Analysis of quantification methods used for cell viability, cell morphology, and synaptic formation in modeling HIV associated dementia in primary neuronal cultures.

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Change is inevitable, changes in neuronal function occur in physiologic and pathologic processes. The ability to reliably analyze and quantify those changes in neuronal morphology and function has been an important part of technical developments in Neuroscience. A key innovation in the Neuroscience was the development of primary neuronal cultures. Primary neuronal cultures allow neurons to be dissociated and studied as individual components. The study of specific pathologic processes associated with neurodegeneration have benefited greatly from the development and characterization of dissociated primary neuronal cultures. Human Immunodeficiency Virus can lead to a neurodegenerative process. Establishing a consistent model for studying the effects of HIV infection in the brain has provided a unique challenge. The use of analysis of quantification of neuronal changes in dissociated primary neurons modeling HIV dementia has proven useful. As the study of this disorder continues the characterization of the model system will become increasing important. This review will focus on analysis of specific techniques used to quantify specific changes in neurons in this model system. As this field moves forward it will be important to specifically focus on techniques involved in cell viability, morphologic changes, and synaptic formation.

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Introduction

In 2005 it was reported that over 38 million people were infected with HIV (2006). Human immunodeficiency virus (HIV) associated dementia affects a significant portion of HIV infected patients (Fischer-Smith and Rappaport, 2005)of that develop the Acquired Immunodeficiency Syndrome (AIDS). HIV invades the CNS early in infection (Kramer-Hammerle et al., 2005). Despite its prevalence, the exact mechanism causing the HIV dementia is not known. This is a degenerative disorder that affects neurologic function but does not directly infect neurons (Kaul et al., 2001). The main reservoir of the virus in the brain appears to be resident macrophages and microglia (Kaul et al., 2001). The presence of multinucleated giant cells and microglia nodules are distinct pathologic findings in HIV associated dementia, which were first characterized in human tissue (Wiley et al., 1986, Merrill, 1991). Despite several over 20 years of research in the field, the effects of HIV on macrophage activation are still unclear. Although, early *in vitro* studies involving dissociated neurons were done to determine the cause of neurodegeneration (Brenneman et al., 1988, Kaiser et al., 1990), more *in vitro* studies are needed to study this debilitating disease. The biggest limiting factor for *in vitro* studies on HIV-induced pathology is the standardization of quantifying cell viability.

Dissociated primary neuronal cultures are a widely used *in vitro* system. In dissociated neuronal cultures investigators can manipulate multiple factors that affect neuron viability and function. Dissociated primary neuronal cultures provide controlled experimental conditions with limited cellular interactions at the expense of some in vivo architecture. The development and expansion of techniques for the reliable quantification of changes in the neuron, whether related to development or pathology, is a unique challenge in using dissociated primary neuronal

cultures. The greatest advantage of dissociated primary neuronal cultures is also a significant weakness. In this system the investigator has complete control over the molecules introduced and possible cellular interactions, which make the system artificial and the results of these experiments are difficult to interpret without accompanying *in vivo* experiments (Johnson, 1995). HIV dementia is a chronic complex neurodegenerative disorder that occurs over years, a time course which cannot accurately be modeled in neuronal culture. Nevertheless, specific aspects of development and neuronal function seen in vivo have been replicated in dissociated primary cultures, such as the ability to form synapses and produce and transmit neurotransmitters. (Ransom et al., 1977a, Ransom et al., 1977b, Dichter, 1980, Snodgrass et al., 1980, Swaiman et al., 1982, Bartlett and Banker, 1984a, b) Various studies have been done to examine cell viability, morphologic changes, and synaptic function in neurons exposed to acute toxins associated with HIV (Kaiser et al., 1990, Chauhan et al., 2003, Kim et al., 2008). The goal of this review is to analyze specific techniques used to quantify cell viability, identify neuronal morphologic changes, and identify synapses in dissociated neurons used to model aspects of HIV dementia.

