MN9D cells is nearly full whereas there is storage available in the N18TG2 cell which does not

synthesize dopamine. In summary, the ability of oleic acid to increase dopamine levels in MN9D cells does not appear to involve a non-specific, detergent-like effect of the fatty acid on the cell's membrane since N18TG2 cells do not accumulate more exogenous dopamine when exposed to oleic acid.

Neurotransmitter uptake by primary dopaminergic neurons is somewhat non-selective in that the cells are also capable of transporting and storing serotonin (Stamford et al., 1990; Mossner et al., 2006). Since this is the case, we examined whether oleic acid treatment of MN9D cells would affect cellular accumulation of serotonin. MN9D cells were treated for 48 hrs with DMSO or 124 μM oleic acid. Some of the cultures were exposed to 2 mM AMT for the last 24 hrs to block further dopamine synthesis. At the end of 48 hrs, the cultures were incubated in 100 μM serotonin for 30 min.

Table 2

Effect of Oleic Acid on the Accumulation of Exogenous Serotonin (5-HT) in MN9D Cells

	Endogenous DA ng/mg Pr	**+ AMT** Endogenous DA ng/mg Pr	5-HT Accumulated ng/mg Pr	**+ AMT** 5-HT Accumulated ng/mg Pr
DMSO	142.07 ± 3.11	5.98 ± 0.34[b]	-	-
DMSO + 5-HT	95.55 ± 2.03[c]	5.33 ± 0.30	149.15 ± 2.77	116.59 ± 2.47[#]
Oleic	441.15 ± 6.97[a]	17.12 ± 0.40[b]	-	-
Oleic + 5-HT	333.99 ± 8.20[c]	17.80 ± 2.42	196.27 ± 5.09*	145.58 ± 9.77[#]

The values represent the mean ± SEM, n = 3 cultures. Accumulated 5-HT is the amount of 5-HT in MN9D cells incubated with exogenous 100 μM 5-HT.
[a]Significantly different from DMSO vehicle control, $p<0.0001$, two-tailed t-test.
[b]Significantly different from corresponding DMSO- or oleic acid-treated MN9D cells not exposed to AMT, $p<0.001$, two-tailed t-test.
[c]Significantly different from corresponding DMSO- or oleic acid-treated MN9D cells not exposed to exogenous serotonin or AMT, $p<0.001$, two-tailed t-test.
*Significantly different from DMSO vehicle control (in the absence of AMT) incubated with 5-HT, $p<0.005$, two-tailed t-test.
[#]Significantly different from corresponding DMSO- or oleic acid-treated MN9D cells exposed to exogenous serotonin, but not to AMT, $p<0.05$, two-tailed t-test.

MN9D cells were capable of accumulating exogenous serotonin from the culture medium. As seen in Table 2, cells treated with oleic acid and exposed to exogenous serotonin, in the absence of AMT, accumulated significantly more (+31.6%) serotonin as compared to DMSO-treated cells. Without prior AMT-induced dopamine depletion, it is apparent that accumulated serotonin is able to partially displace stored dopamine within the cells since endogenous dopamine levels in both DMSO- and oleic treated cells were significantly reduced. Once the cellular dopamine content is depleted by AMT however, exogenous serotonin is unable to reduce endogenous dopamine any further. Serotonin displaced 107.16 ng/mg of dopamine in oleic acid-treated cells which was 2.3-fold more than the 46.52 ng/mg of dopamine displaced in DMSO-treated cells.

Oleic acid produced a 3.1-fold elevation in MN9D dopamine content as compared to DMSO treated cells and AMT exposure resulted in a significant, marked 96% reduction in cellular dopamine independent of treatment group. On a percentage basis, AMT depleted dopamine to a similar extent in both treatment groups, however, the absolute amount of dopamine decrease was 3.1-fold greater in oleic acid-treated (424.03 ng/mg) as compared to DMSO-treated (136.09 ng/mg) cells. Theoretically, despite near total transmitter depletion and three times more transmitter storage available following AMT, oleic acid-treated cells were not able to accumulate more exogenous serotonin than DMSO-treated cells. This is in contrast to increased accumulation of serotonin observed in MN9D cells treated with oleic acid in the absence of dopamine depletion by AMT. In addition, following AMT depletion, less serotonin is accumulated by DMSO- or oleic acid-treated cells. The data suggest that serotonin does not have access to, or cannot accumulate within, the storage compartment that is depleted by AMT. These results differ from those described above (see Table 1, this report) where AMT depletion of endogenous dopamine enhances the accumulation of exogenous dopamine by MN9D cells. Taken together, the findings demonstrate that serotonin and dopamine only partly share a common storage compartment within MN9D cells and suggest that additional, separate storage sites for dopamine are revealed upon transmitter depletion by AMT.

In primary catecholamine neurons, dopamine is stored within vesicles which release the neurotransmitter when stimulated by depolarizing concentrations of potassium. Since oleic acid appears to increase the storage of dopamine in MN9D cells, we examined whether the neurotransmitter was contained in a similar type of compartment that was sensitive to release by potassium depolarization. MN9D cells were treated with DMSO or 125 μM oleic acid for 48 hrs and then incubated with various concentrations of potassium chloride (KCl) for 20 min.

MN9D cells exposed to oleic acid contained 3.1-fold more dopamine and released 2.5-fold more of the transmitter into the media as compared to DMSO-treated cells under basal conditions (Table 3). Increasing concentrations of KCl significantly enhanced the amount of dopamine released into medium from DMSO-treated, but not from oleic acid-treated MN9D cells. Thus, despite containing three times more dopamine, the neurotransmitter is stored within a compartment in oleic acid-treated cells that is not stimulated by depolarizing concentrations of potassium. One possibility is that the increased dopamine in MN9D cells exposed to oleic acid is stored in vesicles of the constituitive, rather than regulated, secretory pathway as has been

reported to occur with non-dopaminergic mammalian cells which have been loaded with exogenous dopamine (Kim et al., 2000).

Table 3

Potassium-Evoked Release of Dopamine from MN9D Cells Exposed to Oleic Acid

	Cellular DA ng/mg Pr	Media DA ng/ml/mg Pr	Media DA % above control
DMSO/water	108.43 ± 1.47	14.37 ± 1.05	-
DMSO/50 mM KCl	108.20 ± 2.13	20.23 ± 7.42	40.8%
DMSO/75 mM KCl	106.18 ± 5.96	26.76 ± 1.08	86.2%*
DMSO/100 mM KCl	105.79 ± 2.03	39.67 ± 6.62	176.1%**
125 μM Oleic Acid /water	332.49 ± 1.42#	36.49 ± 2.28#	-
125 μM Oleic /50 mM KCl	330.50 ± 1.84	42.83 ± 7.18	17.4%
125 μM Oleic /75 mM KCl	333.48 ± 3.51	44.34 ± 6.42	21.5%
125 μM Oleic /100 mM KCl	303.14 ± 14.13	56.35 ± 5.85	54.4%

The values represent the mean ± SEM, n = 3 cultures.
*Significantly different from DMSO/water control, $p<0.005$, two-tailed t-test.
**Significantly different from DMSO/water control, $p<0.05$, two-tailed t-test.
#Significantly different from corresponding DMSO/water group, $p<0.005$, two-tailed t-test.

We reported earlier (see Annual report of December 2005) that the elevation in MN9D dopamine produced by the partially purified, ultrafiltrate (UF) fraction (<5,000 Da), now known to be ethanolamine and phosphoethanolamine, appeared to be due to increased neurotransmitter storage capacity. However, the data in that report were only suggestive and did not reach statistical significance. The experiment has now been replicated as shown below in Table 4. MN9D cells were treated for 48 hrs with PBS or UF. Some of the cultures were exposed to 2 mM AMT for the last 24 hrs to block further dopamine synthesis. At the end of 48 hrs, the cultures were incubated in 100 μM dopamine for 30 min.

Table 4

Effect of X61-Derived Ultrafiltrate (UF) on Dopamine (DA) Storage Capacity of MN9D Cells

	Endogenous DA ng/mg Pr	+ AMT Endogenous DA ng/mg Pr	Net DA Accumulated ng/mg Pr	+ AMT Net DA Accumulated ng/mg Pr
PBS	67.21 ± 1.78	0.00 ± 0.00[b]	21.31 ± 3.00	55.33 ± 2.28[#]
UF	142.36 ± 2.56[a]	7.42 ± 1.49[b]	52.50 ± 3.78*	103.25 ± 6.98*,[#]

The values represent the mean ± SEM, n = 6 cultures. Net accumulated dopamine was calculated by subtracting the amount of dopamine in MN9D cells not incubated with exogenous dopamine from corresponding cultures incubated with 100 μM dopamine.
[a]Significantly different from phosphate buffered saline (PBS) vehicle control, $p<0.0001$, two-tailed t-test.
[b]Significantly different from corresponding non-AMT treated MN9D cells (i.e., PBS or UF), $p<0.0001$ two-tailed t-test.
*Significantly different from PBS vehicle control (in the absence or presence of AMT) incubated with dopamine, $p<0.0005$ two-tailed t-test.
[#]Significantly different from corresponding PBS- or UF-treated MN9D cells exposed to exogenous dopamine, but not to AMT, $p<0.0005$, two-tailed t-test.

Treatment of MN9D cells with UF resulted in a 2.1-fold increase in cellular dopamine as compared to PBS control. Exposure of PBS- or UF-treated MN9D cells to AMT resulted in major reductions in cellular dopamine (95-100%), as compared to non-AMT treated cells. A significant elevation (1.9-2.5-fold) in net accumulation of dopamine was observed in MN9D cells treated with UF as compared to the PBS control whether in the presence or absence of AMT. PBS- and UF-treated MN9D cells also accumulated significantly more dopamine following depletion of endogenous neurotransmitter stores with AMT. Thus, the UF, like oleic acid, increases MN9D dopamine content by enhancing neurotransmitter storage.

The elevation of MN9D dopamine by the crude, low molecular weight ultrafiltrate fraction (UF) (MW <5,000 Da) is not mediated by an increase in tyrosine hydroxylase (TH) activity or TH protein (see Annual Report of December 2005). Since ethanolamine and phosphoethanolamine have now been identified as active components of the low molecular weight UF fraction, an experiment was conducted to assess whether or not these compounds increase MN9D dopamine by stimulating TH activity. MN9D cells were treated with water (vehicle) or with 25 μM

ethanolamine or 25 μM phosphoethanolamine in the presence or absence of 100 μM NSD-1015, an inhibitor of DOPA decarboxylase. TH activity was assessed by measuring the accumulation of DOPA in cells and media following treatment with NSD-1015.

