CARBACHOL- AND ACPD-INDUCED PHOSPHOINOSITIDE RESPONSES IN THE DEVELOPING RAT NEOCORTEX

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Signal transduction via the phosphoinositide (PI) second messenger system has key roles in the development and plasticity of the neocortex. The present study localized PI responses to individual cortical layers in slices of developing rat somatosensory cortex. The acetylcholine agonist carbachol and the glutamate agonist trans-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) were used to stimulate PI turnover. The PI responses were compared to the distribution of the corresponding PI-linked receptors in order to investigate the regional ontogeny of PI coupling to receptors in relation to neural development.

The method for assessing PI turnover was modified from Hwang et al. (1990). This method images the PI response autoradiographically through the localizaton of [^{3}H]cytidine that has been incorporated into the membrane-bound intermediate, cytidine diphosphate diacylglycerol. In each age group (postnatal days 4-30), carbachol resulted in more overall labeling than ACPD. For both agonists, the response peaked on postnatal day 10 (P10) and was lowest in the oldest age group. The laminar distribution of the carbachol PI response from P4-P16 corresponded fairly well with the laminar distribution of [^{3}H]quinuclidinyl benzilate binding (Fuchs, 1995). However, in the subplate layer the carbachol response was strong while receptor binding was minimal. The carbachol response decreased after postnatal day 10, while the overall levels of receptor binding continued to increase.

From P5 – P14, PI-linked metabotropic glutamate receptors are most concentrated in layer IV (Blue et al., 1997), whereas only on P6 was there a correspondingly high ACPD-initiated PI response in this layer. Unlike receptors, the PI response was strong in upper V (P4 – P12) and within layers II/III (P8 - P16). From P4 – P21, the subplate showed relatively high PI labeling compared to receptor binding. The several differences between the distribution of PI response and receptors suggest spatiotemporal heterogeneity of receptor coupling to second messenger systems.

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INTRODUCTION

Several lines of evidence point to the importance of signal transduction via the phosphoinositide (PI) turnover second messenger system in the development of the mammalian nervous system. PI turnover has been shown to affect DNA synthesis (Ashkenazi et al., 1989), neuronal differentiation (Meier et al., 1991), and neuronal survival (Mattson et al., 1988; Wang et al., 1997; Vincent et al., 1997), and has been linked to the adverse effects of ethanol on the developing brain (Balduini et al., 1994; Balduini et al., 1995), as well as the therapeutic effects of lithium in adults (Godfrey, 1989; Belmaker et al., 1996). In addition, PI turnover is involved in long-term potentiation (Izumi and Zorumski, 1994; Pavlinova et al., 1997; Vickery et al., 1997), and transient increases in PI turnover coincide with experience-dependent changes in the developing neocortex (Dudek and Bear, 1989; Bear and Dudek, 1991), implicating synaptic plasticity.

Many of the neurotransmitter receptors that are coupled to PI turnover have been localized in the developing rat cortex (Rotter et al., 1979; Palacios et al., 1988; Pelaprat et al., 1988; van Huizen et al., 1994; Fuchs, 1995; Aubert et al., 1996; Blue et al., 1997), but little is known about when these receptors become functional by inducing a PI response. Measuring the regional ontogeny of the acetylcholine- and glutamate-initiated PI second messenger responses may provide further information on the role of PI turnover in neural development. For example, increased PI activity coincides with the developmental window in which the rat somatosensory cortex is easily modified (Fox, 1992) and the peak of PI activity that occurs during this time frame has been suggested to be involved in synaptic plasticity of the developing brain (Dudek and Bear, 1989). PI turnover may be a necessary component of neuronal plasticity (Pontzer et al., 1990) and the gene expression of a PI-dependent protein kinase (PDK-1) has been shown to be the most intense in the rat neocortex during the first postnatal week (Yoshida et al., 1999).

Using an autoradiographic technique which assesses phosphoinositide turnover in brain slices by imaging the accumulation of the membrane-bound PI cycle intermediate cytidine diphosphate diacylglycerol (CDP-DAG) (Hwang et al., 1990), the present study examines transmitter-stimulated PI activity in specific layers of rat somatosensory cortex. The technique developed by Hwang and his colleagues was modified to increase our overall experimental success rate, as well as to improve inter-animal comparisons. The resulting PI response was then compared to the ontogeny and distribution of PI-linked receptors for glutamate (Blue and Johnston, 1995; Blue et al., 1997) and acetylcholine (Cortes and Palacios, 1986; Fuchs, 1995), which are thought to play a prominent role in the development of the neocortex (Bear and Singer, 1986; Bear and Dudek, 1991). The highest transmitter-induced PI response occurs during the critical period of developmental synaptic modification and has been suggested to regulate the "wiring" of the developing brain (Bear and Dudek, 1991). By examining PI activity in identified cortical layers, this study describes the overall density and the laminar distribution of agonist-induced PI turnover in the developing neocortex when synaptic connections are being formed, eliminated, and modified (Fox, 1992; Fox and Zahs, 1994).

Overview of Phosphoinositide Turnover

Intracellular signals often involve second messenger cascades like the one involving the hydrolysis of membrane-bound phosphatidylinositols (Hokin, 1985; Hirasawa and Nishizuka, 1985; Majerus et al., 1986). A rapid metabolism of phosphatidylinositols is initiated when neurotransmitters bind to specific receptors located on the extracellular surface of neurons (Nahorski et al., 1986). These receptors couple to G-proteins which activate phospholipase C which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) (Berridge, 1984). This sequence of events occurs within the plasma membrane, with the hydrolysis of PIP_2 resulting in two

prominent second messengers. One is inositol 1,4,5-triphosphate (IP_3), which is released into the cytoplasm to mobilize calcium from endoplasmic reticular stores (Berridge, 1984). The second is 1,2-diacylglycerol (DAG), which remains in the plasma membrane and activates protein kinase C (Berridge, 1984). These second messenger molecules are vital in the transduction and amplification of signals derived from external stimuli (Berridge, 1987). They allow environmental signals originating from synaptic transmission to be relayed across the plasma membrane and into cellular compartments. In the case of developing neurons, this information appears to be involved in neuronal differentiation (Aruffo et al., 1987) and plasticity (Dudek and Bear, 1989; Bear and Dudek, 1991).

Acetylcholine- and Glutamate-Initiated PI Turnover and Cortical Development

Most neurotransmitters have metabotropic receptor subtypes that are associated with second messengers, and many have a subtype connected to the PI cycle (Chuang, 1989; Diamant and Atlas, 1989). Certain metabotropic glutamate (mGluRs) and muscarinic acetylcholine receptors (mAChRs) are PI-linked and have been associated with experience-dependent synapse modification during neocortical development (Dudek and Bear, 1989; Bear and Dudek, 1991).

Glutamate is the primary excitatory neurotransmitter in the cortex, and there is a growing body of evidence supporting glutamate as the principal neurotransmitter involved in synaptic plasticity (Collingridge et al., 1983; Bear et al., 1987; Bear et al., 1990; Kirkwood et al., 1995). PI-linked metabotropic glutamate receptors subtypes (mGluR1 and mGluR5) bind an immediate early gene protein (HOMER) that is particularly responsive to synaptic activity in the developing cortex (Brakeman et al., 1997). At the end of the first postnatal week, glutamate-stimulated PI turnover is much higher in the neocortex than at any other time during development or adulthood (Dudek

et al., 1989; Balduini et al., 1991). It has been suggested that this surge in PI activity is involved in synaptic plasticity (Bear and Dudek, 1991) because the critical period of strengthening and eliminating synapses (Fox, 1992) parallels this surge in PI activity. Further, if glutamate-stimulated PI turnover is antagonized during this time frame, the normal ocular dominance shift associated with monocular deprivation is prevented until the antagonist is removed (Dudek and Bear, 1989). This developmental time frame also coincides with an increase in metabotropic glutamate receptors (mGluRs) (Blue et al., 1997) and receptor mRNA (Condorelli et al., 1992), with mGluR expression (Blue et al., 1997) peaking at the same age (P10) as the glutamate-linked PI response found in the present study.

Acetylcholine has also been shown to play a role in brain development and plasticity (Shaw et al., 1984; Bear and Singer, 1986). Whereas glutamate is thought to play a major role in the actual process of synaptic formation and modification, acetylcholine has been shown to be important in the biochemical process that allows ocular dominance columns to form (Bear and Singer, 1986). Acetycholine appears to be necessary, but not sufficient for cortical plasticity to proceed (Bear and Singer, 1986). Muscarinic acetylcholine receptors activate the PI second messengers that in turn induce neuronal plasticity by activating mitogen-activated protein kinases (MAPKs) (Rosenblum et al., 2000). Previous studies have compared the relationship between acetylcholine-induced phosphoinositide activity and the number of mAChRs. In the adult cortex, Fisher and Snider (1987) found the levels of PI turnover to be proportional to the number of occupied muscarinic binding sites. However, while muscarinic receptors increase until adulthood (Evans et al., 1985; Aubert et al., 1996), PI turnover induced by muscarinic agonists has been reported to reach a maximum in the cortex during the first postnatal week (Balduini et al., 1987; Balduini et al., 1991), suggesting an amplified PI response per receptor in the first postnatal week relative to the response per receptor in the adult.

Current Research

Receptor binding and immunocytochemistry can be used to identify ligand recognition sites in tissue, but do not show whether the recognition site is coupled to second messenger responses. The present study has the anatomical resolution to correlate histologically assessed receptor distribution with a functional measure, namely the accumulation of membrane-bound CDP-DAG. Previously, agonist-induced PI hydrolysis has been assessed primarily through studies of cross-chopped tissue slices and synaptosomes. "Cross-chop" studies assess PI turnover by chopping a slice (approximately 400 μm thick) into 350 μm x 350 μm pieces. These pieces are then incubated in vials containing [^{3}H]inositol or [^{3}H]cytidine as precursors. The results are obtained by using chromatography to monitor the corresponding metabolic products, radiolabeled [^{3}H]inositol phosphates (Rooney and Nahorski, 1987; Balduini et al., 1991) or [^{3}H]CDP-DAG (Godfrey, 1989). The "cross-chop" technique has lower morphological resolution than receptor autoradiography and does not distinguish layers of the cortex, leaving open the question of whether different layers show the same developmental pattern. Examining the relationship between receptors and PI-linked responses on a laminar basis is important, given that the functional organization of the cortex is based on unique characteristics of each layer and the interactions between layers. Indeed, the mRNA of two key enzymes in PI signal transduction (CDP-DAG synthase and PI synthase) varies across cortical layers (Saito et al., 1998). The present study allows for comparisons between levels of receptor binding in specific cortical layers and a functional PI response (examining the relationship between the two).

Preliminary reports from this study (Robinson et al., 1993) were the first to assess PI activity in individual layers of the developing cortex. Since that study, another research group has shown transient PI responses in the developing rat neocortex using a

similar technique (Bevilacqua et al., 1994 and 1995). However, they used emulsion-coated slides rather than autoradiography film, and could only localize silver grains to pyramidal neurons or non-pyramidal tissue within a layer. Quantification was restricted to layers with pyramidal neurons; layers I and IV were excluded. The distribution of the PI response obtained in the present study was compared to existing receptor binding data. Also, the magnitude of PI responses from muscarinic and metabotropic glutamate agonists were compared with one another and considered in relation to cortical development.

METHODS

Slice Preparation

Male Long-Evans hooded rats ranging from postnatal day 4 through P30 were deeply anesthetized via Metofane inhalation. At least three slices were used in each of the following age groups: P4, P6, P8, P10, P12, P14, P16, P21, and P30. A total of 43 rats were used in the analysis. The dorsal skull was removed and the whisker barrel region of SI cortex was dissected out. Chilled , oxygenated low Ca^{++}/high Mg^{++} buffer was periodically poured over the cortex during the dissection. The removed brain region was immediately transferred to the stage of a McIlwain tissue chopper and sliced at 400 μm (Hatton et al., 1980). The anesthetized rat was then sacrificed via decapitation.