Quantification of Primary Neuron Viability

Primary dissociated neuronal cultures are valuable for studying cell viability after exposure to specific acute insults. Neuron death is a process associated with HIV dementia progression along with dendritic injury (Masliah et al., 1997, Kaul and Lipton, 2006). This is a complex chronic neurodegenerative disorder that dissociated cultures cannot fully mimic, instead

they allow a snapshot of the affects after an acute incubation. Multiple methods have been used to quantify neuron viability in primary cultures in this system (Singh et al., 2005, Aksenova et al., 2006, Agrawal et al., 2007, Wang et al., 2007) . The four methods will be divided by their mechanisms of action, either membrane permeability or enzymatic activity.

Three of the more common methods of quantifying neuron survival and death are trypan blue (TB) uptake, propidium iodide (PI) uptake, and lactate dehydrogenase release (LDH). These three assays all work under the basic assumption that cellular injury corresponds to changes in membrane permeability(Johnson, 1995). Trypan blue is hydrophilic molecule that can be excluded from healthy cells; thus cells that allow ingress of trypan blue have lost the ability to maintain their cell membrane integrity and are considered non-viable in quantification (Walum et al., 1985). All cells that do not take up the dye cannot be considered healthy. However, as this assay is usually performed at a fixed time point, other cells maybe in the process of dying (Johnson, 1995, Aras et al., 2008). Propidium iodide uptake works under the same basic principles as trypan blue with the following exception: trypan blue crosses a compromised cell membrane and remains in the cytoplasm, while propidium iodide crosses the compromised cell membrane and binds to DNA. The binding of PI to DNA causes the dye to become fluorescent, which can be quantified using an imaging software, spectrophotometer or flow cytometry (Johnson, 1995, Cho et al., 2007). Trypan blue and propidium iodide require a manual analysis as the user is responsible for the selection of the field that is quantified and possibly the counts themselves, providing a greater level of detail about the system but also requiring a higher level of scrutiny. In dissociated neuronal cultures, propidium iodine has a high level of consistency between experiments (Bachis et al., 2003, Bachis and Mocchetti, 2004)

(Kaul and Lipton, 1999). LDH works in a different manner; it is still based on the idea that non-viable cells have a compromised cell membrane. In this assay instead of measuring entrance of a foreign dye in to the cell, measurements are taken of LDH released into the media. Actual measurements of LDH released into the supernatant are based on the reduction of (Nicotinamide adenine dinucleotide) NAD to NADH (Koh and Choi, 1987, Johnson, 1995).

Each of these three assays measures neuronal viability, but in slightly different ways. PI can be used with a neuronal marker, but it can also be used with imaging software and plate readers to allow for automated quantification. TB and PI allow for determination of cell death throughout multiple fields, a feature LDH assays lack. LDH assays do not allow for the identification of the types of cells that are dying, which can be problematic in mixed neuronal cultures. In HIV dementia the exact role of astrocytes in neurodegeneration is still unknown(Nottet, 1995), thus any preparation examining factors associated with HIV would require cell type identification. LDH assays are often used in HIV dementia models, but in these preparations glial growth in the neuronal cultures is inhibited. Though the cultures are all stained with a neuronal marker, the percentage of glial cells in the culture ranges (Trillo-Pazos et al., 2000, Jana and Pahan, 2004). The varied strengths and weaknesses of these assays explain why these techniques are often used in combination, and have been used to assess of neuron viability in HIV dissociated neuronal cultures