Both ethanolamine and phosphoethanolamine markedly increased MN9D cellular dopamine in the absence of NSD-1015 (Table 5). However, TH activity, measured by DOPA accumulation in the presence of NSD-1015, in the cells or media of ethanolamine or phosphoethanolamine-treated cells, was not significantly different from that of the water vehicle control. This finding is in agreement with a lack of effect on TH activity by the low molecular weight UF.

Table 5

Effect of Ethanolamine or Phosphoethanolamine on Tyrosine Hydroxylase Activity in MN9D Cells

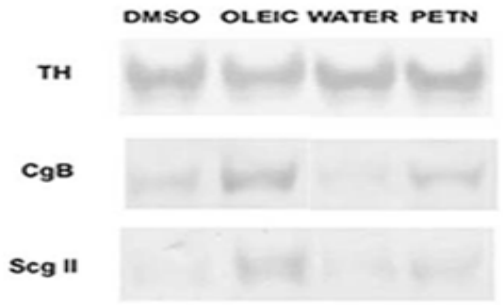
	DA Cells (ng/mg Pr)	DOPA Accumulation Cells (ng/mg Pr)	DOPA Accumulation Media (μg/mg Pr/ml)
Water	98.73 ± 5.58	168.92 ± 8.72	43.28 ± 3.86
Ethanolamine	233.18 ± 5.06*	181.83 ± 10.11	41.15 ± 5.65
Phosphoethanolamine	238.90 ± 3.66*	203.73 ± 13.52	37.58 ± 2.24

The values represent the mean ± SEM, n = 3 cultures.
*Significantly different from water vehicle control, $p<0.0001$.

The neurochemical data, thus far, suggest that the mechanism underlying the dopaminergic stimulatory action of oleic acid and the UF fraction involves an increase in neurotransmitter storage. We previously reported that the expression of chromogranin B (CgB), an acidic soluble protein stored within secretory vesicles (Winkler and Fischer-Colbrie, 1992), was increased in MN9D cells treated with oleic acid (+3.4-fold) or with the low molecular weight UF (+1.8-fold) (see Annual Report of December 2005). In addition, microarray data from that report showed increased gene expression of secretogranin II (Scg II), another vesicular soluble protein (Fisher-Colbrie et al., 1995), in sodium oleate-treated MN9D cells as compared to vehicle control. Studies have now been conducted using immunoblotting techniques to determine whether the protein expression of secretogranin II, as well as other vesicular markers, is altered in MN9D cells following 48 hr exposure to 200 μM oleic acid or 25 μM phosphoethanolamine (PETN). MN9D dopamine content was elevated 3.3-fold by oleic acid (compared to DMSO vehicle control) and 2.2-fold by 25 μM phosphoethanolamine (compared to water vehicle control)(data not shown).

As seen in Figure 9, oleic acid elevated the protein expression of chromogranin B (+3.3-fold) and secretogranin II (+3.5-fold). Phosphoethanolamine also increased the expression of chromogranin B (+1.8-fold) and secretogranin II (+1.5-fold) albeit to a lesser extent than oleic acid. The change in chromogranin B and secretogranin II protein expression produced by these two compounds was of similar magnitude to their effect on MN9D dopamine content. There was no effect of either oleic acid or phosphoethanolamine on the expression of tyrosine hydroxylase (TH) protein consistent with our findings of a lack of effect of these compounds on TH activity (see Annual Report of December 2005 and this report). No change in expression due to oleic acid or phosphoethanolamine was observed for other markers tested, including the vesicular monoamine transporter 2 (VMAT2), synaptosome-associated protein of 25 kDa (SNAP-25), vesicle-associated membrane protein-2 (VAMP2, also known as synaptobrevin), and Raf kinase inhibitory protein (RKIP, also known as phosphoethanolamine binding protein).

Figure 9



Chromogranin B and secretogranin II are acidic proteins contained within large, dense-core vesicles (LDCV) and, in PC12 cells, they are stored along with catecholamines. It has been suggested that chromogranin proteins are important for LDCV biogenesis (Huttner et al., 1991) and elevated expression of chromogranins has been correlated with increased LDCV diameter (Umbach et al., 2005) or the formation of more granules (Huh et al., 2003). If elevated expression of chromogranin B and secretogranin II in MN9D cells exposed to oleic acid or phosphoethanolamine is associated with increased LDCV size or number, this would provide a means for enhancing the storage capacity for dopamine.

Signaling Pathways Underlying the Dopaminergic Stimulatory Effect of X61-Derived Factors

As reported previously, we found that the nuclear hormone receptors, peroxisome proliferator-activated receptor-γ as well as retinoic acid receptors, were partly involved in the oleic acid-induced enhancement of the dopaminergic phenotype of MN9D cells (see Annual report of December 2005). On the other hand, protein kinases did appear to represent a major signaling pathway in mediating the dopaminergic stimulatory effect of oleic acid. Subsequent gene expression analysis was undertaken in MN9D cells treated with sodium oleate in order to

identify key genes related to signaling pathways affected by oleic acid and determine its molecular mechanism of elevating dopamine Among the cell signaling genes showing up-regulated expression was tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*Ywhaz*) also known as 14-3-3ζ. 14-3-3 proteins are binding partners for a large number of cellular proteins involved in diverse cellular processes including signal transduction, cell cycle, transport, apoptosis, development, cell adhesion and transcription (for review, see Fu et al., 2000; van Heusden, 2005). MN9D cells were transiently transfected with small interfering (*si*)RNA for 14-3-3ζ in order to determine whether this signaling pathway was involved in the dopaminergic stimulatory effect of oleic acid or the low molecular weight UF fraction (<5,000 Da) on MN9D cells.

Table 6

Effect of Oleic Acid or UF on Cellular Dopamine Content of MN9D Cells Transfected with *si*14-3-3ζ

	Wild Type	**Control Vector**	***si*14-3-3ζ**
Media	78.61 ± 2.48	73.93 ± 0.56	57.75 ± 1.19*,#
DMSO	78.57 ± 1.76	70.53 ± 1.37[a]	58.38 ± 1.34*,#
124 μM Oleic Acid	401.99 ± 7.35	418.84 ± 10.57	392.42 ± 3.60
PBS	79.78 ± 1.68	73.78 ± 1.69	61.77 ± 1.29*,#
15 μl UF	177.69 ± 7.77	197.66 ± 7.01	172.83 ± 6.95

The values represent the mean ± SEM, n = 3 cultures.
[a]Significantly different from DMSO-treated wild type MN9D cells, $p<0.05$, two-tailed t-test.
*Significantly different from corresponding wild type MN9D treated cells, $p<0.005$, two-tailed t-test.
#Significantly different from corresponding control vector MN9D treated cells, $p<0.01$, two-tailed t-test.

Wild type MN9D cells as well as cells transiently transfected with vector only or with *si*14-3-3ζ were exposed to DMSO, 124 μM oleic acid, PBS or 15 μl UF for 48 hrs. As seen Table 6, transfection of vector alone did not affect MN9D dopamine content under any of the experimental conditions. A small, but significant reduction in cellular dopamine was observed in *si*14-3-3ζ transfected cells as compared to wild type or control vector under basal media conditions as well as with DMSO or PBS exposure. However, transient transfection of MN9D cells with *si*14-3-3ζ did not alter the elevation in dopamine content induced by oleic acid or UF

thus ruling out this signaling pathway as a primary mechanism underlying the dopaminergic stimulatory effect of these substances.

Further Characterization of Biochemical Mechanisms Underlying the Dopaminergic Stimulatory Effect of X61-Derived Ethanolamine/Phosphoethanolamine and Oleic Acid

Ethanolamine, phosphoethanolamine and CDP-ethanolamine, precursors for phosphatidylethanolamine synthesis, all elevated MN9D dopamine to a similar extent. If phosphatidylethanolamine was required for the dopaminergic stimulatory effect then perhaps other substrates for its biosynthesis would be effective in raising MN9D dopamine content. The pyrimidine, cytidine triphosphate (CTP) reacts with phosphoethanolamine or phosphocholine to form CDP-ethanolamine or CDP-choline, and these then combine with diacylglycerol to form phosphatidylethanolamine or phosphatidylcholine. MN9D cells were treated for 48 hrs with 2 mM CTP, cytidine diphosphate (CDP) or cytidine monophosphate (CMP), in the presence or absence of 25 μM ethanolamine or 25 μM phosphoethanolamine. Cytidine alone did not alter the dopaminergic stimulatory effect of phosphoethanolamine and neither cytidine or CTP affected the oleic acid-induced elevation of MN9D dopamine content (data not shown).

Table 7

Effect of Cytidine Phosphonucleotides on Ethanolamine- or Phosphoethanolamine-Induced Elevation of MN9D Dopamine Content

	Water vehicle	**25 μM Ethanolamine**	**25 μM Phosphoethanolamine**
Water vehicle	132.59 ± 2.39	292.89 ± 9.03[a]	270.94 ± 11.55[a]
2 mM CTP	149.75 ± 1.86[a]	309.60 ± 11.24	162.67 ± 3.72*
2 mM CDP	110.52 ± 17.27	337.10 ± 7.89[#]	128.95 ± 2.74*
2 mM CMP	107.85 ± 1.30[a]	247.82 ± 4.51[#]	118.02 ± 3.93*

The values represent the mean ± SEM, n = 3 cultures.
[a]Significantly different from water vehicle control, $p<0.01$, two-tailed t-test.
[*]Significantly different from phosphoethanolamine/water, $p<0.005$, two-tailed t-test.
[#]Significantly different from ethanolamine/water, $p<0.05$, two-tailed t-test.

As seen in Table 7, simultaneous exposure of MN9D cells to phosphoethanolamine and either CTP, CDP or CMP resulted in a marked decrease (CTP) or total inhibition (CDP, CMP) of the ability of phosphoethanolamine to elevate dopamine. In contrast, concurrent treatment of MN9D cells with ethanolamine and CMP produced a slight reduction (-15%) in cellular dopamine whereas a small increase (+15%) in transmitter was observed with ethanolamine and CDP.

Exposure of MN9D cells to cytidine phosphonucleotides alone resulted in small changes in dopamine; 2 mM CTP slightly increased (+13%) while CMP decreased cellular dopamine by 19%.

