Evaluation of Tau Phosphorylation using a Non-Traditional Immunoassay Platform

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INTRODUCTION

Hyperphosphorylation of tau is the precursor to the formation of neurofibrillary tangles which are characteristic in the pathogenesis of Alzheimer's disease[2-4]. By treating embryonic rat cortical neuronal cultures with okadaic acid, a phosphatase inhibitor, this hyperphosphorylated state can be simulated in vitro.

The Gyrolab platform is a bioanalytical automated system that allows rapid immunoassay development. This platform miniaturizes and integrates assay steps for protein quantification similar to a traditional ELISA. While there are several ways to complete assays on the Gyrolab, the methods used in this presentation involve a biotin-capture Ab binding to streptavidin beads. The antigen in the sample or standard binds to the capture Ab, and the detection Ab binds to the antigen. This enables detection by laser-induced fluorescence.

This rat cortical neuron primary culture system has been used previously to investigate the effect of various Tau phosphorylation pathways (JNK, MAP kinase, etc.)[1,5]. **The goal of this investigation was to develop and validate several phospho-Tau assays in an effort to study the effects of okadiac acid and various kinase inhibitors in this model system.**

METHODS

Experimental Layout

1. Rat Cortical Neuron Prep
96 wells, 2000-5000 cells per well
8 day cultures in B27 Neurobasal Media, 200 ul volume

1. Prepare compound solutions at 2x final concentration, 100 ul per well.
2. Remove 100 ul of media from each well of the cell plate.
3. Add 100 ul of the 2x compound solution per well.
4. Incubate 1h 37C.
5. Add okadaic acid at 5x concentration, 50 ul per well (250 ul total volume in well).
6. Incubate 1h 37C.
7. Aspirate all media off quickly. Do not scrape bottom of well.
8. Add Lysis Buffer: 30 ul/well.
9. Tap plate gently.
10. RT 5' (can be frozen at -80 for future use at this point).

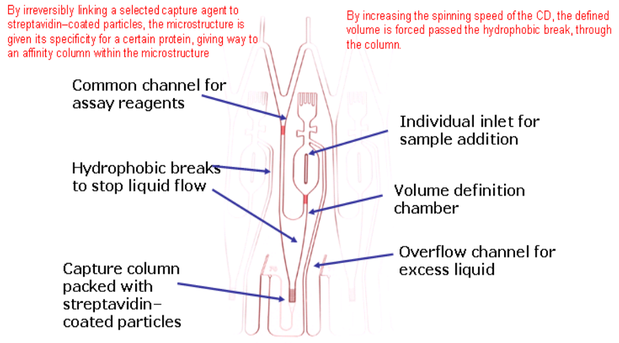
2. Gyrolab Reaction Prep: Analysis by 'ELISA'
2, 96 well PCR Plates ('Reagent' and 'Sample')

1. Add capture antibody (minimum concentration of 100ug/ml (can be diluted with PBS) to 'Reagent' plate.
2. Add detection antibody diluted in Excip F buffer (nM) to 'Reagent' plate.
3. Add standards diluted in Excip CCS buffer to 'Reagent' plate.
4. Add unknown samples diluted 1:1 in Excip CCS buffer to 'Sample' plate.
5. Run plates on Gyrolab instrument (creates a 'sandwich ELISA') to detect specific phospho epitopes of Tau using a 1000nl CD.
6. Sample output is displayed in relative fluorescence units which are normalized to baseline (DMSO treatment) and displayed as '% control'.

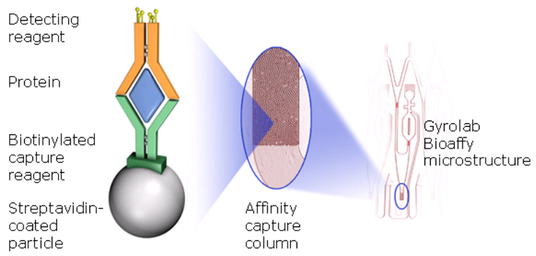
Reagents
•Neurobasal Media, Invitrogen #21103049
•B27 supplement, Invitrogen #17504044
•Glutamax in stockroom, Invitrogen
•Okadaic Acid: Alexis Biochemicals, #ALX-350-011-M001, Make 1.25mM stock in DMSO
•Lysis buffer: Phospho-safe, Novagen #71296;
•Calbiochem Protease Inhibitors, cocktail set III #539134 (use 1:1000 in Phospho-safe)
•Biotinylated capture antibodies
•Fluorphore-labeld detection antibodies (e.g. ALEXA, DyLight)
•Gyrolab proprietary buffers (Excip F and CCS)

Gyros Technology Miniaturizes and Integrates Assay Development through Automation:

One Microstructure - One Data Point

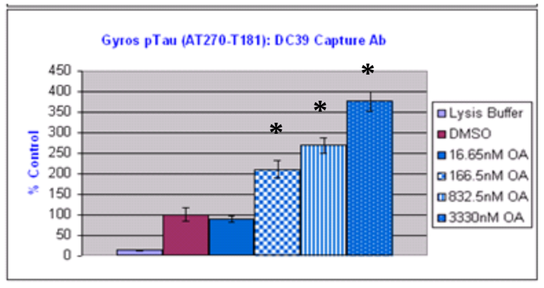


ELISA at the Nanoliter Scale



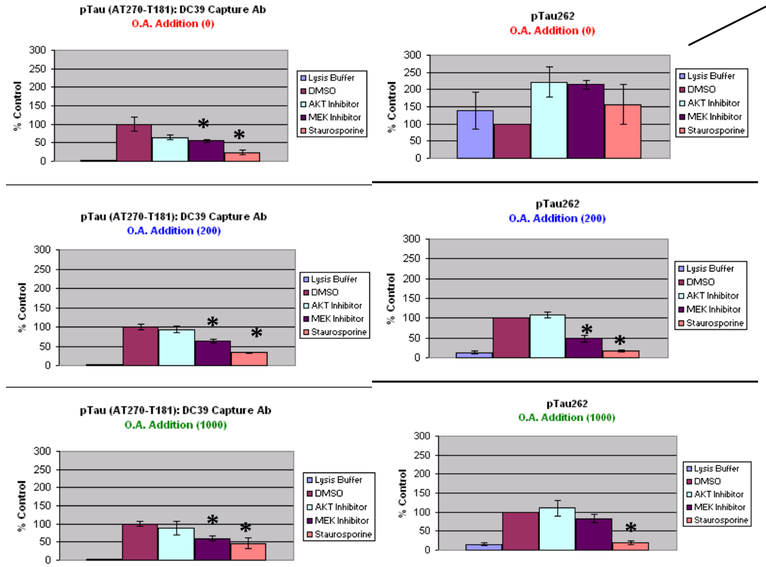
RESULTS: Okadaic Acid Dose Response

7 day rat neuronal cultures dosed with increasing concentrations of OA for 1hr.



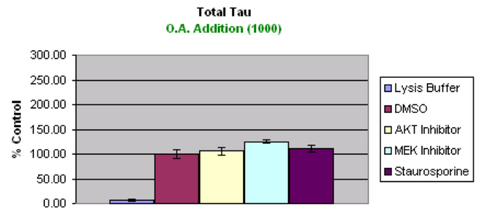
Bars represent the mean and lines the SE from 4 wells for each treatment. Data are normalized to DMSO vehicle baseline. ANOVA's were performed on underlying relative fluorescence signal from immunoassays obtained for each sample followed by multiple t-tests using the Bonferoni correction to determine significant differences between treatment groups post hoc. * Significant difference ($p < 0.05$) from DMSO vehicle control.

Effect of various kinase inhibitors on pTauT181 and pTauS262 in 8 day rat neuronal cultures treated with increasing concentrations of OA for 1 hr.

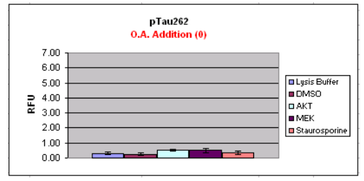


Bars represent the mean and lines the SE from 4 wells for each treatment. Data are normalized to DMSO vehicle baseline. ANOVA's were performed on underlying relative fluorescence signal from immunoassays obtained for each sample followed by multiple t-tests using the Bonferoni correction to determine significant differences between treatment groups post hoc. * Significant difference ($p < 0.05$) from DMSO vehicle control.

Lack of effect of various kinase inhibitors or OA on Total Tau levels in 8 day rat neuronal cultures



Bars represent the mean and lines the SE from 4 wells for each treatment. Data are normalized to DMSO vehicle baseline. ANOVA's were performed on underlying relative fluorescence signal from immunoassays obtained for each sample followed by multiple t-tests using the Bonferoni correction to determine significant differences between treatment groups post hoc.



Note: pSer262 Tau very low in the absence of O.A. Thus small differences lead to apparent changes relative to DMSO treated controls that are not considered biologically significant.

CONCLUSIONS

Increasing concentrations of OA induced a marked increase in pTauT181 and pTauS262. Dosing with various kinase inhibitors also decreased pTauT181 and pTauS262 levels relative to baseline (DMSO). Total Tau levels remained unaffected by treatment. This data was also able to correlate with image analysis observations[6], quantifying epitopes of Tau from a cell culture system. These experiments examined the utility of the Gyrolab platform as an alternative approach to immunoassay development. There are subsequent assays currently in development with the goal of achieving a panel of assays which can further drive the detection of numerous Tau epitopes. The Gyrolab facilitates the ability to compare in vitro models used to support mechanistic studies, new target identification, and validation. The ability to create, validate, and run an ELISA formatted assay enables further understanding of the pathophysiology of Alzheimer's disease.

REFERENCES

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