The second mechanism of quantifying neuronal viability that will be discussed in this review is the MTT assay. The MTT assay is an enzymatic assay that quantifies the reduction of yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) to an insoluble formazan product(Mosmann, 1983, Johnson, 1995, Aras et al., 2008). MTT is reduced to formazan by the mitochondrial protein succinate dehydrogenase. The reduction will only take place in living cells, dead cells cannot reduce MTT, and analysis of this assay is based on the color change from yellow to purple or blue. MTT assays are quantified using a spectrophotometer and have similar strengths and weaknesses to LDH assays. MTT assays can not differentiate various neuronal cell types and so in conditions with multiple cell types this assay alone cannot be used to specify neuronal viability. In previous studies of HIV neuron viability MTT produced fairly consistent results with a minimal about of range from similar conditions (Bachis et al., 2003, Bachis and Mocchetti, 2004)There is a level of automation to this assay that contributes to its reliability. This assay, quick and efficient assay for the quantification of neuronal viability, is often used in combination with any of the other three cell viability assays previously discussed.

Ultimately assessing the utility of these cell viability assays can be difficult given the slight differences in protocols that can alter the results, and these techniques have been proven throughout the literature. For a long term study on cell viability in HIV, MTT and LDH would be the ideal assays as they do not require fixation to perform, the type of long term study needed may be limited ironically to neuronal survival as neurons do live long enough to truly examine the chronic nature of this disorder. That limitation as well as the changes in cell architecture should reinforce the concern about relying on any single test and how the results should be interpreted in the results from an in vivo model.

Morphologic Identification in Primary Neuronal Cultures

Identification in this model system is usually done to confirm the result of another result and in HIV dissociated cultures rarely done as end to itself. In this section the focus will be on cell morphology, including techniques for identification of neurons and methods of quantifying changes in morphology. Initially this section will provide background on specific immunocytochemical markers such as microtubule associated protein 2 (MAP2), class III beta tubulin (Beta-3-Tubulin), and the microtubule associated protein Tau. Not only are all three markers related to microtubules and cytoskeleton structures within neurons, they all also label neurites. This section will also address how these markers are used to quantify morphologic features of HIV dementia in dissociated neurons. Immunocytochemistry is not the only method available to quantify morphologic changes in this system. In the final part of this section the lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and F-actin binding fluorescently labeled phalloidin will briefly be mentioned. DiI and phalloidin allow for the live cell imaging and thus work in a slightly different manner than previous mentioned neuron-specific markers. DiI and phalloidin do not bind specifically to neurons and so identification should be supplemented with immunocytochemistry or based on morphologic features in culture. DiI and phalloidin are currently not extensively used to cultures to study HIV dementia, but their utility suggests that they maybe useful in future studies.

Specific markers of neuronal development have become invaluable tools in studying neurons. Microtubules and associated proteins are structural building blocks of the neuronal cytoskeleton that may regulate stability of axons and dendrites (Matus, 1988). MAP2 interacts

with the tubulin portion of a microtubule to regulate microtubule stability. It is believed that MAP2 is expressed exclusively in neurons (Izant and McIntosh, 1980). Splice variants of MAP2 label more restricted cell population: MAP2c labels neurons and progenitor cells and MAP2ab labels only mature neurons. The specific MAP2 variants are rarely identified when MAP2 is used to identify neurons in culture, but the differences should be known (Garner et al., 1988, Matus, 1988, Svendsen et al., 2001). MAP2 is of particular interest because it not only seems to specifically identify neurons through immunocytochemistry, but it also seems to bind preferentially to dendrites on neurons (Matus et al., 1981, Caceres et al., 1984). These initial findings were all made in whole brain extracts or brain slices, but later studies carried out in dissociated neurons suggested that at the earliest time points of neuronal development MAP2 could bind to both axons and dendrites but after time MAP2 would bind specifically to dendrites (Caceres et al., 1986). In hippocampus dissociated primary neuronal cultures, MAP2 can be identified in all processes after the first few days in vitro (Caceres et al., 1986). This pattern of MAP2 axonal and dendritic staining dissipates by the first week returning to a selective dendritic staining pattern, the relevance of this timeline is important for any MAP2 dendritic staining designed to evaluate developmental landmarks in vitro in the initial days after establishing the cultures (Caceres et al., 1986).