A similar experiment was conducted examining other purine and pyrimidine phosphonucleotides for ability to modulate the dopaminergic stimulatory action of phosphoethanolamine and ethanolamine. ATP, GTP and UTP all significantly reduced (13-38% reduction) the increase in MN9D dopamine content produced by phosphoethanolamine (Table 8). On the other hand, ATP and UTP potentiated the dopaminergic stimulatory effect of ethanolamine by 50-81%. Exposure of MN9D cells to ATP alone resulted in a small, but significant increase (+35%) in cellular dopamine.

Table 8

Effect of Purine and Pyrimidine Phosphonucleotides on Ethanolamine- or Phosphoethanolamine-Induced Elevation of MN9D Dopamine Content

	Water vehicle	**25 μM Ethanolamine**	**25 μM Phosphoethanolamine**
Water vehicle	188.49 ± 5.29	283.61 ± 16.00[a]	310.62 ± 9.38[a]
2 mM ATP	254.27 ± 7.92[b]	512.38 ± 8.05[#]	268.82 ± 7.73*
2 mM GTP	183.02 ± 0.48	313.78 ± 4.87	192.46 ± 3.64**
2 mM UTP	218.06 ± 7.10	426.50 ± 14.24[#]	233.16 ± 4.71**

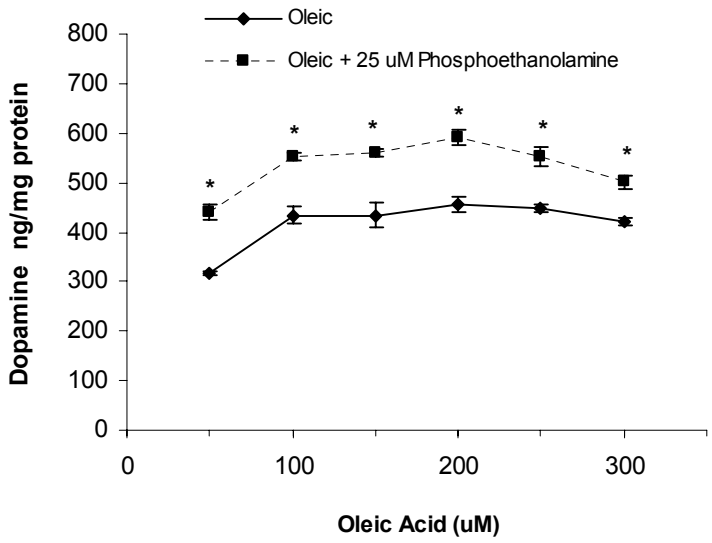
The values represent the mean ± SEM, n = 3 cultures.
[a]Significantly different from water vehicle control, $p<0.01$, two-tailed t-test.
[b]Significantly different from water vehicle control, $p<0.01$, two-tailed t-test.
[*]Significantly different from phosphoethanolamine/water, $p<0.05$, two-tailed t-test.
[**]Significantly different from phosphoethanolamine/water, $p<0.005$, two-tailed t-test.
[#]Significantly different from ethanolamine/water, $p<0.005$, two-tailed t-test.

The ability of purine and pyrimidine phosphonucleotides to inhibit the dopaminergic stimulatory effect of phosphoethanolamine, but not ethanolamine, may simply be due to competition for plasma membrane anion uptake. The finding that ATP and UTP potentiate the dopamine-elevating effects of a saturating concentration of ethanolamine suggests that these phosphonucleotides activate a pathway separate from that mediating the effect of ethanolamine.

It has been reported that exogenous L-serine can inhibit labeled ethanolamine incorporation into phosphatidylethanolamine (McMaster and Choy, 1992) and exogenous choline is effective in inhibiting cellular accumulation of labeled ethanolamine (Massarelli et al., 1986; Yorek et al., 1985). If the dopaminergic stimulatory effect of ethanolamine or phosphoethanolamine on MN9D cells is dependent upon synthesis of phosphatidylethanolamine then the effect could potentially be blocked by L-serine or choline. MN9D cells were exposed for 48 hrs to 10 mM L-serine or 10 mM choline in the presence or absence of 25 μM ethanolamine or 25 μM phosphoethanolamine. Only choline produced a small, but significant (-25%, $p<0.05$), inhibition of the phosphoethanolamine-induced elevation in dopamine (phosphoethanolamine, 310.62 ± 9.38 ng/mg dopamine versus phosphoethanolamine + choline, 232.32 ± 5.51 ng/mg). Choline did not alter the ability of ethanolamine to increase MN9D dopamine content and L-serine did not affect the dopaminergic stimulatory action of either ethanolamine or phosphoethanolamine (data not shown).

We examined whether oleic acid and phosphoethanolamine, which are both derived from X61 cell lysate, increase MN9D dopamine content via a similar biochemical mechanism by exposing MN9D cells simultaneously to a saturating concentration of phosphoethanolamine (25 μM) and varying concentrations of oleic acid (50 μM – 300 μM).

Figure 10



In this experiment, exposure to phosphoethanolamine alone resulted in a 2.2-fold increase in MN9D dopamine content (phosphoethanolamine, 249.18 ± 2.29 ng/mg dopamine; vehicle control, 114.20 ± 5.05 ng/mg). The dopamine-elevating effect of oleic acid by itself saturated at 100 μM (Figure 10). The combined treatment of MN9D cells with 25 μM phosphoethanolamine and oleic acid resulted in significantly greater cellular dopamine levels than oleic acid alone for each fatty acid concentration tested ($n = 3$, $*p<0.05$). Simultaneous exposure of MN9D cells to phosphoethanolamine and oleic acid was additive with respect to MN9D dopamine indicating

that separate mechanisms are most likely involved in the dopaminergic stimulatory activity produced by these two compounds.

It is unclear how oleic acid, ethanolamine and phosphoethanolamine enhance MN9D dopamine content. One possible mechanism involves phospholipid synthesis. Ethanolamine and phosphoethanolamine are substrates for phospholipid biosynthesis. Oleic acid is a major unsaturated fatty acid constituent of phosphatidylcholine in the plasma membrane (Williams and McGee, 1982). Oleic acid stimulates phosphatidylcholine synthesis by enhancing choline-phosphate cytidyltransferase activity (Chander and Fisher, 1988) and the enzyme activity is further activated in the presence of phosphatidylethanolamine (Sleight and Thi Dao, 1990). We examined whether 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which has been shown to reduce cellular levels of diacylglycerol and inhibit CTP:phosphoethanolamine cytidylyltransferase activity (Houweling et al., 2002), could block the dopaminergic stimulatory action of oleic acid or phosphoethanolamine. Treatment of MN9D cells with 10-100 μM AICAR, however, did not diminish the dopamine-elevating effect of oleic acid or phosphoethanolamine (data not shown).

There have been several reports that cis-unsaturated fatty acids promote membrane fusion. The long-chain cis-unsaturated fatty acids, arachidonic acid and oleic acid, induce the fusion of isolated chromaffin granule cells aggregated by calcium (Creutz, 1981). Granule fusion was not observed with elaidic acid, a trans-unsaturated fatty acid, or with the saturated fatty acids, palmitate and stearate. Arachidonic acid increases the fusion frequency of endosomes (Mayorga et al., 1993) and oleic acid stimulates liposome fusion (Melia et al., 2006). Whole cell fusion of erythrocytes is also more readily induced by a number of unsaturated fatty acids including oleic acid, as compared to saturated fatty acids (Ahkong et al., 1973). Biological membrane fusion is also influenced by lipid composition as fusion is facilitated more if membranes contain phosphatidylethanolamine as opposed to phosphatidylcholine (Chernomordik et al., 1995). Of interest is the fact that liposomes composed of oleic acid and phosphatidylethanolamine aggregate and fuse at an acidic pH below 6.5 (Duzgunes et al., 1985), a pH level present within a number of intracellular organelles including endosomes, lysosomes, endoplasmic reticulum and secretory vesicles. In contrast, liposomes composed of oleic acid and phosphatidylcholine do not exhibit aggregation and fusion at low pH. Although we have no direct data, a membrane fusion mechanism offers a reasonable explanation for the increase in dopamine content observed in MN9D cells treated with oleic acid, ethanolamine/phosphoethanolamine or to a combination of oleic acid and phosphoethanolamine. Theoretically, exposure to the fatty acid and/or phospholipid precursor would promote fusion of acidic organelles (i.e., secretory granules) thus enlarging the neurotransmitter storage compartment within these cells and our results demonstrating elevated levels of the granule proteins, chromogranin B as well as secretogranin II, are consistent with this hypothesis.

Effect of Factors Derived from X61 Lysate on Primary Dopaminergic Neurons Using Three-Dimensional Reaggregate Culture

The stimulatory activities derived from the X61 lysate have been routinely assayed during purification by use of the dopaminergic monoclonal cell line, MN9D. The MN9D cell line has

permitted identification of fractions capable of increasing cellular content of dopamine. We have utilized the three-dimensional reaggregate tissue culture system to assess whether such active fractions exhibited a stimulatory effect on primary dopaminergic neurons.

Prior studies using three-dimensional reaggregate cultures demonstrated that a stimulatory effect of exogenous oleic acid on primary dopaminergic neurons was only unmasked when such cells were grown with target cells, as opposed to non-target cells, in the presence of serum-free, defined media (see Annual Report of December 2004). Serum apparently contains levels of fatty acids, as well as other factors, which are sufficient for maintenance of the dopaminergic phenotype.

As we previously reported, the X61 lysate as well as the ultrafiltrate (<5,000 kDa) fraction from the lysate, from which ethanolamine is derived, contains an activity which increases tissue dopamine content, elevates media homovanillic acid levels and enhances cell survival of primary mesencephalic dopaminergic neurons grown in three-dimensional reaggregates in the absence of target cells (Won et al., 2003). Since ethanolamine has now been identified as an active compound contained within the low molecular weight (<5,000 Da), ultrafiltrate fraction from this lysate, we examined whether ethanolamine was capable of elevating the dopamine content of primary dopaminergic neurons.

Table 9

	Reggregate Dopamine	Media HVA
Water	5.85 ± 0.19	43.04 ± 1.92
10 μM Ethanolamine	6.18 ± 0.20	45.48 ± 2.65
25 μM Ethanolamine	6.04 ± 0.22	42.00 ± 1.40

The values represent the mean ± SEM, n = 5 cultures. Reaggregate dopamine is expressed as ng/mg protein. Media homovanillic acid (HVA) is expressed as ng/ml of media per mg reaggregate protein.