Incubation

Each brain slice was collected onto a spatula and transferred into a Millicell-PC (Millipore; Bedford, MA) (Fig. 1) of 3.0 μm pore size. The Millicell platform containing one to three slices was then immediately transferred to a modified 95% O_2/5% CO_2 submersion chamber (12-well Corning tissue plate; Corning, NY) to allow the slice to recover (Teyler, 1980; Nicoll and Alger, 1981). Recovery took place at room temperature. Immediately prior to surgery or during the initial recovery time, 20 μCi of

[^{3}H]cytidine (25 Ci/mmol, Sigma or 23 Ci/mmol, New England Nuclear) was vacuum centrifuged to evaporate the EtOH. Each cortical slice was allowed to recover for 2 x 15 min low calcium (0.5 mM)/high magnesium (10mM) Krebs. This was followed by 30 min in modified Krebs buffer (114 mM NaCl, 4.57 mM KCl, 2.44 mM $CaCl_2$, 1.14 mM KH_2PO_4, 1.2 mM $MgSO_4/7H_2O$, 24.7 mM $NaHCO_3$, 10 mM glucose, 0.1 mM ascorbic acid).

Subsequent incubation procedures were based on those of Godfrey (1989) and Hwang et al. (1990). Each slice was transferred to an individual tissue well (24-well Corning tissue plate) containing 700 µl of Krebs bicarbonate buffer with actinomycin D (0.9 µg) and hydroxyurea (50 mM) added to inhibit the radiolabeled cytidine from being incorporated into nucleic acids. The temperature of the incubation solution started at room temperature and gradually increased to and was maintained at 37^{0}C. After 10 min, [^{3}H]cytidine, resuspended in 200 µl Krebs buffer, was added to the solution in the incubation well. After 30 min, 50 µl of 100 mM LiCl (Sigma) was added to each well for a final concentration of approximately 5 mM. After 10 min, 50 µl of stock agonist, either trans-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD; Tocris Neuramin) or carbamoylcholine chloride (carbachol; Sigma), was added to individual incubation wells. The final concentration of ACPD was 100 µM and the final concentration of carbachol was 1 mM (Hwang et al., 1990). Agonist was omitted from some experiments to establish baseline labeling. The total volume of the incubation solution was 1 ml.

After 45 min, the slices were transferred from the Millicells to embedding molds partially filled with Krebs buffer. The buffer was slowly aspirated while the slice was positioned in the desired orientation against the bottom of the embedding mold. The mold was then placed directly on the steel interior bottom of a –60^{o}C freezer for 10 to 20 sec. This allowed the properly oriented slice to stay affixed to the bottom of the embedding mold when O.C.T. embedding medium (Miles Laboratories) was slowly

added to cover the tissue. O.C.T. was added at this juncture so that the tissue would section more uniformly on the cryostat and to facilitate tissue handling and orienting in the cryostat. The tissue was then immediately stored at -80 ^{o}C.

As a test of the method, three slices from the same animal were allowed to recover for either 60, 90, or 120 min (Fig. 2). This test considered two criteria. The first was tissue viability. If certain layers of cortical cells are more susceptible to death during extended recovery times, the laminar pattern of the PI response should vary with time in recovery. The second variable was inositol depletion. If cells remain viable with extended recovery times, the depletion of inositol during these extended times could possibly lead to an increase in radiolabeling since there is less inositol to remove [^{3}H]CDP-DAG from the membrane. P10 animals (Fig. 3) were chosen for control experiments because this age showed the most robust PI response (mean: 4.2 $\pm$ 0.3 µCi/mg).

Incubation Modifications

Changes were made from the original method (Hwang et al., 1990) to increase tissue viability and labeling consistency. In the present study, slices were placed on a Millicell-PC platform and submersed in the chemical solutions, rather than placed on filter paper with the chemicals applied underneath (Fig. 1). By submerging the slices, the labeling appeared more consistent across the somatosensory cortex when compared to those slices incubated by our research group using the filter paper method. In addition, slices from different animals could be incubated together, thereby facilitating inter-animal comparisons. To reduce cell death due to excess Ca^{++} entry, a low calcium/high magnesium buffer was used during the initial 30 min of recovery.

Sectioning

The frozen, O.C.T. embedded slices were placed on a cryostat chuck and 30-μm coronal cryostat sections were collected on gelatinized slides. After the slides air-dried, they were placed for 5 min in a 37°C nuclease rinse to extract the [^{3}H]cytidine that had been incorporated into nucleic acids. The nuclease rinse contained the following chemicals (from Sigma): RNase A (20 μg/ml), DNase I (20 μg/ml), Tris-HCl (50 mM, pH 7.4), EDTA (2 mM), LiCl (10 mM), cytidine (1 mM), saponin (0.005%), and polyethylene glycol (3%). The slides were rinsed in buffer for 30 sec, dipped in dH_2O (1 sec), allowed to air dry, and then exposed to tritium-sensitive film (Hyperfilm-^{3}H, Amersham) along with tritium standards (ART 123A, American Radiolabeled Chemicals, St. Louis, MO). Calibrations for the standards were provided by R. Hammer and J. Margulies of the U. of Hawaii. The values in nCi/mg of tissue starting with the first standard were 0, 1.5, 7.5, 27.5, 61.3, (number six omitted), 151.8, 250.8, 683.7, 1089.4, 2090.2, 3200.6, 4311.3, (number 14 omitted).

Data Analysis

Following exposure periods of 1-4 days, the film was processed with Kodak D19 for 5 min, water for 30 sec, and Kodak Rapid Fix for 4 min. The autoradiographic image was assessed using computerized video-based densitometry (MCID; St. Catherines, Ontario). At least three slices that were incubated in separate tissue wells with separate solutions from each age group were analyzed. Some of the slices came from the same animal. Slices were chosen based on the consistency of [^{3}H]CDP-DAG laminar labeling across the neocortex. Also, slices with apparent "bleeding" of radioactivity from one

cortical layer to another were discarded because the "bleeding" seemed to result when the integrity of the tissue suffered during freezing and thawing or the nuclease rinse did not remove all of the [^{3}H]cytidine that had been incorporated into nucleic acids. Radiolabel was quantified by calibrating film densities with respect to a best-fit curve based on the tritium standards which had been calibrated in terms of nCi/mg wet weight of tissue. Average density was determined by using an MCID trancept to outline a region of the slice. After the desired area was outlined, the average density measurement was obtained. Laminar profiles were obtained by placing one rectangular transept (300 μm in width) along the depth of the cortex for each slice. Laminar boundaries were determined by superimposing a camera lucida drawing (magnified 40 to 100 times) of the Nissl-stained section with the same or adjacent autoradiographic section (Fig. 4).

RESULTS

There were no obvious effects of time in recovery on laminar distribution or overall levels of radioactivity (60 min recovery, 4.8 μCi/mg; 90 min, 4.6 μCi/mg; 120 min, 4.8 μCi/mg) (Fig. 2). This suggests that during the recovery times utilized in this study, the free inositol pools were not depleted to the extent that the results would be affected (if inositol were not available to remove [^{3}H]CDP-DAG, radiolabeling would increase). P10 sections that were not rinsed in the nuclease rinse had strong, more homogeneous labeling across the entire neocortex whether agonist was used (12.9 μCi/mg) or not (3.8 μCi/mg). Rinsed P10 sections that had been incubated in the absence of neurotransmitter agonists had minimal baseline labeling in the neocortex (87 ± 11 nCi/mg, n = 4 slices) (Fig. 2) as compared to total labeling when carbachol was used as an agonist in P10 animals (4.2 ± 0.3 μCi/mg, n = 5 slices).

Carbachol produced more labeling than ACPD for all age groups studied (Figs. 5 - 8). The S.E.M. for each group was based on three slices. There were significant age-

related changes in density for each agonist (ACPD: $p < 0.001$, $F_{8,18} = 18.17$; carbachol: $p < 0.001$, $F_{9,20} = 29.97$). The highest PI response was in the P10 age group for both carbachol and ACPD, while the lowest PI response elicited by both agonists was found in the oldest age group (P30) (Table 1). For ACPD, values for P10 were significantly greater ($p < 0.05$) than each of the other ages. For carbachol, values for P10 were greater ($p < 0.05$) for all ages except P8. PI activity induced by both carbachol and ACPD steadily increased from P4 until a peak at P10 and then steadily decreased to the low levels of activity found in the P30 rats. The general pattern and the developmental peak for the ACPD animals were similar to those of carbachol.

The laminar distribution of the carbachol PI response matched fairly well with [^{3}H]QNB binding. At the earliest age (P4), the carbachol response was higher in the supergranular layers than in layers IV - VI (Fig. 9). With the exception of dense labeling in layer I, this pattern corresponds to that of [^{3}H]QNB binding. From P6 - P16 the carbachol-induced PI response for the most part matched the receptor binding data in that both showed high densities in layers II - upper IV and lower V - VI with a band of relatively low density overlapping the whisker barrels in lower IV and extending through upper V below the whisker barrels (Figs. 10 -15). However, the carbachol response was strong in the subplate layer, while receptor binding was less dense in this layer (note: one of the three P16 slices did not show labeling in the subplate). The overall levels of receptor binding remained strong through adulthood and maintained a laminar distribution, while the carbachol response decreased after the second postnatal week and became much more uniform throughout the cortical layers after P16 (Figs. 16 and 17).

The PI response elicited by ACPD was strong in upper layer V from ages P4 - P12 and in a strip within layer II/III from ages P8 - P16 (Figs. 18-24). Only on P6 did the PI response elicited by ACPD in layer IV stand out among the other cortical layers (Fig. 19). All three of the P6 slices incubated with ACPD showed layer IV labeling. ACPD

induced labeling in the subplate from P4 - P12. However, one slice from the P8 group and one slice from the P10 group did not show an amplified PI response in the subplate. The PI response and receptor binding data showed similar labeling pattern in older animals, both being generally less dense and more homogeneous throughout the neocortex (Figs. 25 and 26).

Difficulties in obtaining consistent [^{3}H]CDP-DAG labeling across the cortex were encountered. Out of 132 slices, 96 were considered successful. Of the unsuccessful slices, 9 showed very weak labeling and 27 irregular labeling. Only the slices that contained fairly consistent laminar [^{3}H]CDP-DAG labeling across the cortex were used in the results presented here. This consisted of the three most consistent slices from each age group, with at least two of the slices being from different animals. The variability of slices was greater between animals rather than within animals.

DISCUSSION

There was a transient increase in both carbachol- and ACPD-induced PI-turnover that began early in postnatal development, peaked at P10, and gradually declined towards adulthood. This P10 peak coincides with the highest density of ACPD-sensitive receptor sites in the rat somatosensory cortex (Blue et al., 1997) and has been suggested to play a role in the "critical" period for malleability of synapses in rat cortex (Dudek et al., 1989; Bear and Dudek, 1991). This developmental time course further supports the suggestion that muscarinic and glutaminergic receptors could participate in synaptic plasticity in the developing brain (Mattson et al., 1988; Dudek and Bear, 1989; Bear and Dudek, 1991). The findings in this study also agree with some of the developmental observations

obtained from previous measurements of PI turnover using cross-chopped slices (Lee et al., 1990; Balduini et al., 1991) and synaptoneurosomes (Dudek et al., 1989). Carbachol-induced PI responses were shown to peak around P6 - P10 and then decline towards adulthood in rat cortex (Heacock et al., 1987; Dudek et al., 1989; Balduini et al., 1991). Lee et al. (1990) found the carbachol PI response to peak somewhat later, with the response gradually increasing from birth to P21 and then declining to the adult levels. The PI response elicited by ACPD followed the same ontological pattern shown in some biochemical studies (Dudek et al., 1989; Bear and Dudek, 1991), but not all. Balduini et al. (1991) found the glutamate response to peak at birth and then decrease through adulthood. mRNA for the PI cycle enzymes CDP-diacylglycerol synthase and phosphatidylinositol synthase in the cerebral cortex reaches a maximum at P7 - P14 (Saito et al., 1998), perhaps contributing to the increase in carbachol and ACPD-induced PI response activity at these ages.

