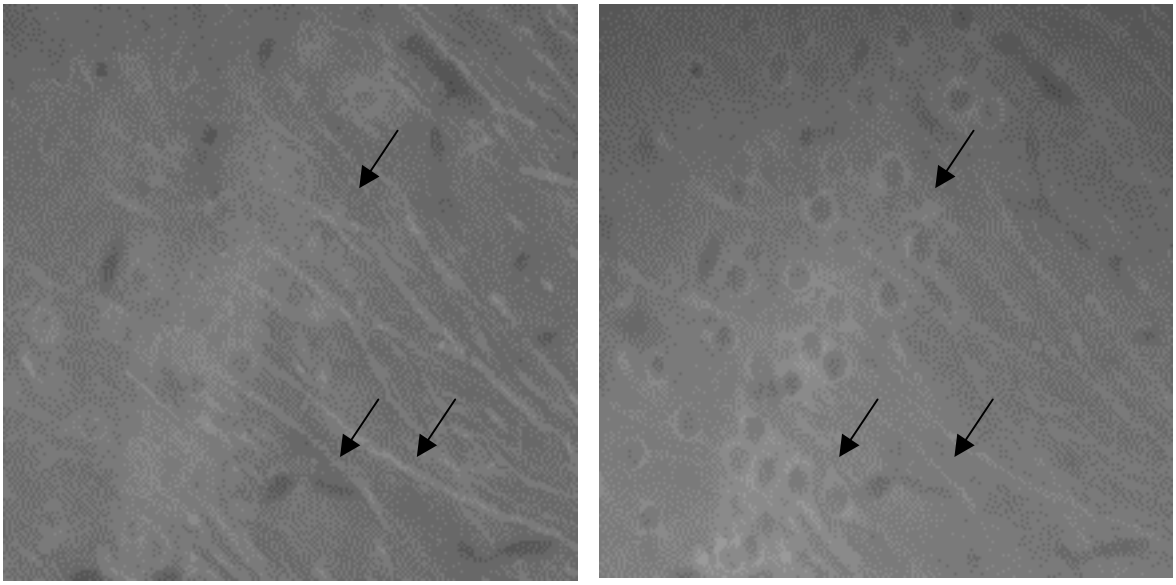
Integrin Involvement in Long-term Potentiation

Long-term potentiation or LTP is an activity-dependent strengthening of synaptic connections that has been proposed as the model for learning and memory. Some of the early experiments investigated the induction of LTP in pyramidal cells of the CA1field of rat hippocampus by presenting high frequency stimulation to the Schaffer collaterals (Purves et al, 2001). The result of such high frequency stimulation was the activation of NMDA receptors on the postsynaptic cell (the CA1 pyramidal cells) allowing the influx of calcium cation (Purves et al, 2001). Calcium influx activates a series of Ca-dependent kinases that initiates a signaling cascade ultimately thought by many to lead to the formation of new synapses through the growth of new dendritic spines (Purves et al, 2001). LTP can also be induced by the activation of Pyk2, a cytoplasmic tyrosine kinase highly expressed in the central nervous system, which upregulates the function of NMDA receptors (Huang et. al., 2003). While tetanic stimulation can lead to the activation of Pyk2 and therefore the upregulation of NMDA receptor function to induce LTP, a mechanism involving integrin activation of Pyk2 has never been characterized.

Integrins are a family of non-covalently bound alpha and beta heterodimer transmembrane receptor that mediates several cellular processes. Mechanically, integrins form cell-matrix and cell-cell adhesion, while chemically they initiate a number of signaling cascades that ultimately influence the proliferation, differentiation, process outgrowth, gene expression, and survival of the cell that expresses them. Recently, alpha 3, 5 and 8 integrin subunits, all of which dimerize with the beta 1 subunit, has been shown to highly express in CA1 and CA3 fields of hippocampus (Pinkstaff et. al., 1999). Alpha 5 integrins have been shown to preferentially localize to the apical dendrites of the CA1 pyramidal cells (Bi et. al., 2001). This preferential localization has one important consequence in our investigation: Pyk2 expression is also preferential to the apical dendrites of CA1 field pyramidal cells (Menegon et. al., 1999) suggesting that a link may exist between the stimulation of alpha 5 integrin and activation of Pyk2 leading to LTP induction.