Reaggregates were prepared from fetal mesencephalon and optic tectum (an area of brain which does not receive a dopaminergic innervation) similar to that used for experiments using the X61 ultrafiltrate fraction. The cultures were exposed to water (vehicle), 10 μM or 25 μM ethanolamine for culture days 1-15. The higher concentration of ethanolamine (25 μM) is saturating and generally produces a maximal stimulation (~ 2.5-fold increase) of MN9D dopamine content. The compounds were replaced every other day along with medium changes. After 15 days of culture, the reaggregates were collected for neurochemical analysis.

Treatment of reaggregates containing primary mesencephalic dopaminergic neurons with ethanolamine did not increase tissue dopamine content or culture media HVA levels (Table 9) as was previously observed for X61 cell lysate as well as the low molecular weight (<5,000 kDa) ultrafiltrate fraction (Won et al., 2003).

The factor(s) in the X61 ultrafiltrate responsible for the dopaminergic stimulatory effect on primary mesencephalic dopamine neurons is, as yet, unknown, but apparently is different from ethanolamine, and most likely, phosphoethanolamine, which also enhances the MN9D dopaminergic phenotype. Phosphoethanolamine has previously been purified from bovine pituitary (Kano-Sueoka et al., 1979) and from postnatal rat brain (Bostwick et al., 1989). Glycerophosphorylethanolamine, a structurally similar derivative, has also been isolated from porcine liver extracts (Nelson et al., 1996). Both phosphoethanolamine and glycerophosphorylethanolamine exhibit growth stimulating activity on mammalian cells in culture (Kano-Sueoka et al., 1979; Nelson et al., 1996), an effect which is not observed with MN9D cells (data not shown). Treatment of primary neuronal cultures with phosphoethanolamine results in enhanced high-affinity choline uptake and acetylcholine synthesis by fetal rat septal cholinergic neurons, but no effect was observed on dopamine uptake by cultures of ventral mesencephalon (Bostwick et al., 1992).

The MN9D cell line has served as a rapid, convenient assay for following dopaminergic stimulatory activity during purification of X61 cell lysate, a screening method which would have been impractical with primary neuronal cultures. Although the chemical moieties, ethanolamine and phosphoethanolamine, identified in the lysate clearly enhanced the dopaminergic phenotype of the MN9D cell, they failed to increase the dopamine content of primary fetal dopaminergic neurons. Our results, and those of Bostwick et al., 1992, suggest that the phenotype of developing, fetal dopaminergic neurons does not respond to ethanolamine/phosphoethanolamine. It still, however, is unclear whether ethanolamine would produce a stimulatory effect on mature dopaminergic neurons *in vivo*. There remains the possibility that phospholipid precursor therapy may have utility in the case of neurodegenerative disease. It has been reported that there is increased demand for membrane phospholipid synthesis in the substantia nigra of Parkinson's disease patients, as evidenced by elevated activity of phospholipid biosynthetic enzymes (Ross et al., 2001). Enhanced phospholipid metabolism in this brain region may be a compensatory response to degenerating nigrostriatal axons. Interestingly, in a rat model of Parkinson's disease, animals that received unilateral 6-hydroxydopamine lesions of the substantia nigra showed increased incorporation of ^{3}H-arachidonic acid into phospholipids of the basal ganglia on the lesioned side as compared to the intact side (Hayakawa et al., 1998). In addition to Parkinson's disease, postmortem brain samples from patients with Alzheimer's disease and Huntington's disease show significant decreases in levels of phosphoethanolamine and ethanolamine with the most severe reductions occurring in brain regions exhibiting neuronal loss for both diseases (Ellison et al., 1987).

KEY RESEARCH ACCOMPLISHMENTS

- Purification and identification of two types of dopaminergic stimulatory factors from the cell lysate of striatal hybrid X61 cells: 1) the organic solvent extractable component was primarily composed of oleic acid and 2) the water-soluble factor was composed of ethanolamine and phosphoethanolamine.

- Demonstration that the ability to increase MN9D dopamine by oleic acid is shared by a number of other long-chain, unsaturated fatty acids with one to four double bonds including arachidonic, linoleic, linolenic, palmitoleic, and cis-13-octadecenoic acid.

- Demonstration that the ability to increase MN9D dopamine by ethanolamine/phosphoethanolamine is shared by a number of other lipid moieties containing ethanolamine including CDP-ethanolamine, lysophosphatidylethanolamine, oleyolethanolamine, palmitoylethanolamide, and dimethylethanolamine. Choline, as well as choline-containing lipids, do not elevate MN9D dopamine content.

- Neurochemical evidence that the elevation of MN9D cellular dopamine by oleic acid as well as ethanolamine/phosphoethanolamine involves an increase in neurotransmitter storage as opposed to an increase in neurotransmitter synthesis.

- Evidence supporting an increase in the size of the storage compartment for dopamine from Western blot analysis demonstrating enhanced expression of catecholamine-associated secretory granule storage markers, chromogranin B and secretogranin II, in MN9D cells exposed to oleic acid or phosphoethanolamine.

- Results demonstrating that nuclear hormone receptors, in particular retinoic acid receptors, play a role in the dopaminergic stimulatory effect of oleic acid.

- Demonstration of enhanced survival of primary murine dopaminergic neurons in reaggregate culture by a low molecular weight (<5,000 Da) ultrafiltrate fraction of X61 cell lysate.

- Demonstration that a stimulatory effect of oleate on primary dopaminergic neurons co-cultured with their target cells is unmasked when the reaggregate cultures are maintained in serum-free, defined medium as opposed to serum-containing medium.

REPORTABLE OUTCOMES

Publications (included in the Appendix)

Abstracts:

Won, L., Bubula, N., Hessefort, S., Gross, M. and Heller, A. Enhanced survival of dopaminergic neurons induced by a partially purified cell lysate fraction from striatal derived hybrid monoclonal cells. Soc. Neurosci. Abstr., Program no. 299.17, 2003.

Manuscripts:

Heller, A., Gross, M., Hessefort, S., Bubula, N. and Won, L. Dopaminergic stimulatory polypeptides from immortalized striatal cells. Ann. N. Y. Acad. Sci. 991, 339-341, 2003.

Won, L., Bubula, N., Hessefort, S., Gross, M. and Heller, A. Enhanced survival of primary murine dopaminergic neurons induced by a partially purified cell lysate fraction from striatal derived hybrid monoclonal cells. Neuroscience Letters 353, 83-86, 2003.

Heller, A., Won, L., Bubula, N., Hessefort, S., Kurutz, J.W., Reddy, G.A., and Gross, M. Long-chain fatty acids increase cellular dopamine in an immortalized cell line (MN9D) derived from mouse mesencephalon, Neuroscience Letters 376, 35-39, 2005.

Patent Application:

Provisional Patent Application Entitled: OLEIC ACID AS A THERAPEUTIC FOR TREATING NEUROLOGICAL DISEASES BY by Alfred Heller, Martin Gross and Lisa Won. Reference ARCD: 411USP1 filed on August 23, 2004.

CONCLUSIONS

Parkinson's disease remains a major world-wide health problem particularly in the aging population. In North America alone, over one million individuals suffer from the disease experiencing severe motor dysfunction with muscle rigidity, bradykinesia and tremor. The motor disabilities are a result of degeneration of a dopaminergic projection arising in the mesencephalon and providing an extensive innervation to the corpus striatum a subdivision of brain subserving extrapyramidal motor function. Therapeutic interventions, therefore, which can prevent or retard dopaminergic cell loss, increase sprouting of dopaminergic axons or upregulate the phenotype of remaining dopaminergic nigrostriatal neurons have the potential for reversing the motor deficits.

Given that Parkinson's disease is a degenerative disorder involving loss of the dopaminergic neurons of the nigrostriatal projection, considerable interest has focused on the possible therapeutic role of trophic agents. We have purified two types of chemically distinct dopaminergic stimulatory factors from the lysate of immortalized, striatal X61 cells. One type of factor could be extracted from the cell lysate by isoamyl alcohol/chloroform and was identified to be composed of primarily oleic acid. The other type of factor was water-soluble and consisted of ethanolamine and phosphoethanolamine. Our experimental results indicate that both types of dopaminergic stimulatory factors elevate the dopamine content of mesencephalic-derived MN9D cells through an increase in storage capacity, possibly via a membrane fusion mechanism, rather than by enhancing neurotransmitter synthesis. These findings suggest a novel mechanism of action by which such factors regulate the dopaminergic phenotype. Despite the fact that developing, fetal primary dopaminergic neurons in reaggregate culture are unresponsive to ethanolamine in terms of increased phenotypic expression, it remains open whether phospholipid precursor therapy may be of some utility in neurodegenerative disorders, such as Parkinson's disease, given the need of neurons to repair damaged cell membranes.

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List of Personnel Receiving Pay from the Research Effort

Nancy Bubula
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Program Number: 299.17 **Day / Time:** Sunday, Nov. 9, 1:00 PM - 2:00 PM

Enhanced survival of dopaminergic neurons induced by a partially purified cell lysate fraction from striatal derived hybrid monoclonal cells.

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We have previously reported that the lysate of an immortalized, monoclonal cell line (X61) derived from embryonic murine corpus striatum is capable of increasing the dopamine (DA) content of a mesencephalic monoclonal hybrid cell line expressing a DA phenotype (MN9D) as well as three-dimensional reaggregate cultures containing primary DA neurons. The present experiment was conducted to determine whether the rise in DA levels in the reaggregate cultures seen with exposure to X61 cell lysate or with a partially purified, low molecular weight (<5 kDa) ultrafiltrate fraction of the lysate (UF4) is due to an increase in the survival of DA neurons. Treatment of mesencephalic-tectal reaggregate cultures containing DA primary neurons grown in the absence of target cells over a two week period with X61 lysate or UF4 ultrafiltrate resulted in significant elevations of reaggregate DA levels (+32%) and of the DA metabolite, homovanillic acid, in the media (1.8 to 3.9-fold). Quantitation of DA cells, visualized using tyrosine hydroxylase immunocytochemistry, in the reaggregates showed an increase in the density of DA neurons in X61 lysate (2.9-fold) and UF4 (2.0-fold) treated cultures. The results demonstrate that X61 cell lysate or partially purified fractions from this material (i.e., UF4) contain a factor(s) capable of preventing DA cell loss which occurs in the absence of target cells. Such a factor(s) may have utility in the investigation and treatment of Parkinson's disease in terms of elevation of transmitter level in surviving cells, prevention of progressive cell loss and as an adjunct to DA cell survival in fetal transplantation.