The present results distinguish cortical laminae, providing better histological resolution than cross-chop studies that have examined the ontogeny of PI turnover. In the present study, the PI response was measured in discrete cortical layers and was compared to the laminar distribution of PI-coupled receptors during ontogeny. Rather than placing the slices on filter paper and letting the filter paper float on 0.1 ml of solution as was done in the original methods paper (Hwang et al., 1990), the present study submerged the slices in 1 ml of solution. This minor modification allowed for the simultaneous incubation of slices from different animals in the same tissue well, and in our hands produced more consistent labeling patterns.

The present study found baseline labeling to be 2.1% of the carbachol-induced

labeling in P10. These findings are consistent with those found in a study (Bevilacqua et al., 1994) that measured the accumulation of [^{3}H]phosphatidyl CMP. They found baseline labeling to be 1.6% that of carbachol-induced PI turnover in P9 rats (44 dpm/μg of protein without agonist, 3221 dpm/μg protein with both lithium and carbachol; 63 dpm/μg of protein without agonist, 3295 dpm/μg protein with both lithium and carbachol). Some cross-chop studies show similar differences between basal levels and carbachol-induced PI turnover in the rat neocortex. Balduini et al. (1991) found cortical basal levels to be 0.09% of that of carbachol-induced PI turnover at P7, whereas Heacock et al., (1993) found basal levels to be 0.1% of carbachol-induced levels in P13 – P18 rats.

The weaker overall response elicited by ACPD may be due in part to differences in the accumulation of inositol phosphates and CDP-DAG when ACPD is used as an agonist (Heacock et al., 1993). The Heacock study showed that the formation of CDP-DAG is less than that of inositol phosphates when ACPD was used as an agonist. The best correspondence between the formation of CDP-DAG and inositol phosphates was exhibited when carbachol was administered to cortical slices. However, studies that measured the accumulation of [^{3}H]inositol-labeled inositol phosphates in the cortex still found the cholinergic response to be more robust than the glutamate elicited response (Balduini et al., 1991; Heacock et al., 1993).

For the most part, the development of the laminar distribution of the carbachol-induced PI response found in this study parallels that of muscarinic receptors. On P4 the PI response to the acetylcholine receptor agonist carbachol showed greater labeling in the cortical plate than in layers V and VI. Between P6 and P16, labeling in II – upper IV remained high while deep IV - upper V remained low. However, in contrast to P4, lower

V - VI had a relatively high density of labeling at these ages. For the most part, these patterns of PI turnover follow the PI-linked muscarinic receptor developmental patterns (Fuchs, 1995; Höhmann et al., 1995). The exceptions were the low PI response in layer I at ages P4 - P16 relative to the receptor binding pattern and the high PI response in the subplate relative to the receptor binding pattern during these ages. The molecular layer is distinguished by being rich in axons, but having very few neuronal cell bodies. White matter has been shown to express very little phosphatidylinositol synthase relative to grey matter (Saito et al., 1998). The unique cytoarchitecture of this layer may somehow account for its lower PI response (for example, PI-linked G-proteins may be relatively sparse or PI-linked receptors not well coupled in axons). It may also be that the expression of phosphatidylinositol synthase is expressed in all of the cortical layers except layer I as shown by Saito et al., 1998. Another possibility for the discrepancy between receptor binding levels and PI response levels in layer I may be associated with the specificity of the ligand used in the binding study. Both M_1 and M_2 muscarinic receptors are labeled by [^{3}H]QNB. However, M_2 receptors are not linked to the PI cycle (Norman et al., 1989). It may be that it is the M_2 receptors that are predominantly labeled by [^{3}H]QNB in layer I.

There was also a discrepancy between receptor binding levels and the PI response in the subplate layer. From P6 - P16 the carbachol response was strong in the subplate, while receptor binding was less dense. The ACPD-induced PI response was also amplified relative to the receptor binding levels from P4 - P12. The high amount of [^{3}H]CDP-DAG labeling in the subplate may be linked to the transient developmental role of this cortical sublayer in guiding ingrowing cortical afferents (De Carlos and O'Leary,

1992).

Receptor-binding autoradiographic studies reveal that metabotropic glutamate receptors (mGluRs) in layer IV of the neocortex are first expressed on P5 (Blue and Johnston, 1995; Blue et al., 1997), which is about the time that thalamocortical synapses become active in the rat (Agmon and O'Dowd, 1992). This concurs with the high level of ACPD-induced PI activity in layer IV at P6 and is consistent with mGluR5 immunocytochemical data in the rat barrel fields showing these receptors to be more prevalent in layer IV than above or below (Blue et al., 1997). The strong ACPD-induced PI response in layer IV at this age coincides with the time that non-NMDA receptors in the cortex first respond to synaptic currents evoked by thalamic stimulation (Agmon and O'Dowd, 1992). This ACPD-induced PI response in layer IV at P6 occurs near the age (P5) that mGluRs are first reported to emerge in the layer IV whisker barrel field (Blue et al., 1997). It is also during the first postnatal week that barrels in the rat rapidly develop with the formation of septa on P5 (Rice et al., 1985). The present study is the first evidence that mGluRs elicit a PI response in layer IV when whisker barrels begin to mature. Although the PI response to ACPD in layer IV was relatively low by P8, receptor binding and immunocytochemical data in layer IV show mGluRs to have their highest densities in between the ages of P5 and P14 (Blue et al., 1997). After P14 there is a significant decrease in mGluR binding in the layer IV whisker barrels (Blue et al., 1997). The high PI response corresponded with the high density of mGluRs in layer IV only during the age when mGluRs are first emerging and whisker barrels are first being formed, suggesting that the ACPD-induced PI response may have a role in the initial functioning of barrels.

The overall PI response to ACPD decreased after P10 as does the density of ACPD-sensitive mGluRs (Blue et al., 1997). However, in addition to the laminar discrepancy between the dense receptor binding and the relatively low level of PI response in layer IV between P8 and P14, receptor binding patterns do not coincide with the PI activity in layer II. There is heavy [^{3}H]CDP-DAG labeling in layer II between the ages of P8 and P16, but relatively little mGluR binding in layer II at this time. The neurons in layer II are primarily small pyramidal cells that form intracortical connections. These are the last cells to differentiate from the cortical plate (Hicks and D'Amato, 1968) and the strong PI response found in layer II may be associated with the large amount of synaptogenesis that takes place in this layer during this developmental time period (Miller, 1986; Bolz and Castellani, 1997). This difference may also provide a further example that receptor binding density does not always equate with the magnitude of response mediated by these receptors. The observation that the PI response is not necessarily proportional to receptor density could be related to various factors. For example, the number of G proteins available to the receptors, the tightness of the coupling between the receptors and the G proteins, or the local variations in the availability of free inositol (Moats et al., 1993; Bersudsky et al., 1994) and sources of diacylglycerol (Ikeda et al., 1986; Bishop and Bell, 1988) could all account for the discrepancy.

Another research group has employed a similar autoradiographic technique to visualize a PI response in developing neocortex (Bevilacqua et al., 1994 and 1995). The main differences in methodology and analysis are summarized in Table 1. Unlike the laminar analysis in the present study (Robinson et al., 1993), the Bevilacqua group used

emulsion-coated slides and quantified PI responses in somatosensory cortex as silver grains in individual neurons or in neuropil. They did not use a counterstain to visualize cell bodies, and could only localize grains to pyramidal cells whose form was distinctly labeled. They omitted layers devoid of pyramidal cells, such as layer IV. It is worth noting that this layer contains most of the thalamocortical synapses (Agmon and Dowd, 1992) and is the layer that constitutes the whisker barrels (Rice et al., 1985). In addition, cortical layer IV has the highest density of PI-related glutamate receptors (Blue et al., 1997).

MATERIALS & METHODS	**Bevilacqua et al., 1995**	**Present Study**
Rat Strain	Albino	Pigmented
Ages, where PO = day of birth	P2,P6,P9,P13,P20	P4,P6,P8,P10,P12, P14,P16,P21,P30
Anesthesia	None	Metofane
Recovery in Low Ca^{++}	None	0.5 mM, 30 min
Oxygenation	Every 15 min	Continuous
Ascorbic Acid	None	0.1 mM
Glucose Concentration	12 mM	10 mM
[$CaCl_2$] (Incubation)	1.3 mM	2.4 mM
[Lithium]	20 mM	5 mM
[ACPD]	0.2 mM	0.1 mM
[Carbachol]	2 mM	1 mM
Embedding	Fixation, EtOH, Dehydration, Resin	O.C.T.
Section Thickness	1 μm	30 μm
[^{3}H]Cytidine	10 μCi per ml	20 μCi per ml
Autoradiography	Emulsion-coated Slides	Autoradiographic Film
Quantification	Silver Grains in Pyramidal Neurons and "Neuropil"	nCi/mg in Cortical Layers

Table 2. Differences between the present study and Bevilacqua et al., 1995.

A comparison of observations made in the present study with those of Bevilacqua et al. (1995) reveals some differences. (Note: the ages from the Bevilacqua study have been converted to match the present study with P0 equaling day of birth.) For example, diffuse, negligible labeling throughout the neocortex after P9 was reported by Bevilacqua and colleagues, while the present study found strong labeling and strong laminar demarcations through P16. Also, the Bevilaqua study did not address labeling in the subplate layer (layer VIb). The present study consistently found heavy labeling in this layer, which plays a pivotal role in the development of the neocortex (Ghosh et al., 1990). Another difference between the two studies was that Bevilaqua et al. (1995) reported carbachol-induced labeling to be heavier in layer V than layers II/III from P2 – P9, whereas the present study found the opposite.

When ACPD was used as the agonist, one of the most noticeable differences between the present study and Bevilacqua et al. (1995) was that the presence of labeling in the subplate layer reported in the present study was not addressed in the Bevilaqua study. In addition, only the present study found labeling in layers II/III on P8. Of special note, there was labeling of layer IV on P6 in the present study. This layer is of particular interest because it is at P5 when mGluR density first becomes greater in layer IV than in surrounding layers and immunostaining of these receptors reveals a whisker-barrel pattern in layer IV (Blue et al., 1997). Bevilaqua et al. did not report any labeling in layer IV at any age, including P6. Another difference was that with ACPD, Bevilacqua et al. (1995) reported uniform labeling along the depth of the cortex on P9, whereas the present study found ACPD-induced labeling to be trilaminar at this age.

The differences in Table 1 may account for some of the differences found in the results from these two studies. Our group has found that the integrity of the tissue can influence the PI response. Using Erythrocine B to measure tissue viability, we have found the handling of the tissue, the oxygenation of the tissue, and the [Ca^{++}] in the recovery buffer to influence the health of the brain slice. Ca^{++} tends to increase tissue damage via excitotoxicity. "Dead" tissue often had a PI response that resulted in an edge effect (darker labeling on the borders of the slice). On the other hand, more homogeneous, fairly dark labeling occurred when measures allowed [^{3}H]cytidine to remain incorporated in tissue via molecules other than CDP-DAG. These include omitting the nuclease rinse or fixation in formaldehyde vapor before the nuclease rinse.

There were similarities between the present study and Bevilacqua et al. (1995). Both studies found the overall labeling from both ACPD and carbachol to peak around the same time (P10) and to be at a minimum at the oldest ages examined (P20, Bevilacqua; P30 present study). In addition, both studies found carbachol to produce more labeling than ACPD for all age groups studied. Both studies found strong ACPD- and carbachol-induced PI responses in layer V within the first ten postnatal days. When carbachol was used an agonist, both studies reported additional labeling in layers II/III and VI between the ages of P6 – P10.

In the present study, developmental decreases in overall PI response may reflect a shift away from PI-linked receptor subtypes as the neocortex matures. Blue and Johnston (1995) found the density of PI-linked metabotropic glutamate receptors to decrease after P14 while NMDA glutamate receptors steadily increase after this age until reaching a stabilization point at P21 that lasts through adulthood. This developmental shift in

glutamate receptor subtypes from the PI coupled mGluRs to the non-PI linked NMDA receptors is consistent with the steady decrease in the PI response after P10 that reaches a low on P21 and P30. However, as described earlier, this does not seem to be the case for muscarinic receptors linked to the PI cycle. M_1 and M_2 receptors increase in the rat neocortex with age (Aubert et al., 1996), while the PI response decreases. This may be interpreted as a decrease in coupling between the receptors and the PI response. Interestingly, the decrease in the muscarinic PI response with age may be linked to the increase in NMDA receptors with age. NMDA has been shown to inhibit carbachol-induced PI turnover (Lee and Huang, 1997).