For the present time, the assumption that alpha 5 is the only integrin that activates Pyk2 is premature without also determining the localization of alpha 3 and 8 integrins in CA1 pyramidal cells. Recent findings have pointed out that the simultaneous expression of alpha 3, 5 and 8 integrins is required for full hippocampal neuronal plasticity (Chan et al., 2003). Therefore, alpha 5 may not be the only activator of Pyk2, but alpha 3 and/or alpha 8 integrins may also play that role. Hence the goal of this project is (1) to determine the preferential localization of alpha 3 and 8 integrins in CA1 field pyramidal cells and (2) to show that either selective stimulation of one particular integrin or collective stimulation of all three integrins (i.e. alpha 3-beta1, alpha 5-beta 1, and alpha 8-beta 1) leads to Pyk2 activation. Preliminary data generated at the genesis of our investigation have already shown that alpha 5 integrins do indeed co-localize with inactive Pyk2 in apical dendrites of CA1 field pyramidal cells (Figure 1). If the same co-localization of Pyk2 with alpha 3 and 8 integrins can be observed then it is quite possible that the activation of Pyk2 also involves alpha 3 and 8 integrin stimulation. Consequently, selective stimulation of a particular integrin and collective activation of all three integrins will be carried out to observe for Pyk2 activation.



a. b.

Figure 1: a. Alpha 5 integrin distribution is preferential to the apical dendritic spines of CA1 pyramidal cells. b. Inactive Pyk2 distribution is also somewhat preferential to the apical dendrites of CA1 pyramidal cells, although its distribution around the cell body is more pronounce then alpha 5. We suspect that during integrin stimulation, inactive Pyk2 from the cell body will be recruited to the dendritic spines. (Arrows) There is definite colocalization of alpha 5 integrin and Pyk2 in apical dendrites of CA1 pyramidal cells.

The attainment of our goals will allow us to answer some fundamental questions about integrin involvement in long-term potentiation. Does integrin stimulation induce LTP by activating Pyk2? If so, which integrin among the three that are distributed throughout CA1 field pyramidal cells are involve in inducing LTP or are all three integrins needed?

At the conclusion of this project we hope to not only determine the preferential distribution of alpha 3 and 8 integrins but to also characterize their potential involvement in the induction of LTP along with alpha 5 integrin. If indeed integrin is also involve in LTP then the data from this project will broaden our knowledge of the known chemical functions of integrins.

Material and Method

Integrin stimulation

The goal of our project requires us to be able to selectively stimulate a particular integrin and then observe for the corresponding Pyk2 phosphorylation. To achieve this we will take advantage of the fact that different integrins bind different ligands. Hippocampal slices will be maintained in culture and the media will then be treated with ligands specific to alpha 3, 5 and 8 integrins. There will be five conditions set up; three conditions involve the selective stimulation of only one of three integrins under each condition, another condition will involve stimulation of all three integrins with non-specific peptide ligand gly-arg-gly-asp-ser-pro (GRGDSP) and the control condition will not have any integrin stimulation.

Preparation of tissue

Cultured hippocampal slices are available in the Gall Laboratory (i.e., prepared by a technician). Following treatment slices are prepared from rat pups at postnatal day 10 and will be used after 10 days in-vitro. Slices will be treated with integrin ligands and/or antibodies to selectively activate different

receptors as described above. Treated hippocampal slices will be removed from cultures and section at 20 microns on a freezing microtome. The sections will be put into .1 M phosphate buffer (PB) and store at 4 degrees Celcius. However, long-term storage of the sections is highly discouraged because it will affect the antibody binding of our integrins and phospho(p)-Pyk2. Therefore, the sections will be mounted immediately on gelatin-coated Super-Frost slides and prepared for immunofluorescence.

