Transiently Selective Activation of Phosphoinositide Turnover in Layer V Pyramidal Neurons After Specific mGluRs Stimulation in Rat Somatosensory Cortex During Early Postnatal Development

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Biochemical analysis of muscarinic- and metabotropic-glutamate receptor stimulated phosphoinositide (PI) turnover in rat cortical preparations during the first three weeks of postnatal development indicates the existence of a transiently increased accumulation of labeled inositol polyphosphates during the first postnatal week (Gonzales and Crews, 1985; Dudek et al., 1989). We now report for first time the visualization of those neurons responding with increased PI turnover to glutamatergic or cholinergic-receptor stimulation in rat somatosensory cortex during early postnatal development utilizing a recently described method (Bevilacqua et al., 1994). Three, 7, 10, 14, and 21 d old rats were studied. Carbachol in the presence of lithium stimulates ³H-CMP-PA accumulation throughout the cortex at all ages studied. In comparison labeled neurons responding to t-ACPD in the presence of lithium were located exclusively in layer V at P3 and P7, but were found labeled throughout the cortex at P10.

Given that glutamate and cholinergic agonist stimulation are both necessary but not sufficient for cortical plasticity to occur, and that muscarinic and mGluRs stimulation both induce a peak in PI turnover response during the same period of experience-dependent neocortical plasticity, PI derived second messengers signals might be involved in the regulation of the molecular mechanisms of neuronal plasticity. Furthermore, our results show the anatomical correlate of receptor-specific PI turnover activation, and indicate that specific agonist induced PI responses are age, and layer specific.

[Key words: phosphoinositide turnover, rat somatosensory cortex, development, critical period, autoradiography, metabotropic glutamate receptor]

Much work has been devoted to the phenomenological understanding of the processes that lead to the establishment of the neocortical structure (Rauschecker, 1991). It is well documented

that the neocortex displays a higher susceptibility to experience-induced synaptic modification during early stages of postnatal development (Hubel and Wiesel, 1970; Van der Loos and Woolsey, 1973; Blakemore and Van Sluyters, 1974; Woolsey and Wann, 1976; Cynader and Mitchell, 1980; Fox, 1992; Schlaggar et al., 1993; Fox and Zahs, 1994). Studies in the rat somatosensory cortex have shown the existence of a narrow period during early postnatal development during which the anatomical and functional structure of the neocortex is highly modifiable in response to changes in the afferent input from the peripheral sensory organs, and after which the cortical circuitry remains relatively unchanged in response to modifications in afferent input (Hubel and Wiesel, 1970; Blakemore and Van Sluyters, 1974; Cynader and Mitchell, 1980). This particular period of postnatal cortical development in mammals is known as the “critical” or “sensitive” period of neocortical development (Hubel and Wiesel, 1970; Fox, 1992; Fox and Zahs, 1994), and has also been exhaustively documented in the cat and primate visual cortex (Hubel and Wiesel, 1970; Blakemore and Van Sluyters, 1974; Cynader and Mitchell, 1980; Le Vay et al., 1980; Daw et al., 1992). Studies of the pathways involved in the regulation of this phenomenon indicate that three neurotransmitter systems are necessary for neocortical synaptic plasticity to proceed, namely: glutamate (Shaw et al., 1984; Kleinschmidt et al., 1987), ACh and noradrenaline (Bear and Singer, 1986).

In rat visual cortex, it has been shown that the critical period for dark rearing and monocular deprivation induced changes of visual cortical function, occurs between days 21–40 of postnatal life (Fagioli et al., 1994). The peak in glutamate induced PI-turnover in rat cortex peaks at 1 week postnatal, and disappears around 35–40 d postnatal. Very recently the correlation of the critical period of cortical modifiability in response to external factors, and inducibility of long term potentiation (LTP) has been compared in the somatosensory and visual cortex. In somatosensory cortex LTP can be induced between days 3–7 postnatal, while in visual cortex it can be induced between days 14–21 (Crair and Malenka, 1995; Kirkwood et al., 1995). Thus, LTP can be induced earlier in the somatosensory cortex, compared to the visual cortex.