MAP2 is used extensively to confirm whether a cell is a neuron in mixed cultures. MAP2 is frequently used as a measure to quantify toxicity by manually counting the number of MAP2 positive cells in randomly selected fields following a treatment (Jeohn et al., 1998). In assays that quantify toxicity through identification of any MAP2 positive staining it is important to distinguish these viability assays from assays quantifying other specific morphologic changes.

It is necessary to distinguish between viability studies based on calculating the total number of cells and morphology studies examining number of dendrites and complexity of arborization. The affects of HIV on dendritic arborization have been examined using MAP2 in dissociated neurons in culture (Iskander et al., 2004)(Zheng et al., 2001, Walsh et al., 2004). The effectiveness of these studies depends greatly on the quantification technique used. Some studies focus primarily on immunocytochemistry and pixel quantification, while others have used an ELISA based assay to quantify MAP2. The immunocytochemical studies prove a wealth of information of morphologic changes related to HIV related stimuli lacking in other studies. Previous studies in other systems have shown MAP2 can be used to quantify specific morphologic changes (Hartbauer et al., 2001, Monnerie et al., 2005). These results depend on the specificity of the antibody but also on the reliability of the marker. Recent findings suggest that oligodendrocyte processes can also be identified with MAP2 (Muller et al., 1997).

Microtubule associated proteins have historically been divided between the high molecular weight molecules to which MAP1 and MAP2 belong, the medium molecular weight molecules like Tau, and the small weight molecules (Matus, 1988). Tau has a molecular weight of between 55,000-65,000 daltons (Weingarten et al., 1975, Matus, 1988). Tau as with MAP2 has multiple isoforms. Tau isoforms 2-3 are believed to be expressed in early development in the brain while isoform 4 is expressed in adulthood and isoform 1 is expressed at much higher levels in the peripheral nervous system than in the brain (Dehmelt and Halpain, 2005). There is some dispute over the localization of Tau expression. Originally it was thought Tau was expressed only in axons(Binder et al., 1985), however, some groups have shown that Tau can be expressed in axons, soma, and dendrites in neurons(Papasozomenos and Binder, 1987). There is

also a question of neuronal type to consider as in axonogenesis in cortical and hippocampus neurons there does appear to be Tau localization in axons. (Binder et al., 1985, Kosik and Finch, 1987, Papasozomenos and Binder, 1987, Matus, 1988, 1990, Dehmelt and Halpain, 2005). These newer findings suggest that Tau can no longer be considered a marker exclusively of axons. There are findings to support this that suggest that in primary rat dissociated hippocampus neurons there are cells that stain multiple processes with Tau, though a single process may stain more intensely than the others (Kosik and Finch, 1987). Interestingly this paper also found that Tau stained post-natal day 1 oligodendrocytes that were MAP2 negative. These findings may serve to further confuse the issue, but importantly they serve as reminders of the caution that must be exercised in analyzing data. Tau is still considered a highly reliable target for identification and staining of axonal components of neurons (Svendsen et al., 2001).

Elevated Tau expression has been associated with certain neurodegenerative disorders and it has been suggested that axonal damage is common among neurodegenerative disorders (Andersson et al., 1999). Axonal damage has been found in association with HIV dementia (Tagliati et al., 1998). The majority of Tau studies have focused on in vivo correlations of Tau with HIV dementia (Brew et al., 2005)(Green et al., 2000)(Andersson et al., 1999)(Ellis et al., 1998). Tau staining has been used extensively in the morphologic assays of neurons in primary cultures, but also in analysis of certain neuropathological processes in vitro (Ferreira and Caceres, 1991, Combs et al., 1998, Takashima et al., 1998, Ruthel and Hollenbeck, 2000). Those studies have not been done in an in vitro model of HIV dementia suggesting an area of the field that is prepared for further exploration.