Immunofluorescence

Immediately following the collection of hippocampal slices, immunofluorescence will be carried out to determine the co-localization of alpha 3, 5 and 8 integrin and p-Pyk2 in pyramidal cells of the hippocampus. The slices will first be incubated with a primary antibody that will only recognize alpha 3, alpha 5, alpha 8 or pPyk2 in the neurons. This establishes the first level of specificity. The pre-requisite for determining co-localization of two antigen of interest is that the two primary antibodies used must be from different species, usually mouse and rabbit. Thus, anti-alpha 5 (or 3 and 8) antibody from rabbit serum and anti-pPyk2 from mouse serum will be diluted one to five hundred in a buffer system consisting of 1 X Tris Buffer Saline, .1% Triton X and 1.5% BSA. The Triton X is a detergent that will make the neurons permeable to anti-pPyk2 antibody, allowing it to come into the cell and bind the p-Pyk2. The BSA will help minimize non-specific binding of the antibody to other proteins.

Following the primary incubation, a secondary antibody incubation will add an additional level of specificity and enhancement of our signal (Figure 2). Two features of the secondary antibody will make this enhancement possible: (1) the secondary antibody only recognizes the primary antibody from one species not both, (2) the Fc region of the secondary antibody contains a tag with a fluorescent marker that only fluoresces at a particular wavelength. The latter feature requires that all secondary antibodies recognizing a primary antibody from rabbit will fluoresce at the same wavelength. Following primary and secondary antibody incubations, the hippocampal sections undergo a final wash in .1M PB to remove any unbound antibody. The slides will be cover-slipped with Permount and view under fluorescent microscope.