Receptors for noradrenaline (Levitt and Moore, 1979; Morris et al., 1980), ACh (Cortés and Palacios, 1986; Spencer et al., 1986; Buckley et al., 1988; Levey et al., 1991), and glutamate (Stephens et al., 1991; Abe et al., 1992; Shigemoto et al., 1992, 1993) are present in the developing rat neocortex, and the expression of some of these receptors has been recently demon-

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Figure 1. Schematic representation of the hypothetical anatomical substrates that could account for the neurotransmitter-induced peak in phosphoinositide detected biochemically during the development of neocortex. *A*, Different neurotransmitters induce labeling throughout all cortical layers. The peak of neocortical PI turnover results from the stimulation of neurons in all cortical layers by several neurotransmitters. *B*, Distinct agonists induce layer specific labeling. The peak of neocortical PI turnover results from the stimulation of neurons in different cortical layers by individual neurotransmitters. *C*, Schematic representation of the PI turnover peak detected biochemically by measuring incorporation of ^3H -inositol into inositol polyphosphates after specific receptor stimulation. Roman numerals indicate cortical layers; WM, white matter; $\blacktriangle$, labeled neurons; the diagonal strip pattern represents neuropil labeling.

strated to be transient (Blue and Johnston, 1994). In addition noradrenaline, ACh and glutamate receptor stimulation increase PI-turnover in rat cortex preparations (Berridge et al., 1982; Gonzales and Crews, 1985; Nicoletti et al., 1986a,b; Balduini et al., 1991). Moreover, it has been shown that muscarinic and glutamatergic agonist stimulated PI-turnover is higher in neonate than in adult cortex (Dudek et al., 1989). Interestingly, a similar peak in PI response can be elicited in kitten neocortex by muscarinic and mGluRs stimulation, and this transient peak parallels the time window of the critical period of kitten visual cortex plasticity (Dudek and Bear, 1989). Therefore, it has been suggested that increased PI-turnover is directly implicated in the cellular mechanism that leads to neuronal neocortical plasticity

(Dudek and Bear, 1989; Bear and Dudek, 1991). If a certain threshold of neurotransmitter induced phosphoinositide turnover is necessary for neocortical maturation to proceed, two mechanisms could account for this transient peak (Fig. 1): either different neurotransmitters stimulate all layers simultaneously (Fig. 1*A*); or neurotransmitter stimulation is layer specific (Fig. 1*B*). Notice that both mechanisms could subserve a transient peak (Fig. 1*C*). Alternatively, a mixed mechanism could also occur.

However, the exact mechanisms by which activation of PI turnover via these neurotransmitter systems is affecting synaptic plasticity is not completely clarified. While it has been suggested that excitatory amino acid (EAA) receptor stimulation controls the magnitude of synaptic plasticity changes occurring in the

neocortex, the catecholaminergic and noradrenergic systems appear to play a permissive role over EAA effects (Bear and Dudek, 1991). Nevertheless, there has been no information so far regarding which individual neurons or cortical layers respond with increased phosphoinositide turnover to each individual neurotransmitter.

In the present article, we report that mGluRs stimulation induces a specific transient phosphoinositide response mainly in layer V neocortical pyramidal neurons at P7 using a novel method recently described by us that allows to visualize which individual pyramidal neurons are activated after specific agonist receptor stimulation (Bevilacqua et al., 1994). By contrast, muscarinic receptor stimulation while also inducing a transient peak, labels pyramidal neurons throughout the cortex at all ages studied. Therefore, mGluR stimulation at early stages of neocortical development is specific for layer V. Furthermore, we propose that layer V pyramidal neurons play an important role in coordinating neocortical synaptic plasticity at early stages of rat postnatal cortical development.