The present study makes contributions to the assessment of PI responses, particularly in cortical development. The technique itself was improved to enhance tissue viability and facilitate inter-animal comparisons. By localizing the PI response to cortical layers, the present study offers better anatomical resolution than previous biochemical studies. The differences between the location of PI turnover-associated receptors and the location of the PI response found in this study suggest regional heterogeneity in the ontogeny of receptor coupling to second messenger systems. In addition, the laminar distribution of the PI responses was found to be agonist- and age-dependent which may prove useful in future research centering on functional development of the neocortex. The transient peaks of ACPD and carbachol-induced PI responses found in this study confirm the transient peaks reported in biochemical studies and further support the suggestion that transient PI responses are involved in cortical development and plasticity (Dudek and Bear, 1989; Dudek et al., 1989; Bear and Dudek, 1991).

FIGURE LEGENDS

Figure 1. The Slice Chamber. Cortical slices are placed on a Millicell-PC platform and submersed in an incubation well containing the continuously oxygenated solutions described in the text. Entering the well is the tubing that supplies the 95% O_2/5% CO_2.

Figure 2. Test of Methods. Time in recovery buffer did not obviously affect laminar pattern or overall levels of labeling (60 min: 4758 nCi/mg; 90 min: 4591 nCi/mg; 120 min: 4806 nCi/mg). Labeling was minimal in slices incubated without agonist (mean: 87 $\pm$ 11 nCi/mg; n = 4 slices).

Figure 3. Carbachol-induced PI response in autoradiographic sections through a slice of P10 primary somatosensory cortex. Labeling was low in a whisker barrel-related pattern extending from deep layer IV through upper layer V. Arrow points to a whisker barrel. Bar represents 1 mm.

Figure 4. Laminar boundaries were determined by comparing the autoradiograph with the same section Nissl-stained or a Nissl-stained section adjacent to the original.

Figure 5. Average ACPD-induced [^{3}H]CDP-DAG accumulation in rat primary somatosensory cortex. The S.E.M. for each group was based on three slices. The PI response steadily increased from P4 until peaking at P10. The response then declined until reaching the low levels of activity found at the oldest age studied (P30). These results follow the same general pattern as a synaptoneurosome study that showed the glutamate-induced PI response to peak at the end of one week of age and decline to adult levels by five weeks of age (Dudek et al., 1989). The results also coincide with immunocytochemical studies that show the developmental expression of mGluR5s in the somatosensory cortex to increase from birth until P10 and then dramatically decline after the second postnatal week (Blue et al., 1997).

Figure 6. Average carbachol-induced [^{3}H]CDP-DAG accumulation in rat primary somatosensory cortex. The S.E.M. for each group was based on three slices. Similar to the ACPD-induced response, the carbachol-induced response increased from P4 to P10, peaked at P10, and then declined to adult levels. However, carbachol produced more over-all labeling than ACPD at each age studied.

Figure 7. Laminar [^{3}H]CDP-DAG labeling induced by the glutamate agonist ACPD in the whisker barrel cortex of rats ranging in age from P4 to P30. At the earliest age (P4) labeling was most pronounced in upper layer V and the subplate. Labeling remained strong in the subplate from P4 until P12, but from P4 to P6 the strong labeling in upper layer V shifted to the whisker barrels of layer IV. From ages P8 – P16 the PI response showed strong laminar activity once again in upper layer V, minimal labeling in layer IV, and labeling was observed in layer II/III. The labeling across laminae in the two oldest age groups (P21 and P30) was less dense and more homogeneous throughout the neocortex.

Figure 8. Laminar [^{3}H]CDP-DAG labeling induced by the cholinergic agonist carbachol in the whisker barrel cortex of rats ranging in age from P4 to P30. At the earliest age (P4), the carbachol response was higher in cortical layers II/III than in the deeper layers (IV – VI). Like P4, from P6 - P16 there was strong labeling in the more superficial layers (II - upper IV). However, unlike P4, from P6-P16 there was heavy labeling in the inferior layers (lower V – VI). In addition, from P6-P16 there was a band of relatively low density in lower IV and upper V. The carbachol response decreased after the second postnatal week and became uniformly distributed throughout the cortical layers by P21.

Figure 9. Autoradiographic image of the PI response elicited by carbachol in the rat whisker barrel cortex on postnatal day 4. The autoradiograph was assessed using computerized video-based densitometry. Laminar profiles were obtained from rectangular transepts placed along the depth of the cortex. Values are expressed as nCi/mg of brain wet weight based on calibrated tritium standards. The X axis shows distances from the top to the bottom of the transept. Below the transept are some white matter and hippocampus that were not included in the density profile. I = cortical layer 1, II/III = cortical layers 2 and 3, IV = cortical layer 4, V = cortical layer 5, VI = cortical layer 6, WM = white matter.

Figure 10. Autoradiographic image of the PI response elicited by carbachol in the rat whisker barrel cortex on postnatal day 6.

Figure 11. Autoradiographic image of the PI response elicited by carbachol in the rat whisker barrel cortex on postnatal day 8.

Figure 12. Autoradiographic image of the PI response elicited by carbachol in the rat whisker barrel cortex on postnatal day 10.

Figure 13. Autoradiographic image of the PI response elicited by carbachol in the rat whisker barrel cortex on postnatal day 12.

Figure 14. Autoradiographic image of the PI response elicited by carbachol in the rat whisker barrel cortex on postnatal day 14.

Figure 15. Autoradiographic image of the PI response elicited by carbachol in the rat whisker barrel cortex on postnatal day 16.

Figure 16. Autoradiographic image of the PI response elicited by carbachol in the rat whisker barrel cortex on postnatal day 21.

Figure 17. Autoradiographic image of the PI response elicited by carbachol in the rat whisker barrel cortex on postnatal day 30.

Figure 18. Autoradiographic image of the PI response elicited by ACPD in the rat whisker barrel cortex on postnatal day 4. The autoradiograph was analyzed using computerized video-based densitometry. Values are expressed as nCi/mg of brain wet weight based on calibrated tritium standards.

Figure 19. Autoradiographic image of the PI response elicited by ACPD in the rat whisker barrel cortex on postnatal day 6.

Figure 20. Autoradiographic image of the PI response elicited by ACPD in the rat whisker barrel cortex on postnatal day 8.

Figure 21. Autoradiographic image of the PI response elicited by ACPD in the rat whisker barrel cortex on postnatal day 10.

Figure 22. Autoradiographic image of the PI response elicited by ACPD in the rat whisker barrel cortex on postnatal day 12.

Figure 23. Autoradiographic image of the PI response elicited by ACPD in the rat whisker barrel cortex on postnatal day 14.

Figure 24. Autoradiographic image of the PI response elicited by ACPD in the rat whisker barrel cortex on postnatal day 16.

Figure 25. Autoradiographic image of the PI response elicited by ACPD in the rat whisker barrel cortex on postnatal day 21.

Figure 26. Autoradiographic image of the PI response elicited by ACPD in the ratwhisker barrel cortex on postnatal day 30.

Figure 1

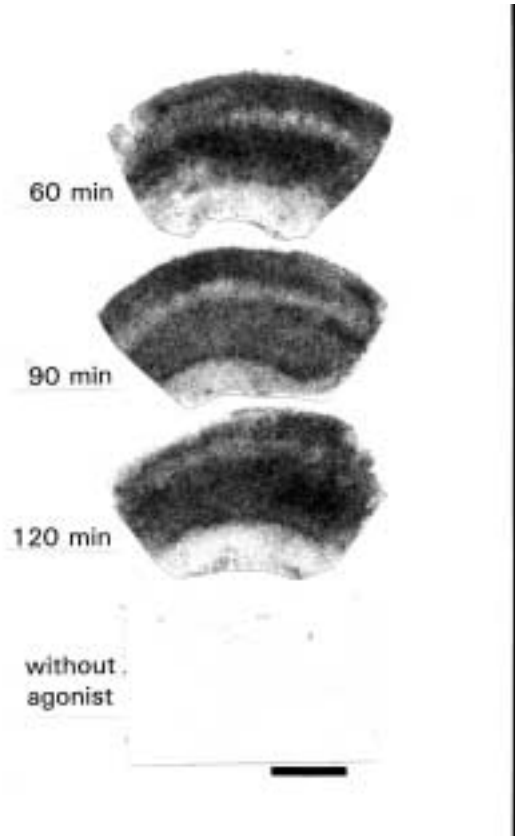
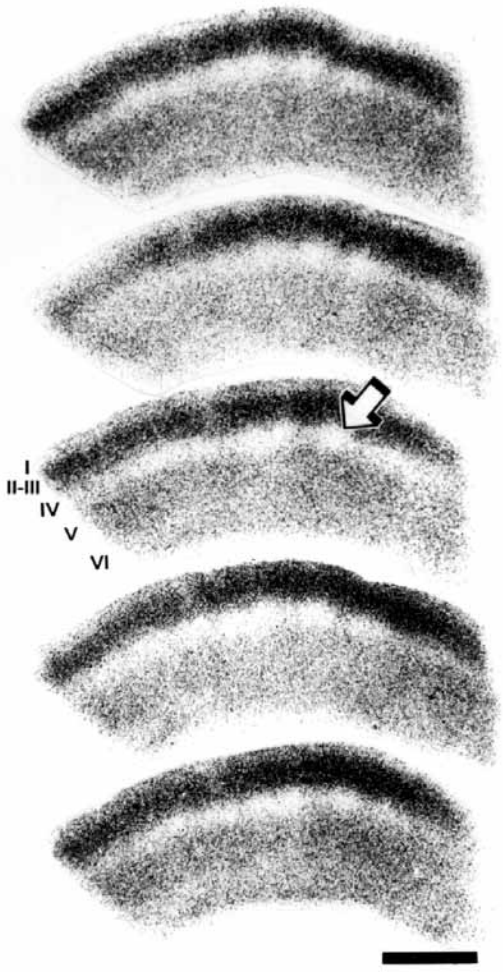


Figure 2



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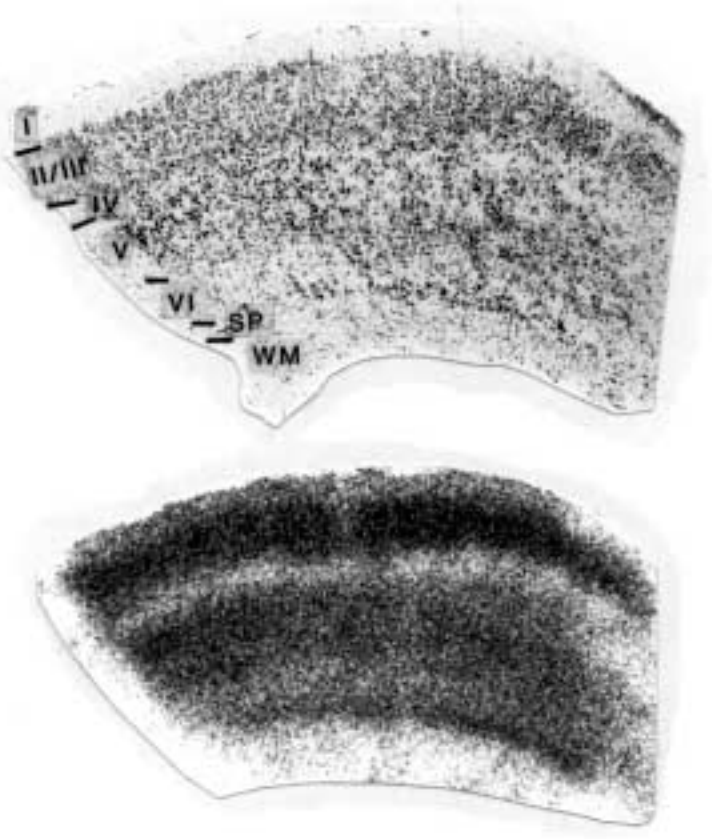