A common neuron specific marker used to visualize neurons is Beta-3-tubulin. Alpha and beta tubulin compose the building blocks of microtubules, part of the cytoskeleton in neurons. There are 6 isotypes of beta tubulin, and of those isoforms, class III Beta tubulin is expressed exclusively in neurons (Burgoyne et al., 1988, Sullivan, 1988, Lee et al., 1990a, Lee et al., 1990b, Ferreira and Caceres, 1992). Beta-3-tubulin is expressed in all neurites in dissociated primary neurons. This makes it useful for analysis of gross morphological changes but not useful for analysis of changes specific to axons or dendrites. Beta-3-tubulin immunocytochemistry is frequently used for the purpose of identification of neurons in culture (Coronas et al., 2000, Svendsen et al., 2001). In addition to being used simply to identify neurons in culture, Beta-3-tubulin is used to determine specific morphologic changes in neurons. (Colombo et al., 1996, Coronas et al., 2000, Roediger and Armati, 2003, Mundy et al., 2008). Beta-3-tubulin has been used to visual morphologic changes in HIV dementia dissociated neuronal cultures (Iskander et al., 2004). In preparing cultures from astrocytomas Beta-3-tubulin has been shown to stain other cell types (Katsetos et al., 2001, Svendsen et al., 2001). This seemingly aberrant staining has not been seen in dissociated primary neuronal preparations and does suggest that the Beta-3-tubulin expression and subsequent staining is dependent on differentiation state (Lee et al., 1990b, Katsetos et al., 2001). This finding requires a general level of caution in preparations of embryonic tissue at stages before neuronal stem cells have differentiated to neurons. Most preparation in this model comes from embryonic tissue.

The examination of neurite outgrowth and retraction addresses a key question in HIV dementia pathology (Zheng et al., 2001, Iskander et al., 2004, Walsh et al., 2004). Few studies have tried to address the question of altered neurite growth in a dissociated neuronal model of

HIV, though studies have suggested that neurite alterations in either dendrites or axons are a potential area of study (Tagliati et al., 1998).

Multiple methods have been used to label neurons in dissociated primary neuronal cultures, and few have been implemented in quantifying morphologic changes in neurons in dissociated HIV dementia studies. In the previous sections three specific markers were outlined. These markers were used associated with immunocytochemistry and related to their ability to identify neurons and illustrate neuron morphology. Carbocyanine dyes and fluorescence bound protein like phalloidin are less intrusive to the primary cultures. Carbocyanine dyes have long been used as neuronal tracers for examination of neuronal pathways in organotypic slice preparations (Godement et al., 1987, Chelvanayagam and Beazley, 1997). Carbocyanine dyes are lipophilic and can be incorporated into the cell membrane of living or fixed neurons (Honig and Hume, 1986, Godement et al., 1987, Honig and Hume, 1989). Multiple carbocyanine dyes are used to study neurons. This review will focus exclusively on DiI, though multiple dyes are used to label neurons. In addition to its utility to trace neuronal pathways, DiI can be used to label axon, soma, and dendrites of dissociated neurons in culture (Honig and Hume, 1986). The ability of DiI to label all of the neuron is particularly important in accessing morphologic changes. DiI has been used to quantifying changes in neurite length in slice preparations. Its use to quantify changes in neurite length in dissociated primary neuron cultures is relatively sparse (Varga et al., 1996, Moolman et al., 2004). In dissociated primary neuronal cultures DiI is used to label and quantify dendritic spines/filopodia (Park et al., 1996, Hasbani, 2001a, b, Alfonso et al., 2005). The ability to use DiI to quantify these structures suggests a continued utility. Identifying morphologic changes in living dissociated neuronal cultures is an application for DiI

that fits with a direction of HIV dementia research. Though previous studies have suggested the filopodia were dendritic spines, but without immunocytochemistry or ultrastructural confirmation the ability to identify these filopodia as spines maybe difficult.