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Dopaminergic Stimulatory Polypeptides from Immortalized Striatal Cells

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KEYWORDS: dopamine; serotonin; trophic factor; cell lines; reaggregate culture; mesencephalon; corpus striatum; nigrostriatal projection

Somatic cell fusion has been utilized as an approach to the immortalization of neurons for the purpose of obtaining monoclonal cell lines expressing neurotrophic factors.[1] Such fusions have permitted the generation of monoclonal hybrid cells derived from neurons of the nigrostriatal projection expressing specific transmitter phenotypes.[1–3] The cells include a striatal cell line (X61) that served as a source of possible trophic agents[3] and a mesencephalic cell line (MN9D) expressing a dopaminergic (DA) phenotype that was used as a test object.[2]

Cell lysates of the striatal X61 line have been shown to contain factors that have a stimulatory effect on both immortalized DA hybrid cells and primary DA neurons.[4] The DA stimulatory activity resides in a low-molecular-weight polypeptide fraction of less than 5 kDa.

The effect of this polypeptide fraction was examined on primary neurons using the three-dimensional reaggregate system that permits culture of mesencephalic DA and serotonergic (5-HT) cells in the presence or absence of appropriate target cells.[1] In the absence of target cells (mesencephalic-tectal aggregates), no axonal arbors are formed and most monoaminergic neurons disappear, presumably secondary to cell death. Some neurons survive and form fairly large processes, which appear to be dendritic in character and make autotypic connections with other DA neurons. We have already reported on the effect of the crude X61 stimulatory factor on DA neurons in such cultures in terms of increased DA content.[4] Here we describe the effect of the partially purified stimulatory factor on the morphology of both DA and 5-HT neurons by means of immunocytochemical methods.

A partially purified preparation (UF4) from X61 cell lysate was added (20 μL/mL) to the aggregate culture medium from day 1 to day 15 of culture. Aggregate sections were examined for DA neurons using an antibody against tyrosine hydroxylase, and

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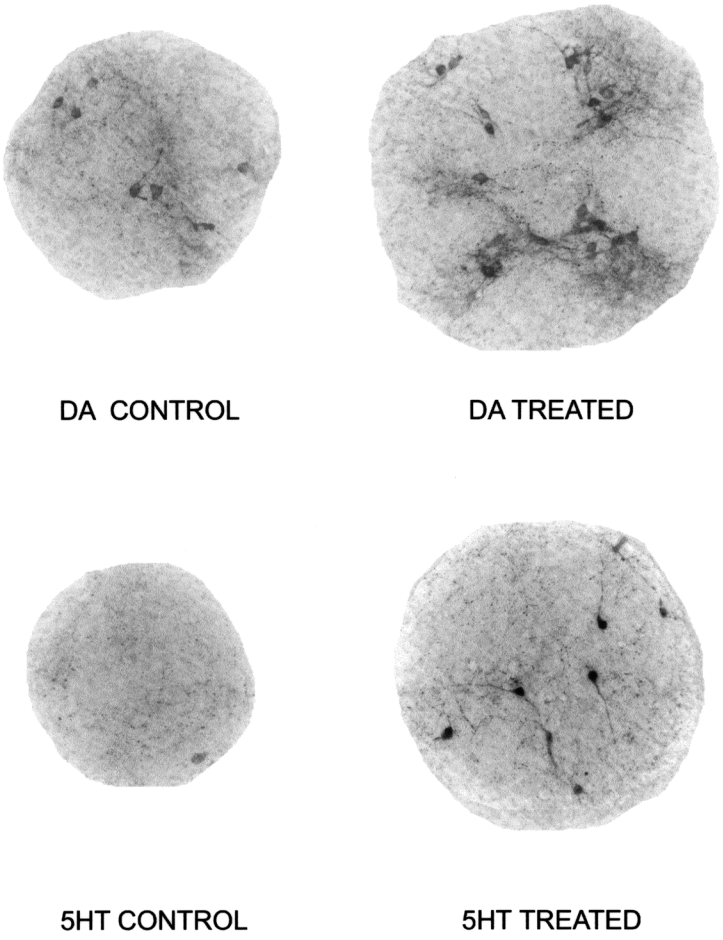

FIGURE 1. Immunocytochemical visualization of monoaminergic neurons in mesencephalic-tectal aggregates following treatment with UF4, a partially purified fraction obtained from the lysate of an immortalized clonal hybrid cell (X61) derived from embryonic murine corpus striatum.

for 5-HT neurons by an antibody against 5-HT. The results of this experiment are shown in FIGURE 1. With UF4 treatment, substantial numbers of densely stained DA neurons with extensive processes are observed. By contrast, while DA neurons are present in the untreated controls, such dense groupings of heavily stained cells with extensive processes are, at best, extremely rare. UF4-treated aggregates also contain 5-HT neurons and axons that are more densely stained than the cells observed in the untreated controls. Neurochemical analysis of the aggregates revealed a 30% increase in aggregate DA ($P < 0.001$) and a 52% increase in aggregate 5-HT follow-

ing treatment with the UF4 partially purified preparation. Homovanillic acid, a major metabolite of DA, was increased by 75% ($P < 0.001$) in the media from UF4-treated aggregates.

A low-molecular-weight polypeptide fraction, obtained from lysate of immortalized monoclonal cells derived from the striatum, is, therefore, capable of increasing DA levels of both a monoclonal cell line (MN9D) and primary DA and 5-HT neurons in three-dimensional reaggregate culture. In addition, the polypeptide fraction increases the immunocytochemical staining of both cell bodies and processes of the monoaminergic neurons. Purification and sequencing of the active polypeptides will permit assessment of their efficacy in the reversal of the motor dysfunction that occurs secondary to degeneration of the DA nigrostriatal projection.

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Enhanced survival of primary murine dopaminergic neurons induced by a partially purified cell lysate fraction from mouse-derived striatal hybrid monoclonal cells

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Abstract

Lysates of X61, a striatal-derived cell line, and a partially purified preparation from the lysate (UF4) contain a factor(s) capable of increasing the dopamine content of a mesencephalic-derived dopaminergic cell line (MN9D) and of cultures containing primary dopaminergic neurons. Treatment of cultures containing dopaminergic primary neurons grown in the absence of target cells over a 2 week period with X61 lysate or UF4 resulted in an elevation of dopamine levels of the cultures and of media homovanillic acid as well as a 2.0-fold (UF4) to 2.9-fold (X61 lysate) increase in the density of dopaminergic neurons in treated cultures. The results suggest that the activity factor derived from X61 is capable of preventing dopaminergic cell loss which occurs in the absence of dopaminergic target cells of the corpus striatum.

Keywords: Dopamine; Nigrostriatal; Trophic factor; Parkinson's disease; Cell lines; MN9D; Reaggregate culture

We have previously reported that the lysate of an immortalized, monoclonal cell line (X61) derived from the striatum is capable of increasing the dopamine (DA) content of a mesencephalic monoclonal hybrid cell line derived from the mesencephalon expressing a dopaminergic phenotype (MN9D) [3]. This lysate also increases the DA content of three-dimensional reaggregate cultures containing primary dopaminergic neurons [3]. The reaggregate cultures consist of fetal mesencephalon co-cultured with optic tectum, an area of brain which does not receive a dopaminergic innervation. Under these circumstances, fewer dopaminergic neurons are observed, possibly secondary to cell death, as compared to the quantitative survival of such cells in reaggregate cultures containing dopaminergic striatal target cells [2]. The present experiment was conducted to determine whether the rise in DA levels in the reaggregate cultures seen with exposure to X61 cell lysate or with a partially purified, ultrafiltrate fraction of the lysate (UF4) [4] was associated with an increase in the survival of dopaminergic neurons in the absence of target cells.

Reaggregate cultures were prepared from embryonic day 14 C57BL/6 mouse brains by dissecting the mesencephalic tegmentum (containing developing dopaminergic neurons) and the tectum (a region to which dopaminergic neurons do not project) (see Ref. [6] for method details). The tissues were dissociated into single cell suspensions and counted using a hemacytometer. A total of 3.25 million mesencephalic cells were dispensed along with 6.5 million tectal cells into 25 ml Erlenmeyer flasks containing 3.5 ml of initial culture medium composed of Basal Medium Eagle's, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin (5000 units penicillin/5000 μg streptomycin) and 0.025% (w/v) deoxyribonuclease I. The flasks were prepared and placed into a rotatory incubator where reaggregates formed over the course of 24 h. After the first 24 h, the medium was removed and replaced with fresh medium containing horse serum instead of fetal bovine serum. At this time, the reaggregates were treated with X61 cell lysate (12.5 μl/ml; $n = 5$ flasks), UF4 (20 μl/ml; $n = 6$ flasks) or with appropriate volumes of phosphate buffered

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saline (PBS) vehicle (12.5 μl/ml for X61 cell lysate ($n = 5$) or 20 μl/ml for UF4 ($n = 6$) treatment). The culture media were changed every 2 days and fresh amounts of X61 cell lysate, UF4 or PBS were added. After 8 days in culture, reaggregates from the flasks for a given treatment group (X61, UF4 or PBS) were combined and redistributed to five or six experimental flasks. The pooling of reaggregates and their re-distribution into experimental flasks reduces the variance among flasks subjected to the same experimental treatment [6]. The cultures were maintained in this manner for 15 days at which time reaggregates and media were collected and biochemical and histological examinations were conducted. For this experiment, culture media were prepared using sera which had been previously dialyzed to remove endogenous serotonin [6].