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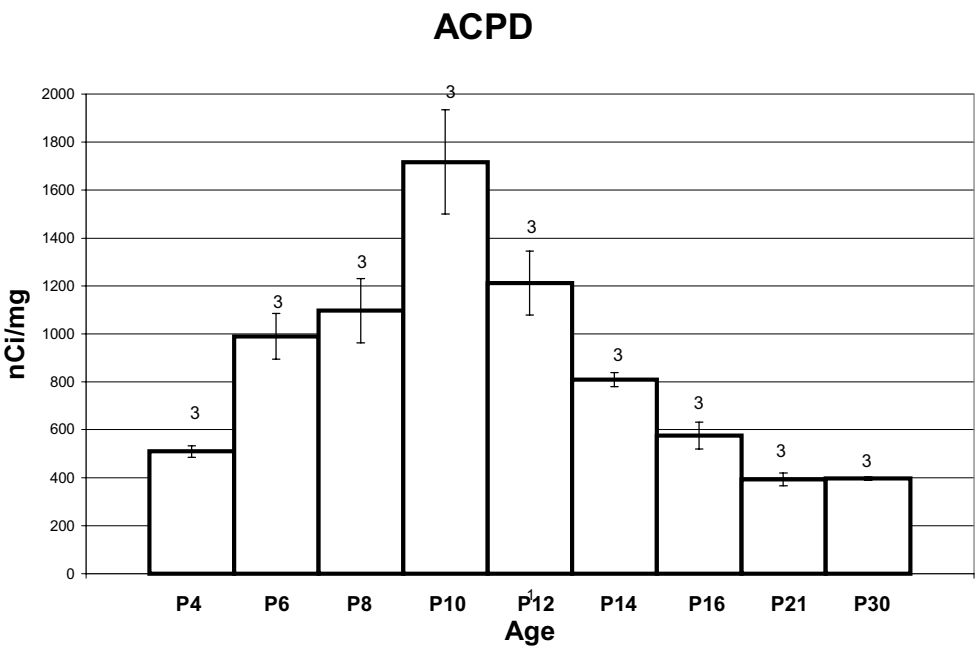

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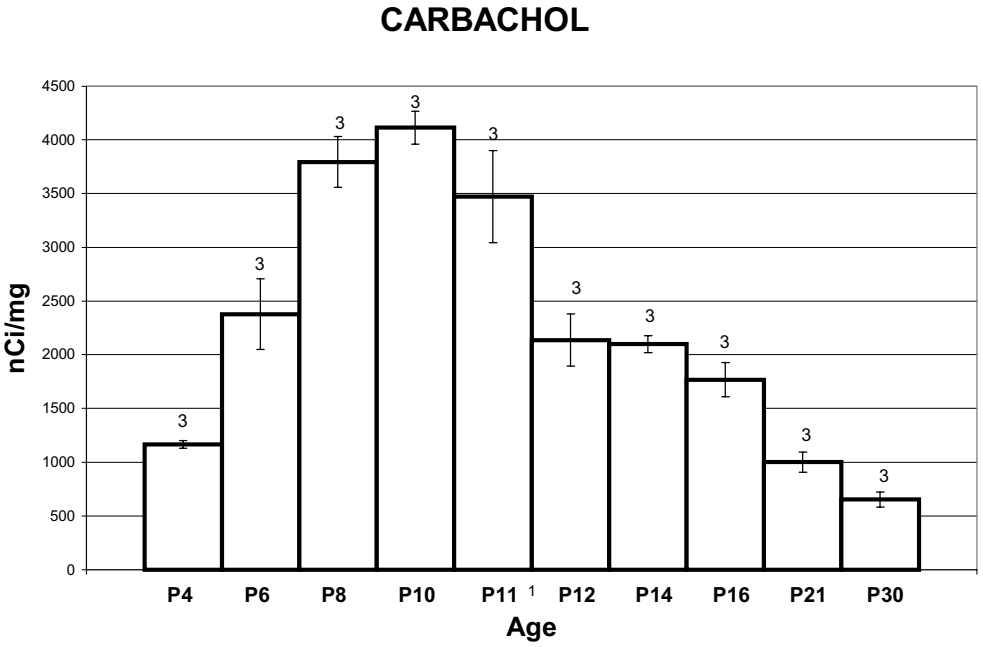

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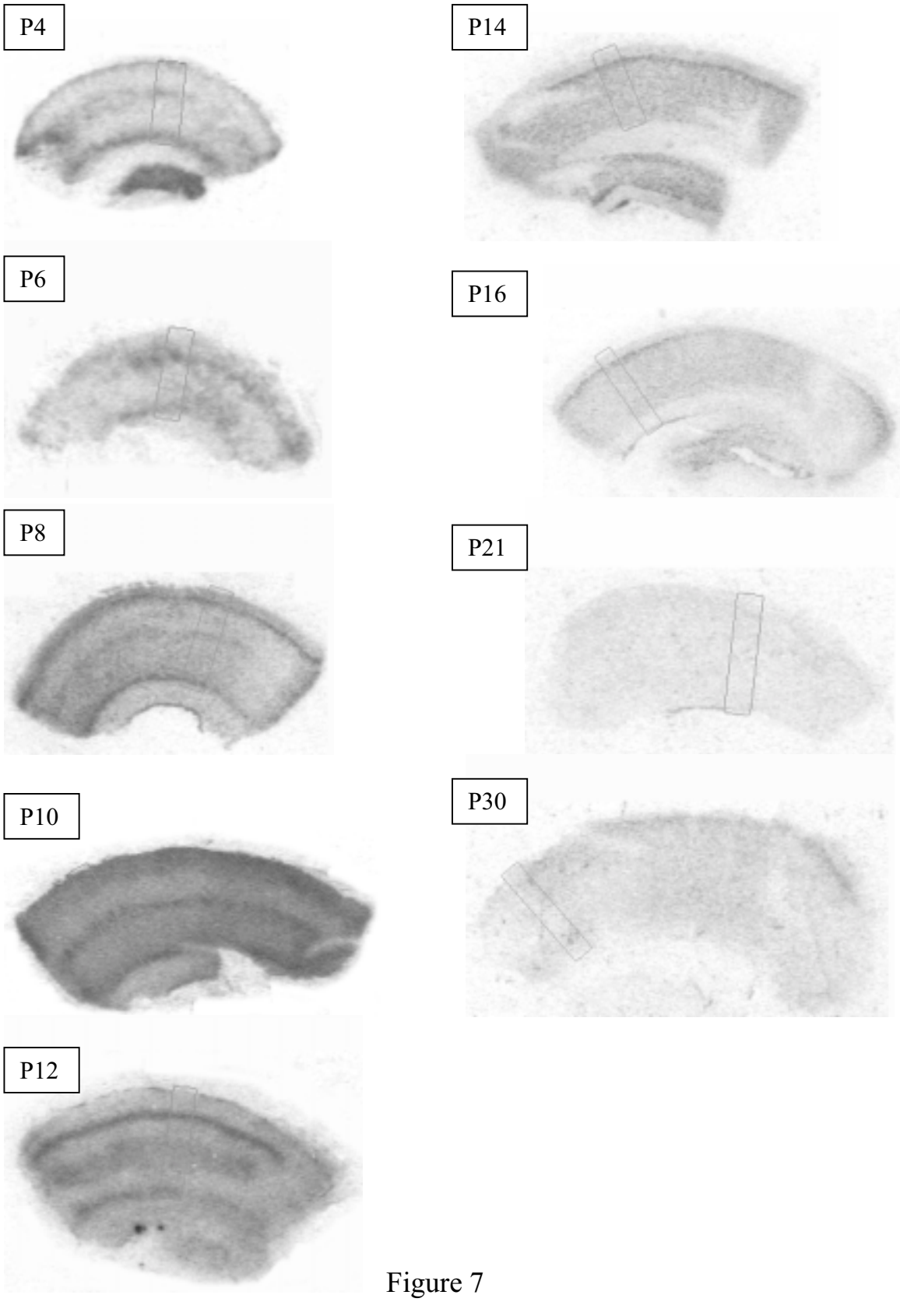
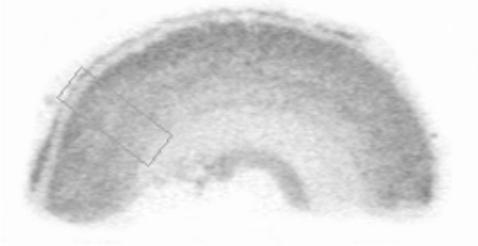


Figure 7

CARBACHOL

P4



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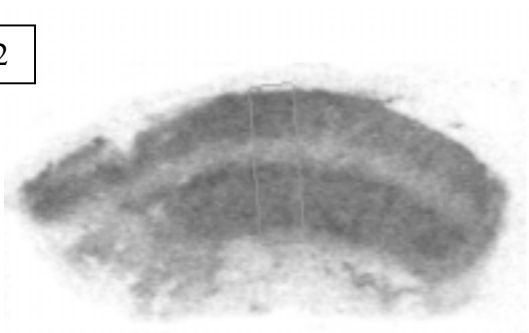

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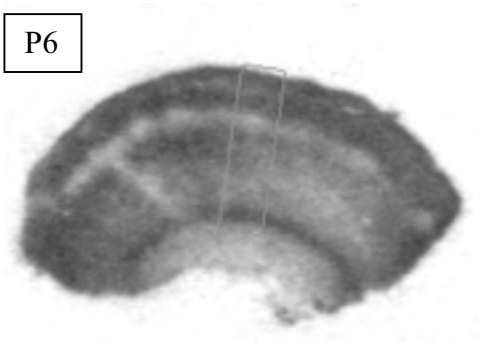
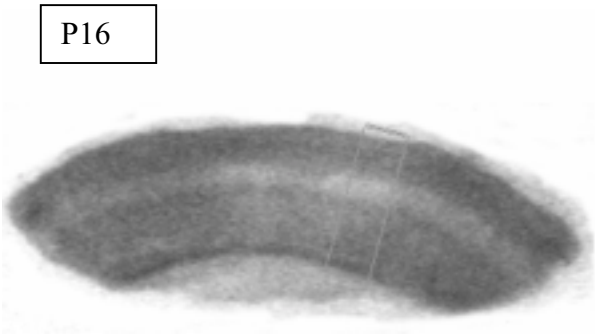


P16



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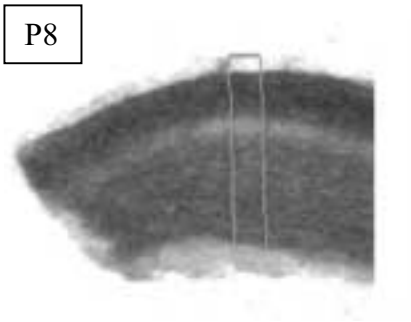

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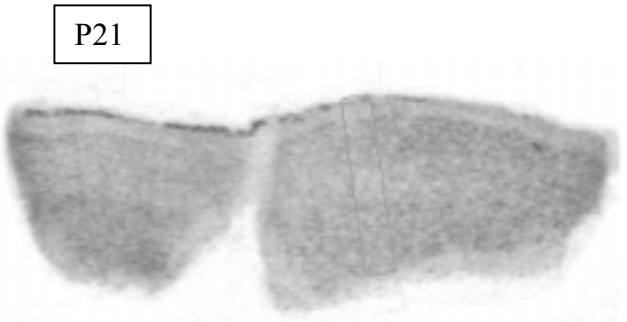
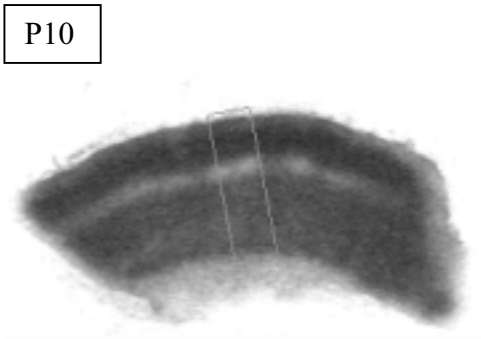