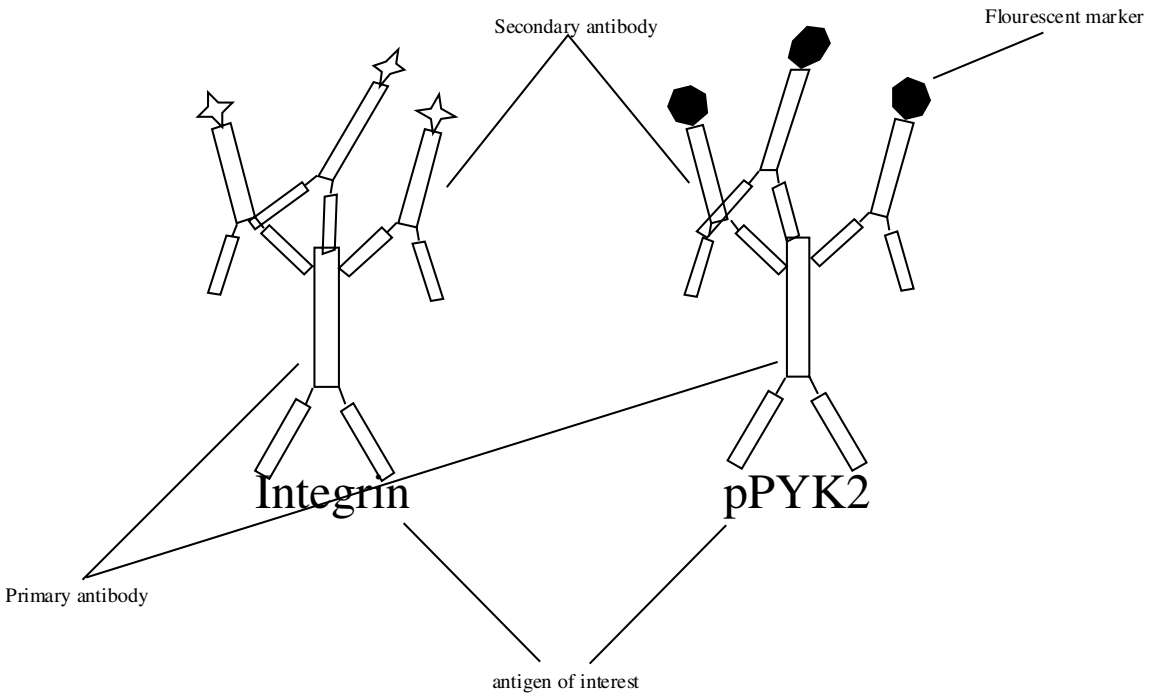


Figure 2: The primary antibody will specifically bind to the antigen or protein of interest (integrin or pPyk2) providing the first level of specificity. For colocalization of integrins and pPyk2 the two primary antibody will be from different species, usually mouse or rabbit. The secondary antibody provides an additional layer of specificity and enhancement of the primary antibody. It will either recongnize mouse or rabbit antibody but not both. Attached to the Fc region of the secondary antibody is a flourescent marker that flouresces at different wavelenghts of light under thefluorescent microscope. For example, all anti-mouse secondary antibody will have a flourescent marker attached that flouresces at a wavelength different from anti-rabbit antibody.

Visualization of Integrins and pPyk2 distribution

Visualization of our immunofluorescent localization will be done under a fluorescence microscope that can selectively detect the specific fluorescent marker (Figure 3). Pictures will be taken with a digital camera set for one, then the other, wavelength to determine if the two antigens labeled are colocalized (Figure 1).

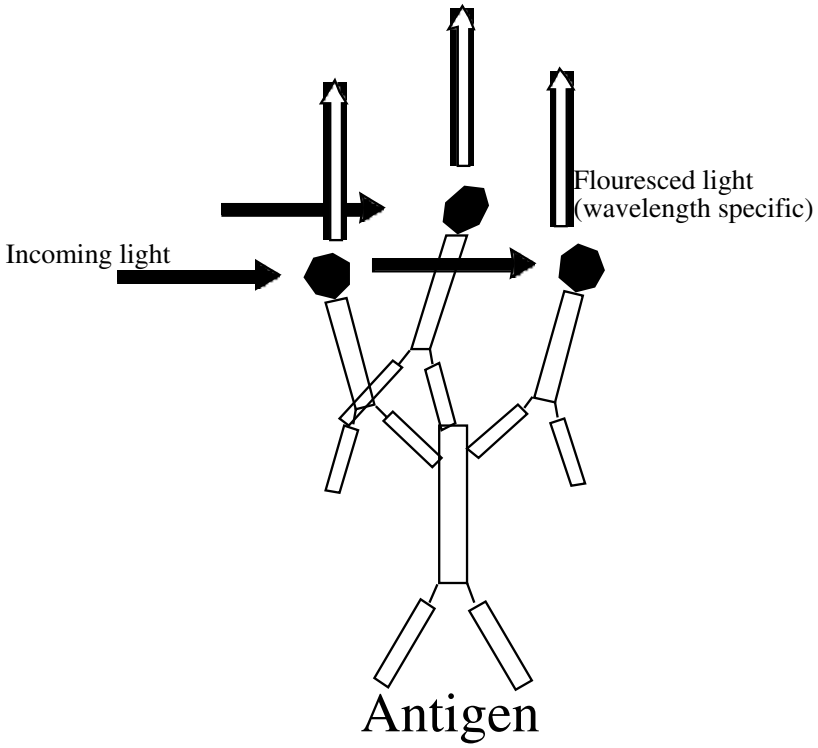


Figure 3: Immunofluorescent takes advantage of the fact that the fluorescent tag attached to the Fc region of the antibody flouresces at one specific wavelength. This feature is very useful for determining co-localization of two proteins where both proteins can be observed under different wavelenghts of light.