X61 cell lysate was prepared by sonicating X61 cells in PBS at room temperature. Purification of the active material from the lysate was facilitated by the finding that the dopaminergic stimulatory activity can be largely converted from a high molecular weight protein fraction to a much lower molecular weight protein fraction when the lysate is allowed to incubate at room temperature for up to 2 days. Amicon YM-5 membranes (5000 Da mol. wt. cut-off) were utilized to separate a low molecular weight stimulatory fraction (UF4) from the X61 lysate. After sonication, the lysate was concentrated by pressure filtration and allowed to 'autodigest' at room temperature overnight. The next morning, the lysate was diluted with PBS to its initial volume and re-concentrated by pressure filtration. After an additional overnight 'autodigestion' and re-dilution on day 2, the lysate was re-concentrated once more, generating the UF4 ultrafiltrate fraction. The conversion of the activity from high to lower molecular weight fractions, in all likelihood, represents an enzymatic breakdown of higher molecular weight material. Active lower molecular weight fractions are stable at room temperature despite the finding that the crude cell lysate activity is labile to boiling [3]. The heat lability may represent a precipitation or coagulation of protein entrapping the activity. Dopaminergic stimulatory activity (i.e. the ability to increase cellular DA content) of the ultrafiltrates obtained from each daily filtration was assessed as previously described [3] using a mesencephalic-derived, dopaminergic cell line, MN9D. Of the ultrafiltrates obtained from each daily filtration, the UF4 ultrafiltrate (following 48 h incubation at room temperature) was the most stimulatory and, therefore, was the preparation used to treat the reaggregate cultures described below. The stimulatory activity in UF4 is approximately equal to that of the initial X61 lysate, and UF4 is approximately 60-fold purer based on absorbance at 230 nm.

Samples of culture media and reaggregates were collected at 15 days from each flask for HPLC analysis of DA and homovanillic acid (HVA) levels. The protein content of the reaggregates was determined spectrophotometrically [12].

The density of dopaminergic cells in the reaggregates was visualized using immunocytochemical methods and estimated based on the procedure previously described by Heller et al. [6] and validated by computer simulation methods [8]. The cultures were fixed with 4% paraformaldehyde, embedded in gelatin and sectioned (50 μm) with a vibratome. Tyrosine hydroxylase (TH) immunocytochemistry was performed on free-floating tissue sections using standard peroxidase anti-peroxidase techniques. Briefly, the number of TH-positive cells from a given flask was estimated by counting the cells from a random selection of 30 sections. A digital image was captured of each of the 30 reaggregate sections used for counting with a Nikon Coolpix 995 digital camera. The area (in pixels) of each reaggregate section was obtained using Adobe Photoshop 7.0. The pixel area from each reaggregate section was converted to square microns and multiplied by 50 (section thickness) to obtain the sectional volume. The density of TH cells was obtained by dividing the number of TH cells counted by the sum of sectional volumes from the 30 reaggregate sections.

In the case of reaggregates treated with X61 cell lysate or the UF4 ultrafiltrate, it was apparent from gross inspection that many of the reaggregate sections contained considerably more TH-positive neurons than the corresponding PBS controls (shown for UF4 treatment in Fig. 1). The results of the quantitative cell counting and neurochemical analysis of reaggregate tissue and media are presented in Table 1.

It is clear from this study that the X61 cell lysate as well as the UF4 ultrafiltrate are capable of preventing a loss of dopaminergic neurons in the absence of dopaminergic target cells in reaggregate culture. The neurochemical changes observed most probably are a result of the increased density of neurons expressing a dopaminergic phenotype in the treated reaggregate cultures. While DA is only increased by 25–30%, this may be a reflection of the immaturity of the neuronal population of the reaggregates. In addition, since no target cells were present in mesencephalic-tectal reaggregates of the current study, axonal process formation

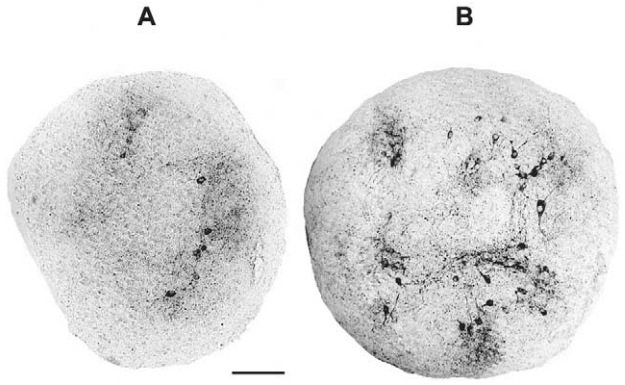

Fig. 1. Sections of mesencephalic-tectal reaggregates with dopaminergic neurons visualized by tyrosine hydroxylase (TH) immunocytochemistry. Microscopic examination of section A (PBS-treated reaggregate) showed that it contained 11 TH labeled cells while section B (UF4-treated reaggregate) contained 35 such cells. Scale bar: 100 μm.

Table 1
Effect of X61-derived dopaminergic stimulatory factor(s) on survival of dopaminergic neurons

	TH cell density	Reaggregate DA	Media HVA
Ultrafiltrate treatment			
UF4	563 ± 131/mm^3*	10.4 ± 0.02***	92.9 ± 1.0***
PBS	196 ± 48/mm^3	7.9 ± 0.22	53.1 ± 0.1
Cell lysate treatment			
X61 cell lysate	839 ± 85/mm^3**	8.5 ± 0.1***	185.0 ± 20.3***
PBS	415 ± 99/mm^3	6.4 ± 0.3	47.5 ± 1.7

Values given are the mean ± SEM of $n = 5$ for the UF4/PBS and $n = 6$ for X61 cell lysate/PBS treatment. Reaggregate dopamine (DA) is expressed as ng/mg protein. Media homovanillic acid (HVA) is expressed as ng/ml of media/mg reaggregate protein. Significantly different than PBS control: *$P < 0.025$, **$P < 0.01$, ***$P < 0.001$.

is at best minimal, limiting DA storage [10]. The dopaminergic neurons within the mesencephalic-tectal reaggregates are, however, clearly neurochemically active and release DA as evidenced by the 1.8- (treatment with UF4) to 3.9-fold (treatment with X61 cell lysate) increase in media content of the DA metabolite, HVA, which provides an estimate of released transmitter (Table 1).

Anatomic loss of dopaminergic neurons of the nigrostriatal projection is a central feature of Parkinson's disease (for review see Ref. [9]). It is clear that one cause of such degeneration is a loss of contact of the mesencephalic dopaminergic neurons with their striatal targets as evidenced by the fact that lesions which transect the projection of dopaminergic neurons to the striatum result in a loss of cellular components of the substantia nigra [11].

It is a reasonable assumption that in the absence of striatal target cells (mesencephalic-tectal reaggregates) there is an actual loss of the dopaminergic neurons as occurs in vivo following various insults, but the possibility that the cells are present, but no longer express a dopaminergic phenotype cannot be excluded. In either case, there are clearly fewer neurons expressing a dopaminergic phenotype in mesencephalic-tectal as compared to mesencephalic-striatal cultures. The three-dimensional reaggregate system, therefore, provides a reasonable model system for the examination of agents capable of preventing dopaminergic cell loss following either loss or separation from dopaminergic target cells.

X61 cell lysate or partially purified fractions from this material (i.e. UF4 ultrafiltrate) contain an activity capable of increasing the DA content of dopaminergic MN9D monoclonal hybrid cells or of three-dimensional reaggregates containing primary dopaminergic neurons in the absence of targets [3]. As demonstrated here, it would appear that this latter effect represents an actual increase (2.0- to 2.9-fold) in the density of dopaminergic neurons surviving in the cultures. The results obtained with the X61-derived dopaminergic stimulatory factor(s) are quite similar to a previous experiment in which we demonstrated that the addition of fetal striatal membrane preparations to mesencephalic-tectal reaggregates resulted in an increased survival of dopaminergic neurons, in some cases almost to the extent of the survival achieved with striatal cells [5]. The result suggested that 'dopaminergic survival factors' associated with membranes were present in the striatum. We did not pursue this finding since the reaggregate system was much too lengthy an assay procedure for the purposes of purification of the cellular-derived activity. Instead, we turned our attention to the use of somatic cell hybridization methods to produce the current monoclonal hybrid dopaminergic and striatal cells [1,7,13]. The basic hypothesis was that the monoclonal striatal cells, such as X61, would produce dopaminergic 'trophic' factors, and the monoclonal dopaminergic cells, MN9D, would serve as a test object for such factors. The data provided here would appear to justify that approach.

The cell-saving activity present in X61 cell lysate is obviously of considerable interest. We have previously demonstrated that the X61 lysate does not contain a significant number of the known trophic factors for dopaminergic neurons [3]. The UF4 ultrafiltrate factor(s) represents dopaminergic stimulatory activity which is less than 5000 Da. The activity in UF4, capable of increasing MN9D DA content, has now been purified some 50,000-fold by gel filtration and charcoal treatment (unpublished observations) and will be tested with regard to its ability to prevent dopaminergic cell loss in the absence of target cells in three-dimensional reaggregate culture as seen with ultrafiltrate (UF4) preparations.

The exact chemical nature and mechanism of action of the X61 cell lysate activity is still under investigation. Demonstration and concentration of the activity was made possible by the availability of an appropriate test object, the dopaminergic MN9D cell. The effect of the lysate activity on such monoclonal cells is to increase DA content. As demonstrated here, the biological effect of the partially purified lysate activity includes the ability to prevent the loss of primary dopaminergic neurons which occurs in the absence of target cells. The lysate activity has obvious utility in the investigation and treatment of Parkinson's disease in terms of elevation of the transmitter level in surviving cells, prevention of progressive cell loss and as an adjunct to dopaminergic cell survival in fetal transplantation.

Acknowledgements

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Long-chain fatty acids increase cellular dopamine in an immortalized cell line (MN9D) derived from mouse mesencephalon

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Abstract

The lysate of an immortalized monoclonal cell line derived from the striatum (X61) contains a dopaminergic stimulatory activity that is capable of increasing the dopamine content of an immortalized mouse mesencephalic cell line (MN9D) which expresses a dopaminergic phenotype. Purification of an isoamyl alcohol extract of this lysate and subsequent identification by NMR spectroscopic analysis demonstrated that the dopaminergic stimulatory activity contained within the lysate was a mixture of 80–90% *cis*-9-octadecenoic acid (oleic acid) and 10–20% *cis*-11-octadecenoic acid (*cis*-vaccenic acid). The effect of oleic acid on MN9D dopamine is a prolonged event. MN9D dopamine increases linearly over a 48 h period suggesting the induction of an increased dopaminergic phenotype in these dividing cells. The ability to increase MN9D dopamine by oleic and *cis*-vaccenic acids is shared by a number of other long-chain fatty acids including arachidonic, linoleic, linolenic, palmitoleic, and *cis*-13-octadecenoic acid. The possibility that oleic or other relatively innocuous fatty acids might affect dopaminergic function in primary neurons is intriguing with respect to possible therapeutic approaches to the treatment of dopaminergic cell loss and the motor sequelae of Parkinson's disease.