P10



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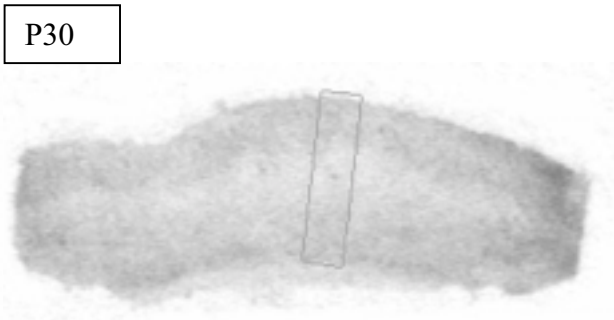
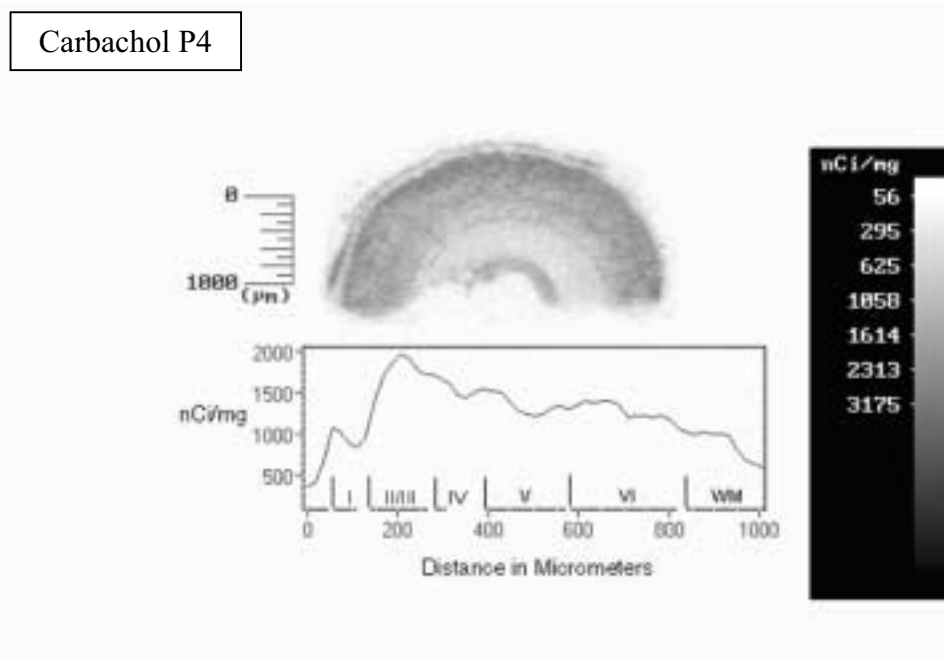


Figure 8

Carbachol P4



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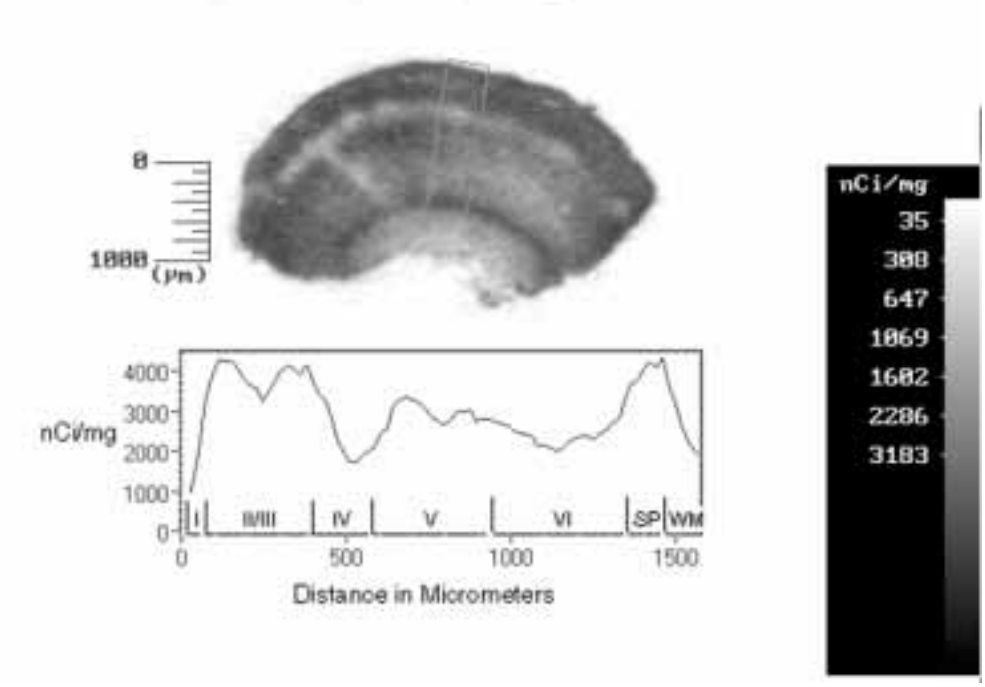
Carbachol P6



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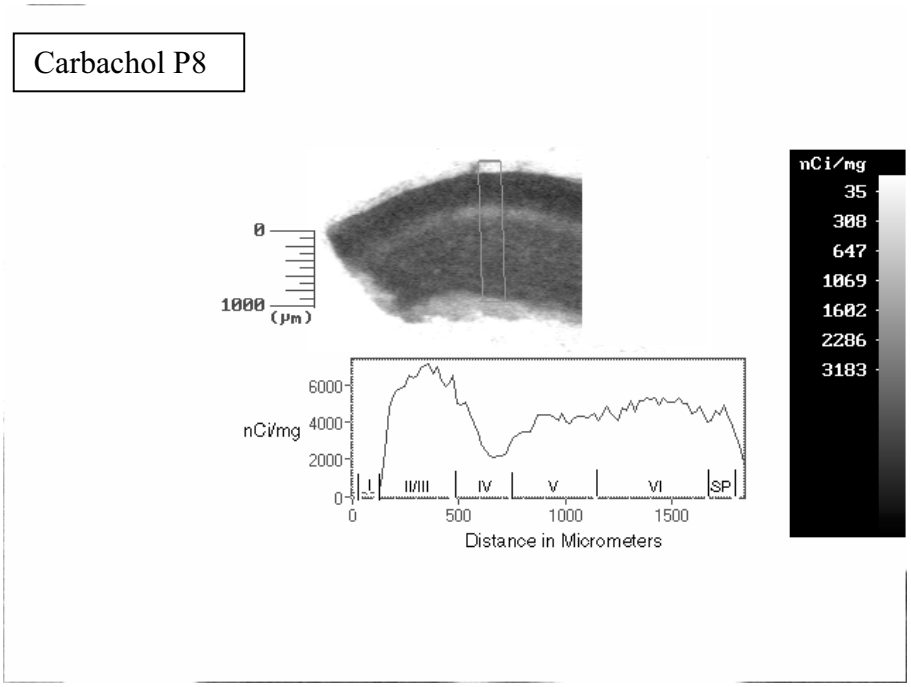
Carbachol P8



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Carbachol P10

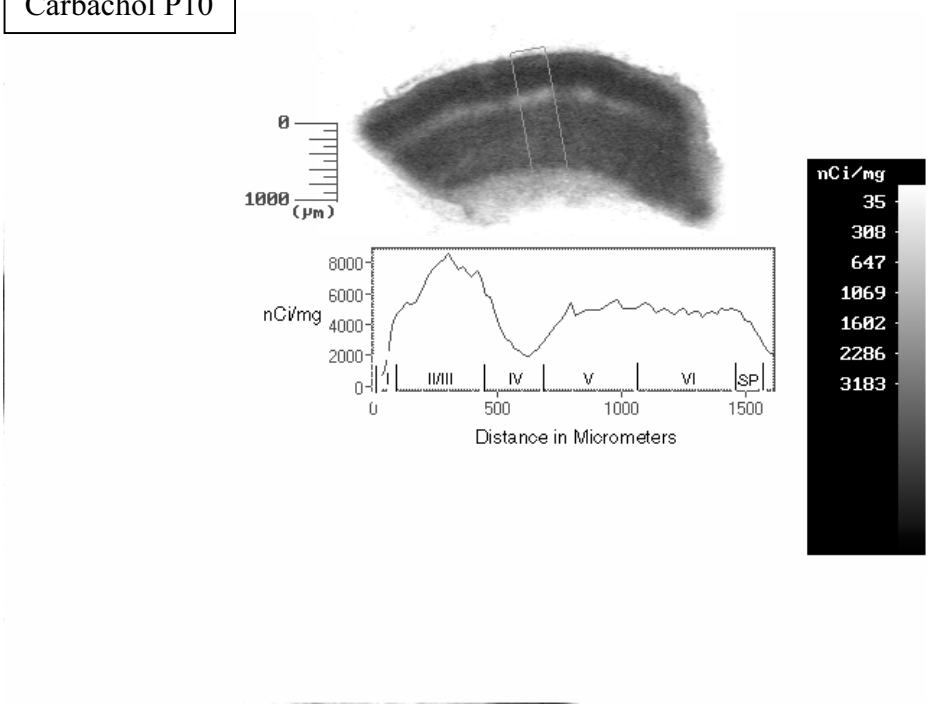
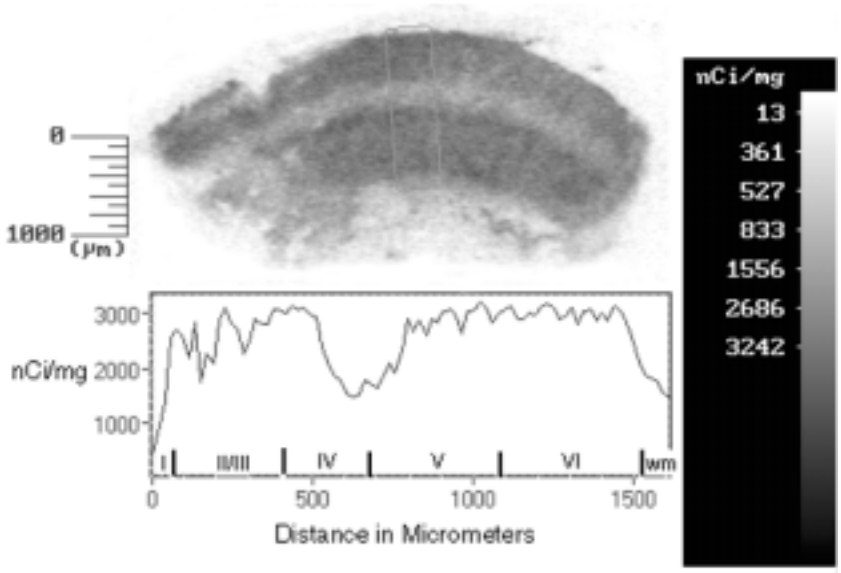