Phalloidin and DiI are of interest for their ability to stain most elements of a neuron and phalloidin in particular for its ability to label synaptic structures. Phalloidin is an organic molecule that binds to actin. Fluorescent bound phalloidin has been used extensively to localize actin in dissociated cells (Wulf et al., 1979, Rashid and Cambray-Deakin, 1992, Ferreira et al., 1993, Brown et al., 2000). Actin has been shown to localize strongly with post synaptic densities in dissociated primary neuronal cultures. Actin's presence in these post synaptic densities suggest that phalloidin can be used to label these structures (Matus et al., 1982, Kaech et al., 1997, Fischer et al., 1998). Phalloidin is not often used to identify neurons or label whole neurons for changes in neurite length in dissociated primary neurons. The ability to quickly and reliably label neurons and specific structural elements is very important in dissociated cultures. Identification of specific structural elements over the course of long term neuronal culture in a HIV dementia model could contribute to identifying changes in neuronal function associated with HIV dementia.

Neurite Length

Quantifying changes in developing neurons in vitro has been challenging. One of the initial observations about dissociated neurons is the shape that they take and how over a very

short period of time that shape changes (Bray, 1973). Although no two neurites share the exact same neurite extension pattern, neurons of the same type have similar patterns (Bray, 1973). Within 24 hours of plating, neurites can be seen growing (Bray, 1973). After a small window of time the neurites appear to take on characteristics of dendrites and an axon and can be labeled specifically by immunocytochemical markers (Matus et al., 1981, Caceres et al., 1984, Binder et al., 1985, Caceres et al., 1986). The labeling of these processes is the first step to quantifying changes associated with them, specifically changes in length. This section will focus on quantification of neurite length in dissociated primary neurons as a determination of development. Though there are a myriad of changes that can occur to alter the morphology of neurites, this review will focus on quantification of neurite length in dissociated primary neurons relating to total length of neurites, length of individual neurites, and degree of arborization. There are a limited number of studies looking at neurite length and complexity in association with HIV dementia in dissociated neurons (Dou et al., 2005)(Iskander et al., 2004)(Walsh et al., 2004)(Zheng et al., 2001). There are difficult issues associated with this quantification; these HIV studies use fetal human tissue which can be a significant barrier to some labs. These experiments used quantification techniques based primarily on pixel counts of MAP2. In this regard the techniques used don't fit traditional methods of length quantification based on identification of neurites and individual measurements of the neurites. These experiments provide gross information about the complexity of these neurites but lack the details that other methods provide.

In some of the earlier work involving quantifying neurite length, manual tracing was often used (Denis-Donini et al., 1983). Manual tracing allows the user to have maximal control

over the identification and measurement of neurites. Manual tracing allows the user to identify branches, quantify arborization in neurites, as well as define individual neurites for length measurement. In manual tracing, as in all types of neurite length quantification techniques, the final measurements are given in some unit (preferably microns), but the key is the calibration and example of a calibration is pixels per micron. The inclusion of a calibration and measurements are what define the results as quantitative as compare to qualitative (Radio and Mundy, 2008). Besides the labor and time intensive nature of manual tracing, there is a question of subjectivity in both the selection of neurons and the tracings themselves that must be considered.

Recently there has been a trend toward the use of semi-automated and fully automated programs for neurite length measurements. These programs allow the user to monitor the tracing of neurites through the plugin NeuronJ for the ImageJ platform an example of a semi-automated tracing program (Meijering et al., 2004). In NeuronJ the user traces the digital image of their neuron and provides a calibration so that an absolute distance can be measured. This program has been used to quantify length in dissociated primary neurons (Vo et al., 2005, Oliva et al., 2006, Edman et al., 2008). In a semi-automated program like NeuronJ the level of user input on the measurement is still significant and there are concerns with subjectivity in the selection of neurons to image and in the measurements themselves. As with manual tracing it is possible to avoid certain confounding issues by blinding the user to the conditions. In NeuronJ users can define individual neurites, quantify their length branch points and neurite arborization. NeuronJ is a program that works with fluorescently labeled neurons, either through expressed of GFP or immunocytochemistry (Vo et al., 2005, Oliva et al., 2006). There are also fully automated programs that trace and quantify neurite length. As semi-automated programs increased the

speed of analysis over manual tracings, a similar phenomenon is seen when comparing fully automated programs to semi-automated programs.