Keywords: Dopamine; Fatty acids; MN9D; Nigrostriatal; Parkinson's disease; Trophic factor

Immortalized monoclonal cells of the mouse nigrostriatal projection have been developed as an approach to the identification of substances which could regulate dopaminergic function and cell survival [1,14]. The dopaminergic MN9D cell line of mesencephalic origin and the X61 cell line of striatal origin were obtained by somatic cell fusion with the N18TG2 neuroblastoma which is lacking the hypoxanthine phosphoribosyltransferase enzyme [1,14]. We have previously demonstrated that striatal-derived monoclonal cells (X61) contain dopaminergic stimulatory substances which increase the dopamine content of MN9D cells [3]. Striatal cell lines (X61) provide a source for such substances and the mesencephalic-derived MN9D cell line provides a rapid assay method for detecting active molecules capable of modulating cellular dopamine. The crude cell lysate of X61 cells, as well as a partially purified ultrafiltrate preparation (UF4) of that lysate, also increases the dopamine content of primary dopaminergic neurons grown in reaggregate culture in the absence of target cells (i.e., mesencephalic cells co-cultured with tectum, a non-target region for dopaminergic neurons) as well as levels of homovanillic acid in the culture medium [15]. In such cultures, in which the majority of dopaminergic neurons are lost due to the absence of target cells, treatment with the crude lysate or UF4 ultrafiltrate results in a 2- (UF4) to 2.9- (X61 lysate) fold increase in the density of dopaminergic neurons in the treated cultures [15].

The UF4 ultrafiltrate contains active substances, probably peptides, of low molecular weight and high water solubility. It was, however, apparent that the bulk (two-thirds) of

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dopaminergic stimulatory activity present in the processed X61 cells resided in a fraction which did not pass through a YM-5 ultrafiltration membrane and was lipid soluble. The present study was conducted to determine the chemical nature and activity of this major fraction.

The UF4 ultrafiltrate was obtained from sonicated lysates of X61 cells which were allowed to "autodigest" at room temperature for 2 days and concentrated by pressure filtration through an Amicon YM-5 membrane (5000 Da molecular weight cut-off). Some form of digestion occurs in this process as evidenced by the fact that 2 days of incubation at room temperature results in the conversion of some (approximately 30%) of the dopaminergic stimulatory activity in the X61 cell lysate from a high molecular weight form to a size that can pass through a YM-5 membrane (see [15] for details).

The low molecular weight UF4 ultrafiltrate fraction contained significant dopaminergic stimulatory activity as assessed by effects on MN9D cells. However, the majority of the activity from the "autodigested" X61 cell lysate did not pass through the YM-5 ultrafiltration membrane. Approximately two-thirds of the activity resided in the material remaining on the high molecular weight side of the Amicon YM-5 membrane and is referred to as "X61 concentrate". This X61 concentrate was subsequently extracted with 2 M NaCl followed by a 1:1 mixture of isoamyl alcohol/chloroform. The isoamyl alcohol/chloroform extract was shown to contain materials capable of increasing MN9D dopamine levels. This activity is not extractable from fresh X61 cell lysate, but appears to require the autodigestion step with time for the activity to become liberated from some cell component and be available for organic extraction.

The isoamyl alcohol/chloroform soluble stimulatory activity was taken up by a C18 reverse phase column from a mixture of 70% acetonitrile/30% (0.05% trifluoroacetic acid in water) and then eluted by a linear gradient from the mixture to 100% acetonitrile. The active fractions from the column showed some absorbance at 215 nm, but the bulk of absorbance was seen in fractions devoid of activity, suggesting that the reverse phase separation resulted in considerable purification of the activity. The active fractions from the reverse phase column were then applied to a Phenomenex 5 μm, 50 Å Phenogel gel filtration sizing column. The dopaminergic stimulatory activity eluted from the Phenogel column in 100% acetonitrile within a single absorbance peak. The Phenogel fractions containing dopaminergic stimulatory activity were subjected to mass spectrographic analysis. Two peaks of high intensity were observed with molecular weights of 283 and 565.

NMR spectroscopy demonstrated that the single elution peak from the Phenogel column contained two chemical moieties, the greater of which constituted approximately 80–90% of the material (Fig. 1). The 600 MHz ^{1}H 1D NMR spectrum of the sample showed one major set of peaks and no significant minor peaks, suggesting a sample purity greater than 95%. The ^{13}C 1D NMR spectrum, however, showed two sets of peaks, indicating that the sample contained two species

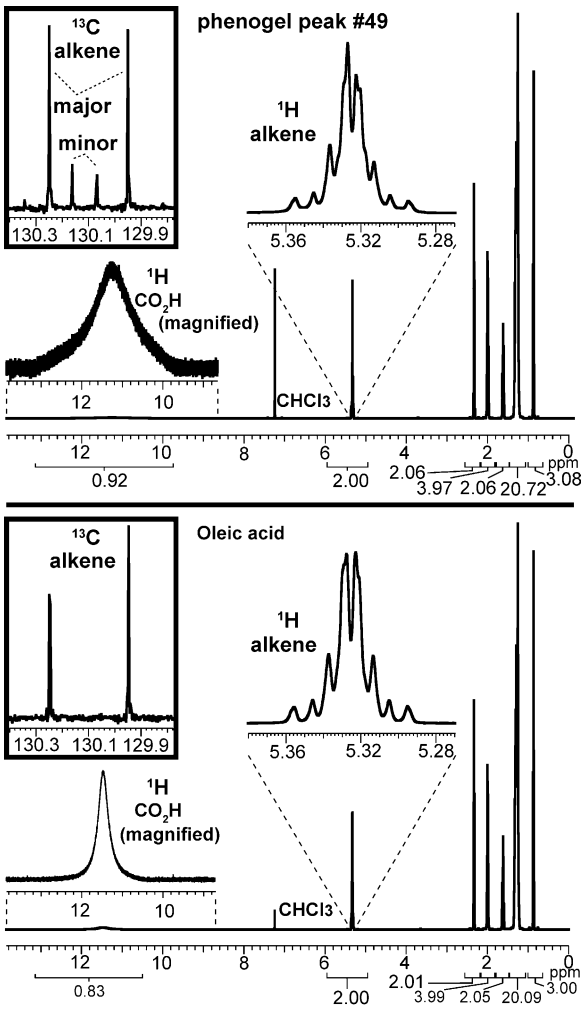

Fig. 1. The NMR spectra of peak #49 from the Phenogel column purified material (top) as compared to commercial oleic acid (bottom). The ^{1}H spectrum is shown at the bottom of each panel, and the peak integral values of the intensities are indicated below the axes. The carboxylic acid and alkene regions of the ^{1}H spectra are magnified and expanded. The alkene regions of the ^{13}C spectra are shown as insets. It is apparent that the Phenogel #49 sample contains one major and one minor species. The minor species was identified by NMR analysis to be *cis*-vaccenic acid.

so closely related that they gave rise to virtually identical ^{1}H NMR spectra. The minor component made up approximately 10–20% of the total sample, according to their relative intensities in the ^{13}C spectra. The positions of the ^{1}H and ^{13}C peaks in these spectra ruled out the possibility that the sample contains protein, peptide, DNA, RNA, carbohydrate, glycolipid, steroid, or other cholesterol-related molecules. The positions are instead consistent with those expected from a fatty acid. Comparison of the major species' spectra with ^{1}H and ^{13}C NMR spectra of various fatty acids showed that they were very similar to those of *cis*-9-octadecenoic acid (oleic acid). It remained uncertain whether the unsaturated bond was of *cis* or *trans* configuration. Pure *cis*-9-octadecenoic acid and *trans*-9-octadecenoic acid (elaidic acid) (Aldrich), and their ^{1}H and ^{13}C NMR spectra were compared to those of the sam-

ple. We found that the major species' peaks were essentially identical to those of the *cis* isomer (i.e., oleic acid) (Fig. 1).

A variety of compounds structurally related to oleic acid were obtained and examined by NMR spectroscopy to determine the identity of the minor compound in the sample. We found that the ^{1}H and ^{13}C NMR spectra of *cis*-vaccenic acid (*cis*-11-octadecenoic acid), which has the same empirical formula as oleic acid, were essentially identical to those of the minor species in the sample. Thus, our NMR data support the conclusion that the purified sample comprises a mixture of approximately 80–90% *cis*-oleic acid and 10–20% *cis*-vaccenic acid.

The NMR spectral analysis with known synthetic entities established the chemical composition of the majority of the purified material to be *cis*-9-octadecenoic acid (oleic acid). The mass spectroscopic analysis is in accord with the NMR data in that the molecular weight of 283 corresponds to that of oleic acid and/or *cis*-vaccenic acid and the larger sized 565 molecular weight species may well represent a dimerization of these long-chain fatty acids.

In order to determine whether the Phenogel purified material and synthetic oleic acid produce similar effects on MN9D cellular dopamine, MN9D cells were plated into six-well culture plates and cultured in Dulbecco's Modified Eagle's medium containing 5% (v/v) Fetal Clone III and 1% (v/v) penicillin–streptomycin (5000 units penicillin/5000 μg streptomycin). The cells were exposed to increasing concentrations of oleic acid or the Phenogel purified material, diluted in dimethylsulfoxide (DMSO), for 48 h and then collected for analysis of cellular dopamine content using high performance liquid chromatography. Protein content of the cultures was determined spectrophotometrically [11].

In agreement with the NMR data, the Phenogel purified material and synthetic oleic acid showed identical concentration–response in terms of increasing MN9D dopamine as shown in Fig. 2. The concentration–response of MN9D cells to oleic acid has been repeated in two other experiments and the results obtained were identical. Exposure of MN9D cells to concentrations of oleic acid or the Phenogel purified material greater than 124 μM produced either less of a dopaminergic stimulatory effect or was actually toxic to the cells. Thus, the response at 124 μM was considered to be maximal. The maximal effect of oleic acid and the Phenogel purified material in this experiment represents an approximate five-fold increase in dopamine level over controls. The EC_{50} for oleic acid is approximately 5.5×10^{-5} M. The effects of oleic acid are not secondary to either an increase in MN9D cell proliferation or differentiation. This issue was tested directly in a separate experiment in which dopamine levels, cell number and the state of cell differentiation were examined. MN9D cells were exposed to DMSO or 124 μM oleic acid for 48 h. An increase in dopamine of 8.3-fold was observed in this experiment. The protein content of MN9D cells exposed to oleic acid was essentially identical to that of the DMSO group and the number of cells in the oleic acid-treated group (0.89 ± 0.13 million cells, mean ± S.E.M., $n = 6$) was not significantly different from DMSO vehicle (1.05 ± 0.10 million cells, mean ± S.E.M., $n = 6$). The oleic acid-exposed cells showed none of the characteristics of differentiated MN9D cells, i.e. a reduction in cell number or increased process outgrowth [1].