Materials and Methods

Chemicals and reagents. Carbamylcholine chloride (carbachol), hydroxyurea, actinomycin D, myo-inositol, ribonuclease A, and deoxyribonuclease I were obtained from Sigma. Lithium chloride, was purchased from Fisons, UK. Trans-dicarboxyl-ACPD [(±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid] (t-ACPD), was obtained from Tocris Neuramin, UK. Paraformaldehyde EM grade was obtained from Agar Scientific LTD, UK. Glutaraldehyde 25% EM grade was purchased from Taab Laboratories, UK. Osmium tetroxide was obtained from Johnson Matthey PLC, UK. Proteins were measured by BCA Protein Assay Reagent Kit, Pierce, USA. Dowex 1×8 resin (200–400 mesh, chloride form) was obtained from Sigma, U.K. 5'-³H-cytidine (20.85 Ci/mmol), and D-myo-2-³H-inositol (23.45 Ci/mmol) were purchased from New England Nuclear, U.K.

Animals and tissue slice preparation. Wistar rats were from an in-house bred colony originally obtained from Charles River, U.K. The age of the animals, either sex, was determined by controlling the date and hour of birth ± 12 hr. The day in which the animal was born was considered as postnatal day 1 (P1). Animals were sacrificed by cervical dislocation followed by immediate decapitation; brains were then removed and dissected on ice. The tissue was sliced coronally at 350 μm. Somatosensory cortex slices were obtained by sectioning apart the frontal and occipital lobes by coronal incisions made approximately 2 mm anterior to bregma and 4 mm posterior to bregma, respectively, followed by a transverse section at the level of the rhinal fissure. The remaining cortex was then carefully separated from the underlying tissue with spatulas and placed onto a flat surface. A final incision, approximately 2 mm lateral to the mid-line was performed in order to remove the forelimb, hindlimb somatosensory regions and cingulate cortical areas.

³H-PtdCMP accumulation for morphological studies. ³H-PtdCMP accumulation for morphological studies was performed as in Bevilacqua et al. (1994). Briefly, after dissection slices were recovered in Krebs-Ringer bicarbonate buffer (KRB), pH 7.4 at 37°C, gassed periodically with O₂/CO₂, 95%/5%, respectively, with the following composition: NaCl, 118 mM; NaHCO₃, 25 mM; KCl, 4.7 mM; MgSO₄·7H₂O, 1.2 mM; CaCl₂·H₂O, 1.3 mM; KH₂PO₄, 1.2 mM; glucose, 12 mM. Tissue slices were left to recover for 60 min. The buffer was periodically gassed with O₂/CO₂ (95%/5%). Slices were then washed once in fresh KRB and transferred individually or in groups into 5 ml flat bottomed plastic vial insert tubes containing 3.0 μCi of ³H-cytidine in 300 μl final volume of KRB. Inhibitors of nucleic acid synthesis were added to the incubation buffer at this step, simultaneously with ³H-cytidine at the following concentration: hydroxyurea, 50 mM and of actinomycin D, 1 μg/ml. Slices were allowed to incorporate the radioactive label for 30 min. in a gently shaking bath, at 37°C. Drugs were added after 30 min of labeling at the following concentrations, LiCl, 20 mM; carbachol, 2 mM; t-ACPD, 0.2 mM, samples were then incubated for further 30 min. To stop the reaction, the incubation medium was aspirated and slices were washed several times with ice cold KRB. The slices were then fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 30 min, and postfixed in 1% osmium tetroxide (OsO₄) in 0.1 M PBS