With the increased usage of high throughput screening (HTS), fully automated quantification programs like Neuritetracer have become increasingly valuable (Pool et al., 2008). Though HTS allows characterization and quantification of changes to neurites under a large number of conditions quickly, a program like Neuritetracer has a utility in the examination of individual neurons in culture. Neuritetracer in a manner similar to NeuronJ, as it works with fluorescently labeled neurons. Neuritetracer is an automated program that can identify a cell and quantify the total neurite length, if properly labeled distinguishing the neuronal soma from the neurites. The automated aspect of this program can greatly increase the amount of image analysis. It also eliminates the issue of bias in neurite measurements. Use of these techniques in the analysis of neurite changes associated with HIV dementia in dissociated neurons could improve the current understanding of the field about the nature of neuron injury in this system. Users should still be cautious as selections of fields is user dependent and is a potential area of subject concern. In these systems the measurements of individual neurites as well as neurite arborization may be difficult to standardize and a combination of techniques is always recommended. The information would provide greater detail to work that already has been done and provide new avenues current studies.

Synapse formation, identification, and quantification

Synapse formation is a significant benchmark in neuron development. In some of the earlier work examining the physical characteristics of dissociated neurons in culture the

distinction between axons and dendrites was identified (Bartlett and Banker, 1984a, Caceres et al., 1986). After examining if neurites from dissociated neurons become dendrites and axons, the next level of study was to examine nature of their interactions and specifically if these cells were able to form synapses. Synapses have been found in dissociated neurons (O'Lague et al., 1978, Bartlett and Banker, 1984b, Fletcher et al., 1994, Guinamard et al., 1999). The discovery of synapse in dissociated primary neurons provides a benchmark to quantify development in these cells and under specific conditions look at possible neurodegeneration as it relates to synapse formation. Identification of these synaptic structures could provide an interesting proxy for changes in neuron function. It is possible that synaptic degeneration is the primary cause of neuronal dysfunction and any apoptosis seen is a later process in HIV dementia (Bellizzi, 2005) (Gelman, 2006). Quantifying changes in synaptic structure may prove useful insight into subtle changes in dissociated neurons exposed to HIV by products.

Interest is starting to increase in examining synaptic function specifically in HIV dementia (Bellizzi et al., 2006). Synapsin I is a protein that is widely expressed throughout the nervous system and is localized to presynaptic vesicles (De Camilli et al., 1979, De Camilli et al., 1983, Fletcher et al., 1991). Synaptophysin is associated with synaptic vesicles (Wiedenmann and Franke, 1985). Synapsin I and Synaptophysin are expressed in dissociated neurons from E18 and expression increased as neurons mature (Fletcher et al., 1991). Synaptophysin had a very similar staining pattern as synapsin suggesting that in dissociated neurons these two proteins could be used to identify synapses in culture (Fletcher et al., 1991, Fletcher et al., 1994). Inclusion of a post synaptic marker in this review may address synaptic changes not associated with the pre-synaptic structure. PSD-95 is a protein associated almost exclusively with the post-

synaptic membrane in neurons (Sheng and Sala, 2001). With Synaptophysin and Synapsin I, PSD-95 has been confirmed to be expressed in dissociated primary neurons and is localized to post-synaptic sites (Halpain et al., 1998, Kim et al., 1998). Recent studies have quantified PSD-95 staining in dissociated primary neuronal cultures modeling HIV (Viviani et al., 2006)(Kim et al., 2008). In these studies PSD-95 was used to quantify synaptic changes associated with components of this disease processes.