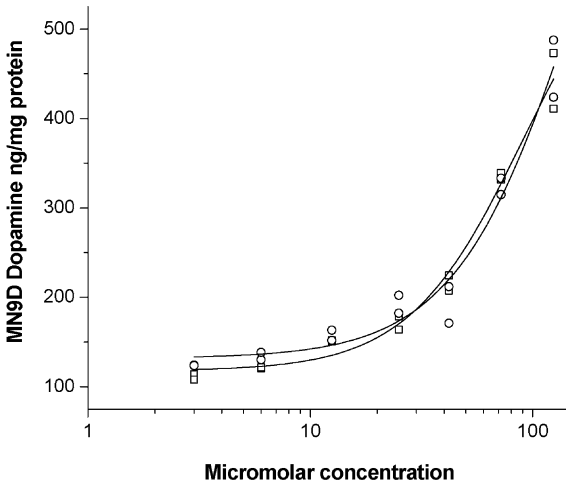

Fig. 2. Effect of 48 h exposure to increasing (log scale) concentrations (3–124 μM) of *cis*-9-octadecenoic acid (oleic acid, squares) or to the Phenogel column purified material from X61 cells (circles) on MN9D dopamine levels. MN9D dopamine level of DMSO vehicle control = 109.9 ng/mg protein.

Given that the assays were carried out in serum-containing medium and the known capacity of oleic acid to bind to serum proteins [8,12], the free EC_{50} for oleic may be considerably lower than this estimate. The level of oleic acid required to increase dopamine levels under serum-free conditions cannot be tested with MN9D cells since they do not grow well under conditions of low serum or serum-free medium. The MN9D cells, in addition, only permit the examination of effects on the catecholaminergic phenotype. The issue of whether oleic acid affects other transmitter phenotypes will require primary neuronal cultures.

While *cis*-vaccenic acid, the minor constituent of the Phenogel purified fraction, is active (see below), a comparison of the concentration–response curve of *cis*-vaccenic with oleic acid on MN9D dopamine, suggests that *cis*-vaccenic acid has a slightly lower potency.

A comparison of the effect of oleic versus the *trans* isomer (elaidic acid), using 11 concentrations ranging from 0.3 to 353 μM, on MN9D dopamine revealed that elaidic acid showed only minimal activity (less than 10% elevation in dopamine even at the highest concentration of 353 μM) (data not shown).

A number of more detailed studies have been initiated on the effect of oleic acid in increasing MN9D dopamine. The first of these studies, a time course on the effect of the Phenogel purified material and oleic acid, demonstrates a linear increase in MN9D dopamine over 48 h (Fig. 3). The effect of both the known compound and the Phenogel purified material in this experiment is impressive, resulting, after 48 h, in at

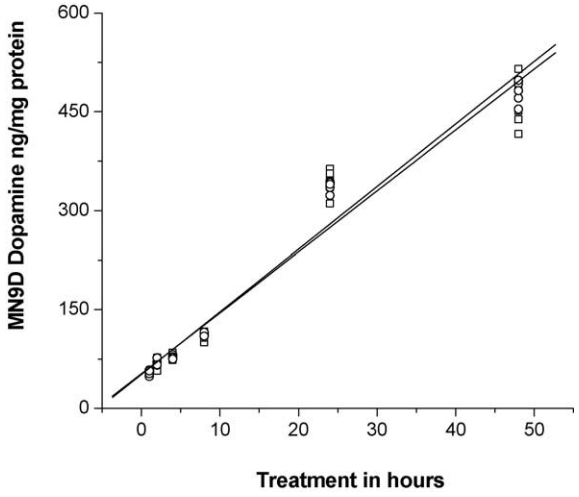

Fig. 3. Time course of the effect of exposing MN9D cells to 124 μM of *cis*-9-octadecenoic acid (oleic acid, squares) or 124 μM of the Phenogel column purified material from X61 cells (circles) on cellular dopamine levels. $N = 6$ cultures per time point. MN9D dopamine level (ng/mg protein) of DMSO vehicle control for the various time points: 1 h, 54.1; 2 h, 63.3; 4 h, 63.6; 8 h, 65.3; 24 h, 77.3; 48 h, 67.5.

least an 8.5-fold increase in MN9D dopamine as was seen in the experiment on cell proliferation described above. Given that the MN9D cells, which are fusion products of mesencephalic cells and the N18TG2 neuroblastoma, are doubling every 24 h, this result would suggest that the effect of the active chemical is to induce an increase in the dopaminergic phenotype of MN9D cells. If that is the case, then the effect should be present in the daughter cells as they appear.

As part of this study, a limited structure–activity analysis was conducted using a variety of common long-chain unsaturated fatty acids. It is clear from this study, that the ability of oleic acid to increase MN9D dopamine content over a 48 h period is shared by a number of other long-chain fatty acids containing one to four double bonds, as shown in Table 1. Concentration–response curves were determined for each of the indicated compounds and compared to oleic acid using seven concentrations over a range of 3–124 μM. The majority of the compounds tested were active, but in most cases, a maximal response was not obtained even at 124 μM. For this reason, Table 1 provides the cellular MN9D dopamine level (ng/mg protein) following 48 h of treatment with 124 μM of each respective compound, and the amount of increase this represents over the DMSO vehicle control.

Table 1
Effect of unsaturated long-chain fatty acids on MN9D cellular dopamine

Compound	Dopamine (ng/mg protein)	Fold-increase over DMSO control
Oleic acid	332	4.8
Arachidonic acid	365	5.3
Linoleic acid	300	4.3
Linolenic acid	297	4.3
Palmitoleic acid	280	4.0
cis-Vaccenic acid	264	3.8
cis-13-Octadecenoic acid	134	1.9
Petroselenic acid	92	1.3
Oleic anhydride	81	1.2

MN9D cells were treated for 48 h with 124 μM of each of the compounds indicated above and then cellular dopamine content determined. All compounds were dissolved in dimethylsulfoxide (DMSO). Dopamine level of DMSO vehicle control = 69 ng/mg protein.

Two of the compounds tested showed only minimal effects: oleic anhydride, formed by the fusion of two molecules of oleic acid with the splitting out of water, and petroselenic acid, an 18 carbon monoenoic acid in which the double bond is at position 6, in contrast to oleic acid with a double bond at position 9. The remaining seven long-chain fatty acids were all active and included both monoenoic acids (oleic, palmitoleic, *cis*-vaccenic, and *cis*-13-octadecenoic acid) and polyenoic acids (linoleic with two double bonds, linolenic with three double bonds and arachidonic with four double bonds).

Thus, it is clear that a variety of long-chain fatty acids are capable of increasing the dopamine content of a mesencephalic-derived immortalized monoclonal cell line expressing a dopaminergic phenotype. While the number and variety of fatty acids tested is too limited to reach systematic conclusions regarding the optimal structure necessary to produce an increase in MN9D dopamine, the ability to significantly elevate MN9D dopamine content appears to depend on the presence of a carboxylic acid group and the relative position of an unsaturated double bond. Both petroselenic and oleic acid are C18 fatty acids. Petroselenic acid, which shows minimal effects (see Table 1), has a *cis* double bond that is located three carbons further from the terminal carboxylic acid than that of oleic acid, thus suggesting that the length of the side chain may be a critical determinant of effect.

The MN9D cell line has served as a useful test object for monitoring the presence and purification of dopaminergic stimulatory activities from lysate of immortalized monoclonal cell lines (X61) derived from the striatum. In addition, at least with respect to the small, water-soluble activity (UF4) which appears to be peptide in nature, effects on the MN9D line were predictive of the ability of this substance to increase the dopamine content of primary dopaminergic neurons and prevent their loss in the absence of striatal targets [15]. Whether the increases in MN9D dopamine seen following treatment with known long-chain fatty acids described here will be replicated on primary dopaminergic neurons is obviously a critical question and is currently being examined using three-dimensional reaggregate culture in a similar manner to the previous studies [3,15].

The striatal lines were developed specifically for the purposes of providing a substantial source of monoclonal cells which could be probed for substances that might influence dopaminergic function, either with respect to dopamine levels, cell survival, or the maintenance of the phenotype. It is worth noting that while an isoamyl alcohol/chloroform extract of a lysate concentrate of the X61 cell yielded a fraction capable of markedly increasing MN9D dopamine, that fraction on purification turned out to contain

long-chain fatty acids (oleic and *cis*-vaccenic acid) which could have been extracted from any number of sources. The presence of these active moieties in the X61 cell line, however, directed our attention to their isolation and purification.

Although this is the first description of an ability of long-chain fatty acids to increase cellular dopamine, there are many reports of effects of oleic and other unsaturated fatty acids on neuronal function. Oleic acid is an activator of protein kinase C activity [4,7] and, as has been reported, its synthesis has been linked to neuronal differentiation during development [6,13] and the promotion of axonal growth and induction of MAP-2 expression (microtubule associated protein-2), a marker of dendritic differentiation [9]. Arachidonic acid markedly stimulates, in a dose-dependent fashion, the spontaneous release of dopamine in purified synaptosomes from rat striatum and inhibits dopamine uptake [5]. In addition, reduced dietary intake of omega-6 (arachidonic acid) and omega-3 (docosahexanoic acid) fatty acids in piglets during the first few weeks of postnatal life has been shown to result in lower brain monoamine concentrations which can be reversed upon supplementation with adequate levels of these fatty acids [2]. We are not aware of any reports of beneficial effects of dietary unsaturated fatty acids on the pathogenesis or clinical course of Parkinson's disease.

While the treatment of the motor sequelae of Parkinson's disease has received intense study for over four decades with some very notable successes [10], it remains clear that additional treatment modalities would be helpful and are being sought. It is intriguing in this regard that a long-chain fatty acid such as oleic acid, which is essentially a benign dietary material, can markedly increase the dopamine content of a cell expressing a dopaminergic phenotype. If it should prove to be the case, that oleic acid or other active fatty acids increase the dopamine content of primary dopaminergic neurons, as we previously reported for the crude X61 lysate and partially purified ultrafiltrates (UF4), the long-chain fatty acids may provide an interesting addition to pharmacological approaches to the investigation of the disease and its treatment.

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