(pH 7.4) further 30 min. Afterwards, slices were dehydrated in increasing concentrations of ethanol with a final step in propylene oxide. Infiltration in Durcupan resin was performed overnight. The next day, slices were flat-embedded on glass slides in fresh Durcupan resin and left to polymerized at 56°C during 48 hr. The coverslips were then carefully removed from the polymerized layer of resin. Small pieces of resin containing whole hippocampal or neocortical slices were reembedded in Durcupan resin blocks. Semithin sections (1 μm thick) were cut on a Reichert-Jung Ultracut ultramicrotome and placed on acid cleaned slides. Slides were then dried on a hot plate. Coating with Ilford K5 autoradiographic emulsion (1:2 in 2% glycerol in distilled water) was performed under Ilford 904 safelight. Sections were exposed for 5–7 d at 4°C. Developing of the coated slides was performed with Kodak D19 for 4 min at 16°C and the slides were then fixed in 30% aqueous sodium thiosulfate for 8 min 16°C. Sections were counterstained with Richardson's Blue (Azure II/ methylene blue/ borax 1% each w/v in distilled water), dehydrated and mounted with DPX (BDH, UK). Photomicrographs were obtained using an Olympus Vanox AH-2 photo microscope in bright- and dark-field utilizing Ilford FP4 film for bright-field micrographs and Kodak Technical Pan 2415, for dark-field micrographs. Due to a number of factors (e.g., the use of 1 μm semithin resin sections, the regular disposition of apical pyramidal cell dendrites versus the random distribution of nonpyramidal cell dendrites, absence of cell type specific counterstains used), morphologically, we can only reliably identify the accumulation of ³H-PtdCMP within neocortical pyramidal cells (Bevilacqua et al., 1994).

Assay of ³H-phosphatidyl CMP accumulation and ³H-inositol lipid hydrolysis. For biochemical measurements of agonist neurotransmitter-induced phosphoinositide turnover 350 × 350 μm cross-chopped slices were used instead of the coronal slices used for morphological studies. Stimulation of phosphoinositide turnover was performed as for morphological studies (see above). The following modifications were introduced; 5'-³H-cytidine and D-myo-2-³H-inositol were used both at 0.33 μCi/100 μl of KRB. All the other reagents including agonists were used at the same concentration. The reaction was terminated by addition of ice-cold trichloroacetic acid (TCA) to a final concentration of 0.5 M, the samples were then left to in ice for 20 min before the analysis of ³H-PtdCMP or ³H-inositol phosphates.

³H-PtdCMP measurement. The acid extracted pellet was then washed once with an aqueous solution of TCA (5% w/v) containing 1 mM EDTA and once in ice cold distilled water. To each sample, 750 μl of methanol/chloroform/12 M HCl (80:40:1 by vol) were added. Samples were then allowed to stand for further 20 min until the addition of 250 μl of chloroform. The solvent mixture from each tube was then collected in a separate vial and phases were split after the addition of 450 μl of 0.1 M HCl by centrifugation for 2 min at 2000 r.p.m. Aliquots (200 μl) from the lower, lipid containing phase (chloroform soluble fraction) were placed in counting vials, and the solvent was left to evaporate. Scintillation cocktail (Ultima Gold, Packard, UK), was then added and samples were counted.

³H-Inositol phosphate analysis. Samples (0.4 ml) of supernatant were mixed 0.1 ml of 10 mM EDTA (pH 7.5 with NaOH), and 0.2 ml of 1,1,2-trichlorotrifluoroethane/tri-n-octylamine, (1:1, v/v) to remove acid; the mixture was vortexed and spun at 2000 r.p.m. to help phase separation. Aliquots of the upper phase (200 μl) were then analyzed on Dowex 1×8 chromatography resin columns. ³H-inositol phosphates and ³H-glycerophosphoinositol phosphates were then separated and counted as described elsewhere (Batty et al., 1985; Simpson et al., 1987).

Quantification of autoradiographic labeling in cortical tissue slice. The same slides were used to obtain photomicrographs and to quantify the density of grains present over different components of the tissue. To this end the Quantimet 500+ Imaging Processing and Analysis System, Leica, Cambridge, U.K. was used. The area occupied by the silver grains (grain area) was assessed against the nonlabeled background tissue, within the perimeter of a given cellular or neuropil area of tissue. In order to calculate the area occupied by each cell, the cell perimeter was outlined and the area included within the line drawn was computed.