Figure 12

Carbachol P12



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Carbachol P14

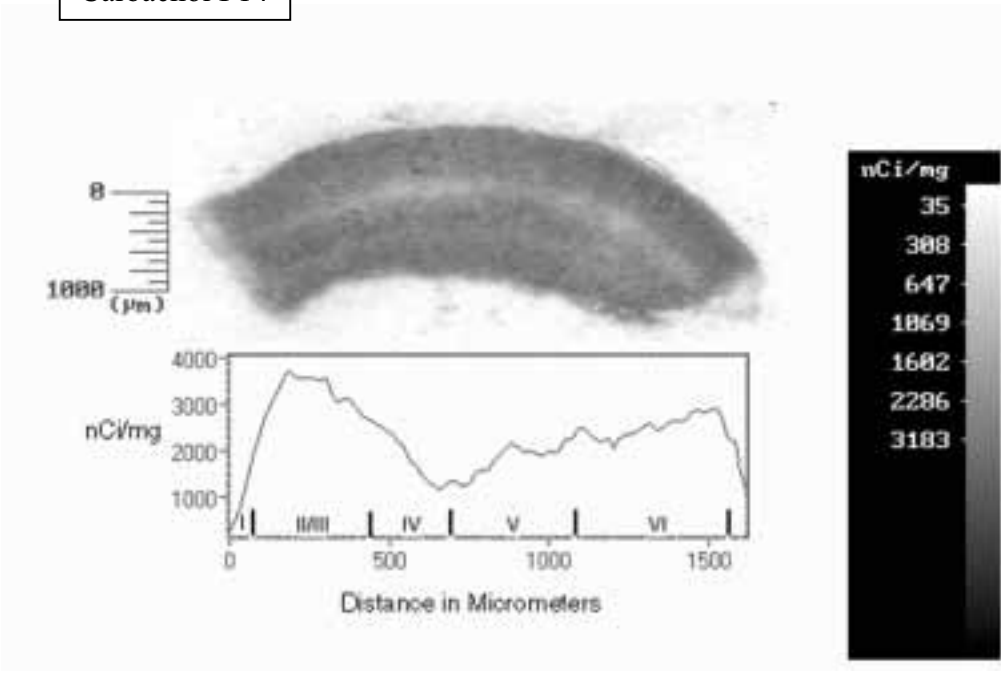


Figure 14

Carbachol P16

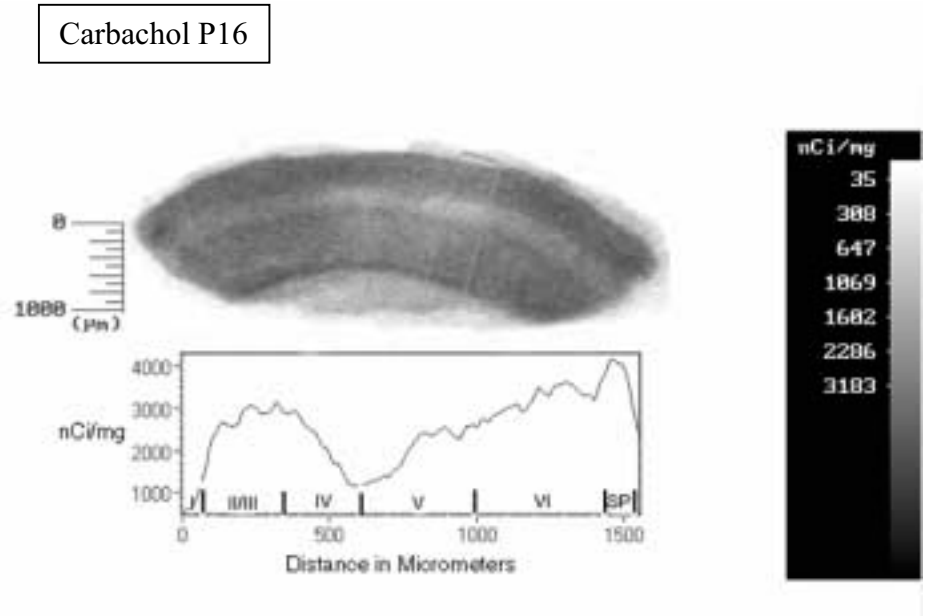


Figure 15

Carbachol P21

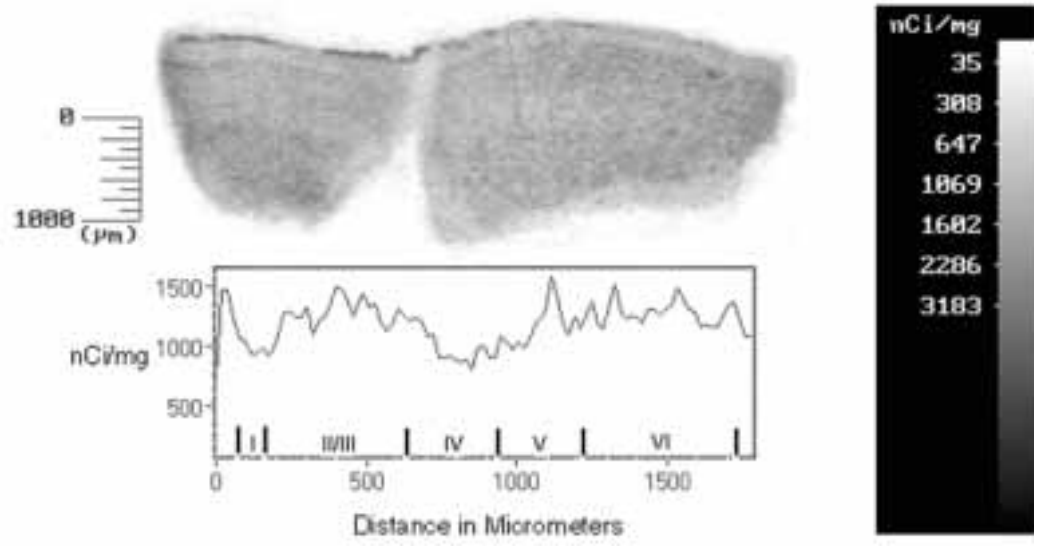


Figure 16

Carbachol P30

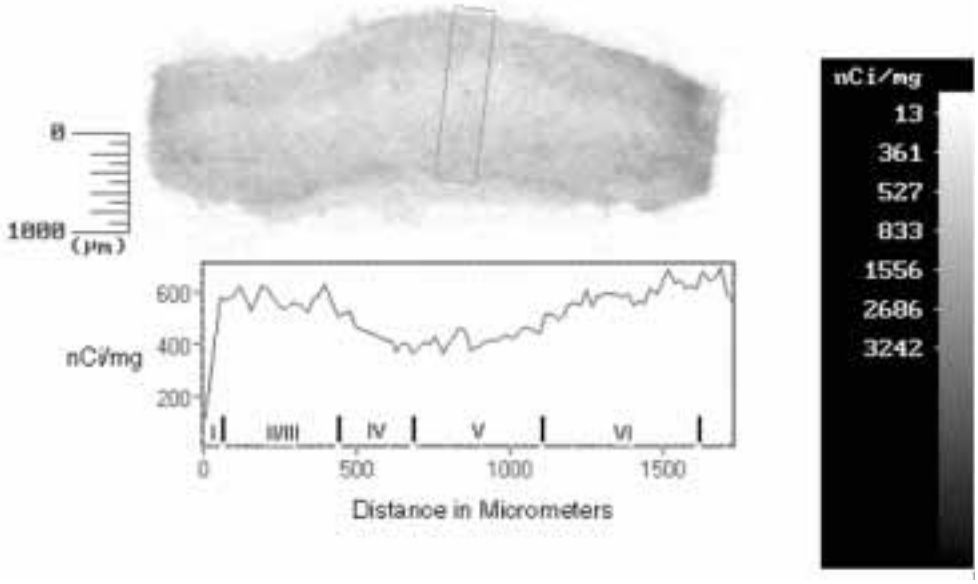
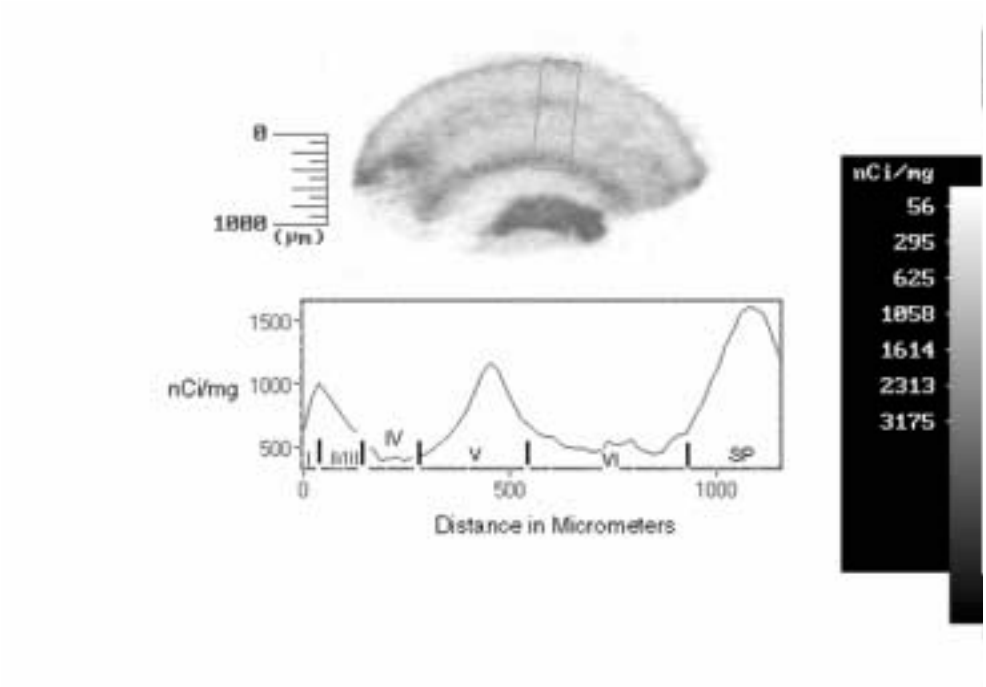


Figure 17

ACPD P4



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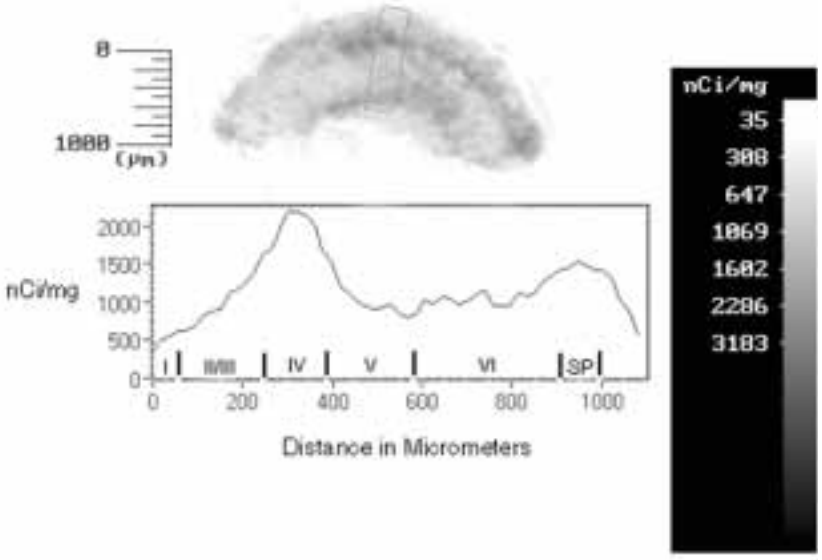
ACPD P6



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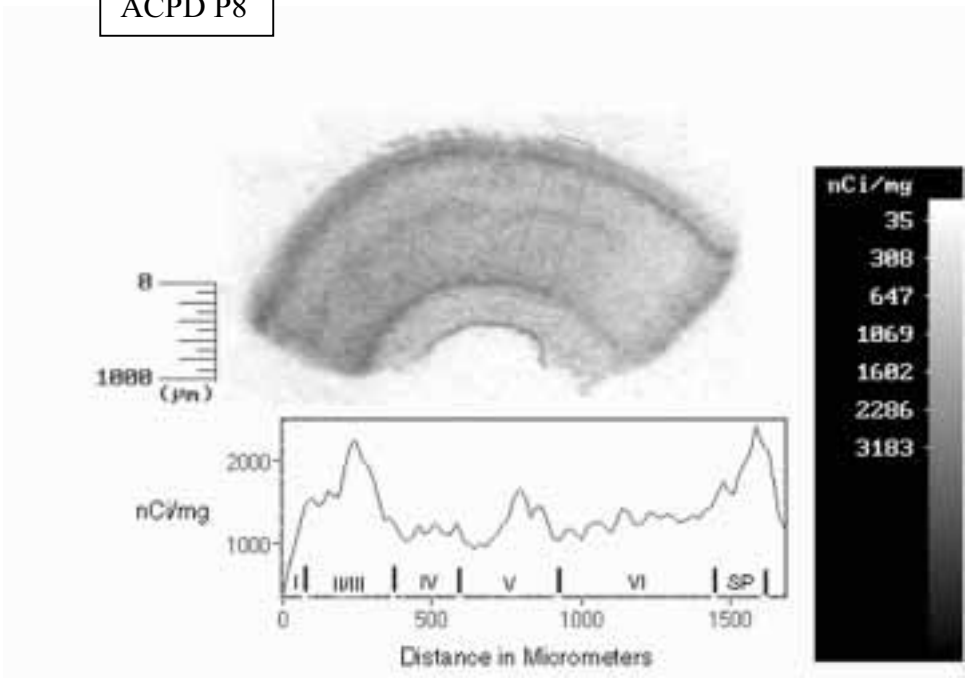