The method of quantification depends heavily on the mode of identification. In previous sections DiI and phalloidin were discussed and briefly mentioned for their ability to identify dendritic spines, a structure associated with the synapse. DiI staining is believed to be accurate but unlike the markers listed above or phalloidin there is no specificity to DiI in these regions. Traditionally Golgi stains are the gold standard for identification of dendritic spines. Golgi staining is not performed on dissociated primary neurons, as the technique is generally considered harsh for a monolayer of tissue. In the papers that have quantified dendritic spine numbers with DiI (Hasbani, 2001b, a) they have manually summed the number of protrusions that fit a morphologic set criteria. Manual counts are an option whether using Phalloidin, DiI, Synaptophysin, Synapsin, or PSD-95. Manual count reliability and subjectivity that could affect the counts should be considered in the study design. There are automated programs that can be used to quantify dendritic spines such as NeuroLucida, which has been used to quantify synaptic structures labeled with Synaptophysin (Rami et al., 2006). MetaMorph is another program used in quantifying synaptic structures in vitro (Kim and Richardson, 2008, McKellar and Shatz, 2008). These programs often allow the user a high level of accuracy and reliability. The threshold for signal vs. noise can be defined by the user so that a true signal will not be missed.

All of these techniques require fluorescence and some level of microscopy, synaptic structures like dendritic spines often require higher level of magnification to image when compared to assessments of neurite length. The ability of these techniques to allow the field to analyze neuronal damage that is subtler than cell death may provide insight into a disease process that often takes years to manifest clinically.

Conclusion

Dissociated primary neurons have and will continue to provide scientists a fertile ground to continue to isolate and answer questions at the heart of neuroscience. The greatest strength of this system is also its greatest weakness; the system simplicity of the system avoids the confusion of the in vivo complexity. Though it has been shown that dissociated primary neurons can grow neurites and form synapses similar to what is seen in vivo (Varon and Raiborn, 1971, Ransom et al., 1977a, Ransom et al., 1977b, Dichter, 1980, Snodgrass et al., 1980, Swaiman et al., 1982, Bartlett and Banker, 1984a, b), questions remain regarding whether these processes can truly be studied in vitro in chronic diseases. HIV dementia provides a particularly interesting challenge, in addition to the traditional difficulties of working with dissociated neurons there are issues unique to this system. It maybe difficult for investigators to acquire and culture human tissue, and in that case rat and mice cultures are often used but they bring a separate set of questions. Ultimately as investigators continue to advance in the techniques available some of these concerns may disappear and a deep body of HIV related dissociated neuronal experiments will emerge. The positives are that the techniques are available to quantify several neuronal

properties (Meijering et al., 2004)(Viviani, Gardoni et al. 2006; Kim, Martemyanov et al. 2008). The reliability of specific results can be questioned at times, but generally these techniques are being used within the field. This review sought to define a small set of techniques available to examine the functions of cell viability, neuron identification, neurite growth, and synaptic formation in dissociated primary neuronal cultures. The success or failure of any system lies in the reliability of that system hopefully this system will continue to become assessable at the level of morphologic analysis. At the moment examinations of acute changes to neurons in this system can extensively studied, but investigators should always remain cautious when accompanying in vivo experiments are not available. True long term cultures that could be studied for years instead of weeks would be a significant break through in exploring a complex and chronic neurodegenerative disorder such as HIV dementia. Time will tell if an accurate model system of these diseases is possible in dissociated primary neurons. The changes that pathologic changes that accumulate over decades at the moment cannot be replicated in neurons that survive for a few months (Ray et al., 1993). That discrepancy defines the challenges of moving forward trying to establish and maintain dissociated primary neuronal cultures that more accurately mimic the chronic nature of certain neurodegenerative diseases. Hopefully investigators can study the disease process of HIV dementia in dissociated neurons and use multiple techniques to answer questions dealing with cell viability, neuron morphology, and synaptic structure with new confidence based on the techniques that are currently available.

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