Quantification of pPyk2 activation

The amount of p-Pyk2 activation can be assessed in two ways by (1) visual inspection of immunofluorescent staining (i.e., is there more staining with treatment) and (2) Western Blot analysis of phospho-protein levels. Western Blot analysis will be used as the final measure because increases in levels of p-Pyk2 can be quantified.

Student responsibilities and level of preparedness

A qualified lab technician will perform any procedures with animal subjects and, in particular, will prepare cultured slices. However, I will be responsible for treating the cultured slices with the appropriate ligands to selectively stimulate the integrin of interest. Furthermore, I will be responsible for determining the co-localization of the integrins of interest and pPyk2 using our established immunoflourescent protocol. I will also be responsible for taking necessary pictures of our immunoflourescent staining using a digital camera mounted onto the fluorescent microscope. Depending on the outcome, I will also be involved in measuring Western blots and evaluating band densities.

To fulfill my responsibilities I have been trained and am able to carry out immunofluorescent staining and Western blot analysis. I have done each of these procedures more then once and am confidant in my ability to carry out these procedures to obtain my results. I am also capable of using the fluorescent microscope to visualize and photograph our immunofluorescent staining.

Project timeline

Our project began this summer; therefore we have already determined the parameters of our investigation. For example, we have determined the appropriate concentrations of buffer and antibody to use, or the appropriate incubation conditions and time for our sections and so on. Thus, all of the small but necessary details of this project have been worked out. All that is needed now is to perform the

experiments proposed. Once we are able to purchase the appropriate antibodies the localization of our integrins of interest can be determine within days for each particular antigen. To evaluate three integrins it will take a few weeks (maybe 4). Following this the visualization and quantification of Pyk2 activation can be accomplish once again within days for initial localization studies and about 2 weeks for colocalization studies. The matter that remains is to replicate our entire experiment several time to be absolutely sure of our accomplishments. Overall, the work will take 10 to 15 weeks.

PROPOSE BUDGET

REAGENTS	**AMOUNT**	**PRICE**
Antibodies-		
anti-alpha 3	100 micro-liter	$ 250.00
anti-alpha 8	100 micro-liter	$ 250.00
anti-beta1 neutralizing antibody	100 micro-liter	$ 250.00
Secondary fluorescent antibody	100 micro-liter	$ 250.00
		TOTAL: $1000.00

REFERENCES

Andrea Menegon, Ferran Burgaya, Pierre Baudot, D.D. Dunlap, Jean-Antoine Girault, and Flavia Valtorta,FAK and Pyk2/CAKB, two related tyrosine kinases highly expressed in the central nervous system: similarities and differences in the expression pattern, European Journal of Neuroscience, 1999, Vol 11, pg. 3777-3788.

Chi-Shing Chan, Edwin J. Weeber, Sindhu Kurup, J. David Sweatt, and RL Davis, Integrin Requirement for Hippocampal Synaptic Plasticity and Spatial Memory, The Journal of Neuroscience, August 6, 2003, 23(18), pg. 7107-7116

Hynes R., Integrins, versatility, modulation, and signaling in cell adhesion, Cell, 1992, Vol 69, pg. 11-25

Jason K. Pinkstaff, Jon Detterich, Gary Lynch, And Christine M. Gall, Integrin Subunit Gene Expression is Regionally Differentiated in Adult Brain, The Journal of Neuroscience, March 1999, 19(5), pg.1541-1556

Dale Purves, George J. Augustine, David Fitzpatrick, Lawrence C. Katz, Anthony-Samuel LaMantia, James O. McNamara, s. Mark Williams, Neuroscience 2nd Edition, Sinauer Associates Inc. 2001,pg. 541-550

Xiaoning Bi, Gary Lynch, Jun Zhou, and Christine M. Gall, Polarized Distribution of alpha5 Integrin in Dendrites of Hippocampal and Cortical Neurons, The Journal of Comparative Neurology, 2001, Vol 435, pg. 184-193