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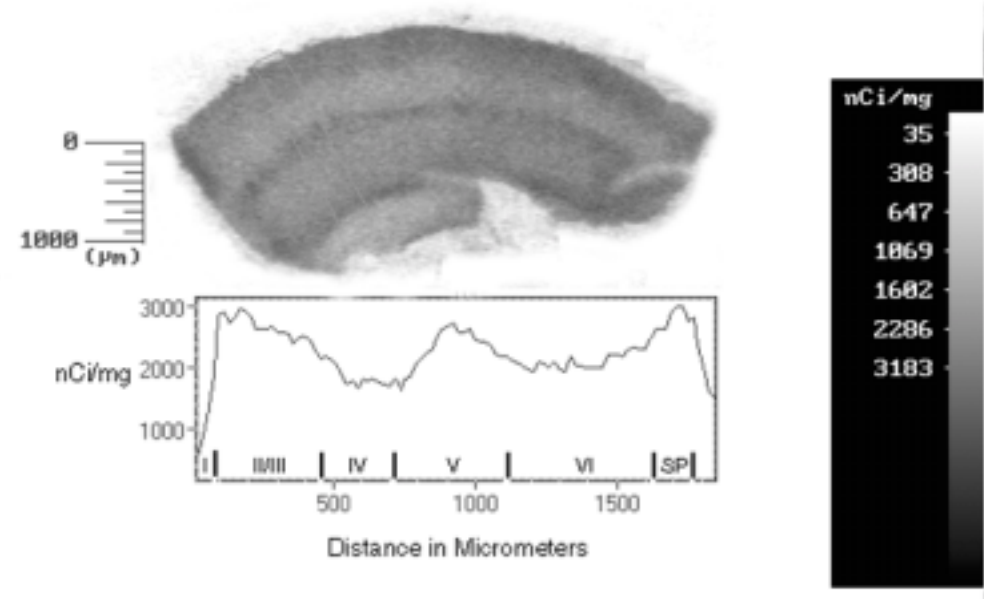

Figure 21

ACPD P12

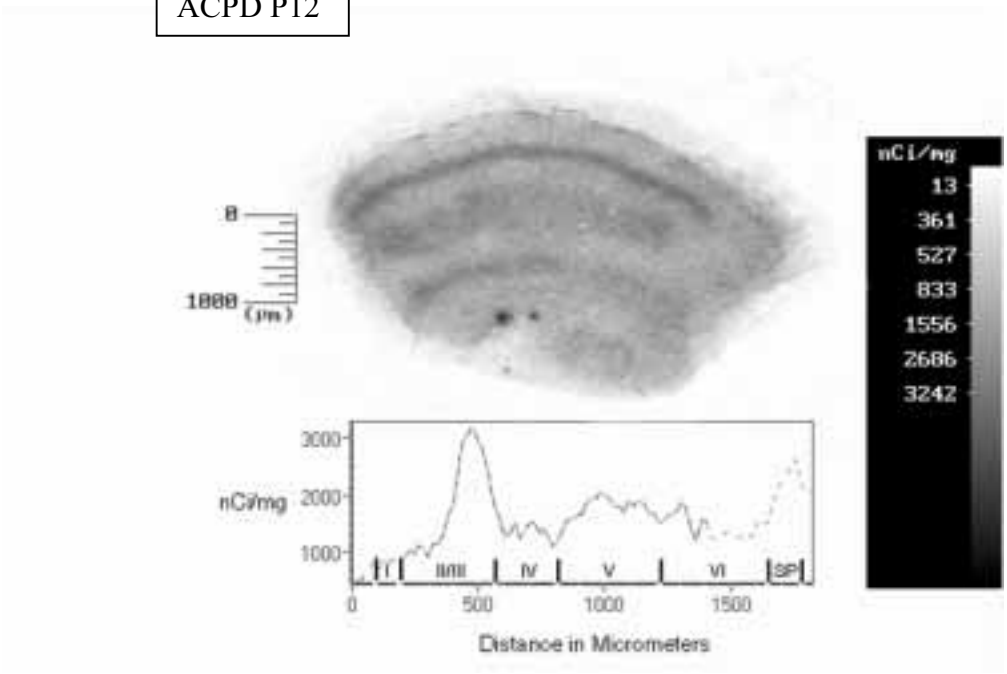

Figure 22

ACPD P14

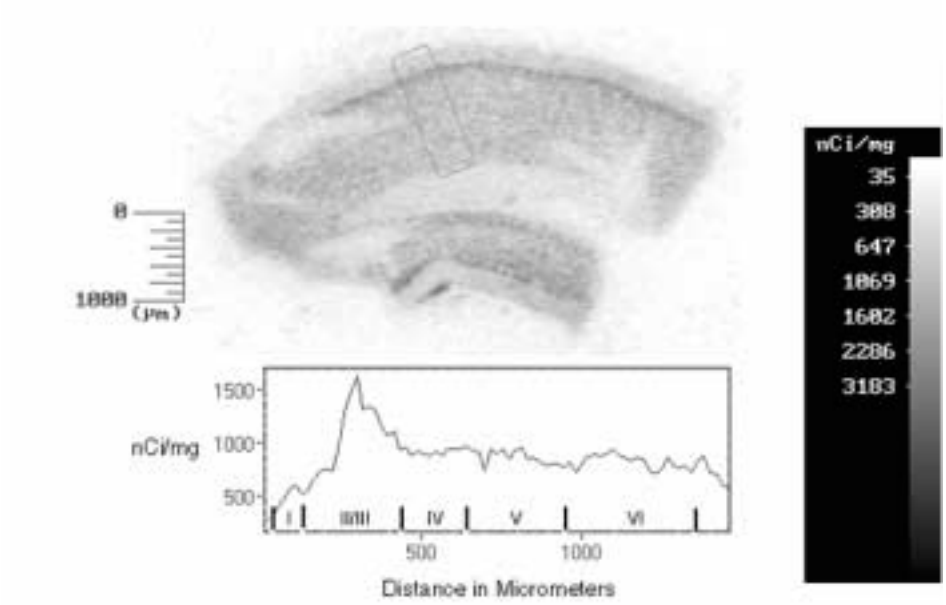

Figure 23

ACPD P16

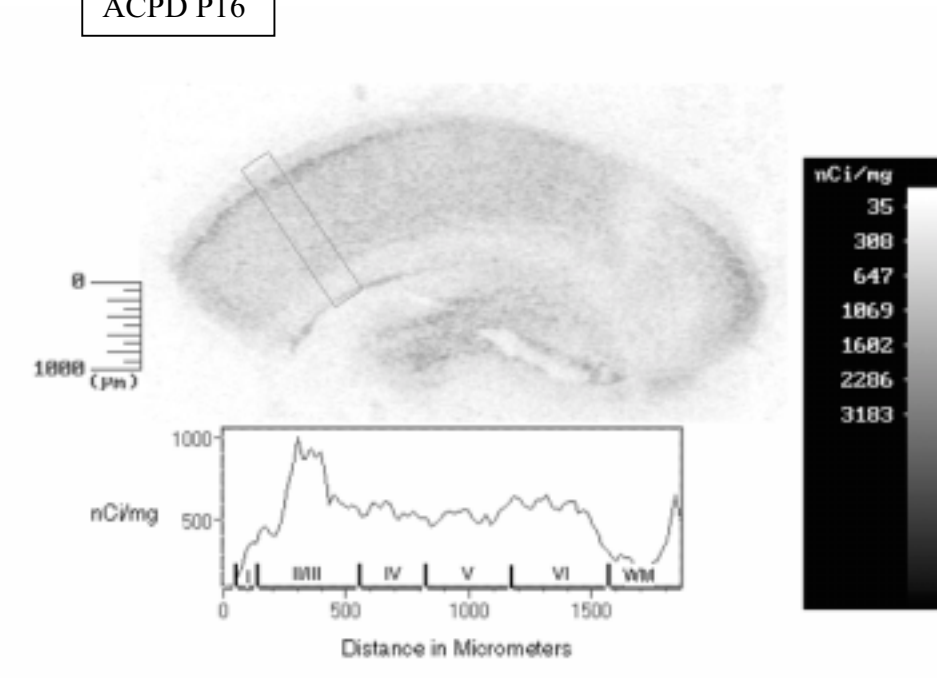

Figure 24

ACPD P21

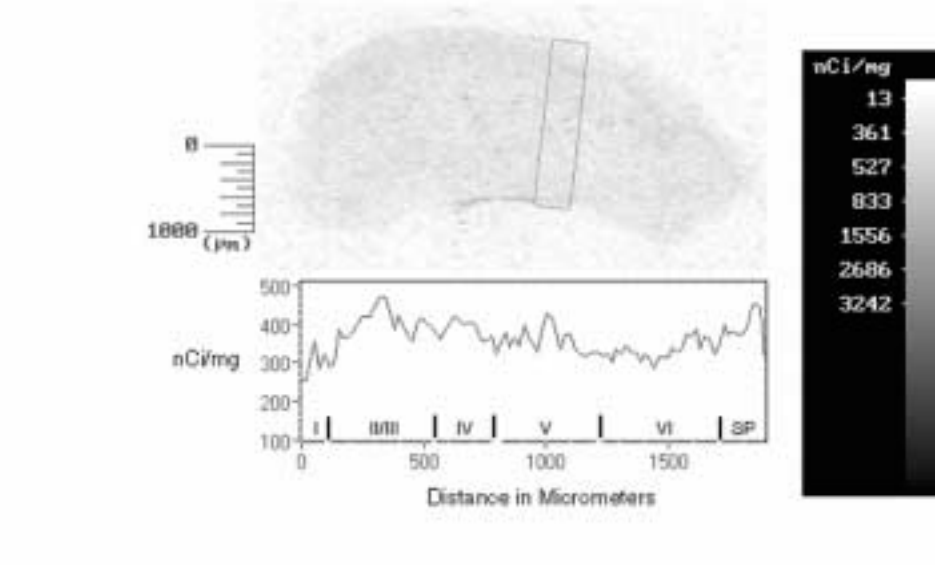

Figure 25

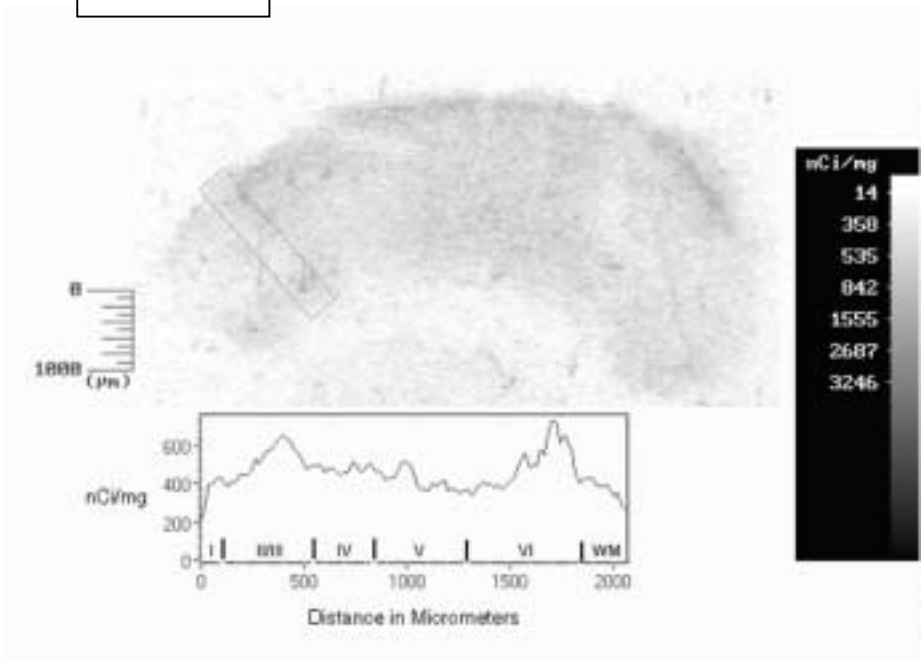
nCi/mg
14
350
535
842
1555
2687
3246
0
1000
(µm)
nCi/mg
600
400
200
0
I
II/III
IV
V
VI
WM
0
500
1000
1500
2000
Distance in Micrometers

Figure 26

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