Statistical analysis of data. Two measurements were used to calculate the density of silver grains over neuronal bodies and neuropil: (1) The total amount of silver grains, calculated as the area occupied by the silver grains expressed in μm²; (2) The area of a cell or neuropil under study also expressed in μm². The area occupied by neuronal cell bodies was determined by outlining manually each cell perimeter on the computer screen and the enclosed area was then calculated (cell area, CA). In the case of the neuropil, the area under study was outlined on the

Table 1. Summary of results obtained from analyzing the density of silver grains over neuronal bodies and neuropil in two or three different experiments performed at each age studied

Layer	Age					
	3 d		7 d		10 d	
	Cells	Neupl	Cells	Neupl	Cells	Neupl
<i>t</i> -ACPD						
I	—	0:2	—	0:3	—	0:3
II/III	0:2	—	0:3	0:3	2:3	3:3
V	2:2	0:2	3:3	0:3	1:3	3:3
VI	1:2	0:2	0:3	0:3	2:3	2:3
Carbachol						
I	—	1:2	—	3:3	—	2:3
II/III	1:2	—	3:3	3:3	2:3	3:3
V	2:2	1:2*	3:3	2:3#	2:3	3:3
VI	1:2	1:2	3:3	3:3	3:3	2:3

Each fraction shown represents the ratio between positive experiments against the total number of experiments quantified for each age, and for both neurons and neuropil (neupl). For example, 0:3 means that in none out of three experiments evaluated did the density of grains differ from controls as evaluated by Student's *t* test for paired samples (15); 2:3 means that in two out of three experiments evaluated, the density of silver grains differed from lithium controls, etc. Differences were considered statistically significant when $p < 0.02$.

* The negative experiment has a borderline result, $p < 0.025$.

The negative experiment has a borderline result, $p < 0.058$.

computer screen excluding cell bodies (neuropil area, NA). The density of grains was then calculated as the ratio (*R*) between the grain area (GA) over the cell or neuropil area, according to the equation $R_{\text{neuron}} = \text{GA/CA}$ or $R_{\text{neuropil}} = \text{GA/NA}$. As *R* values were usually below 0, they were multiplied by 100 in order to facilitate the understanding of the quantification. Thus, *R* values represent the number of grains contained within a cell body or a given area of neuropil. Two to three experiments from each age considered were evaluated. In each experiment three different conditions were analyzed: lithium- (control), *t*-ACPD plus lithium-, and carbachol plus lithium-induced labeling. The density of grains was counted for neurons in cortical layers II–III (or cortical plate in P3 old animals); V and VI, and for the neuropil in areas I, II–III (CP), V and VI at P3, P7, and P10. The mean of 10 *R* values (*XR*) from each cortical layer was calculated. The mean for each layer was then compared to the corresponding layer in lithium controls from the same ex-

periment. Significance was calculated by Student's *t* test for paired samples and significance was considered as $P^* < 0.02$. A layer was considered labeled when its *XR* value was significantly higher than the *XR* value in lithium controls. Therefore labeling was considered negative when the value was equal or lower than the value in lithium controls (Tables 1, 2).

Results

Morphological studies

The great majority of the labeled cells were pyramidal-like neurons according to the shape of the cellular body and the main apical dendrite. With the methodology used in the present study, it was not possible to detect labeling in small neurons (i.e., layer I, IV neurons) or glial cells. Thus, the results reported in the following paragraph refer to pyramidal-like neurons in layers II, III, V, and VI. Labeling was occasionally seen in non-pyramidal like cells. Nevertheless, in the case of cortical layer I, due to the low density of cell bodies, the density of silver grains in the neuropil could be quantified independently and changes in the density of silver grains in this layer are reported for the different ages studied.

At P3 the labeling detected in individual cortical layers was agonist-dependent. Control slices, incubated with lithium 20 mM alone, showed a light labeling distributed homogeneously throughout the cortex (Fig. 2*a*) and at high power magnification, few grains were observed over the perikarya and dendrites of neurons in the cortical plate (Fig. 2*b*), layer V (Fig. 2*c*) and layer VI (Fig. 2*d*). By contrast, Carbachol 2 mM in the presence of lithium 20 mM (Fig. 2*i–l*), induced a strong labeling that was distributed homogeneously throughout the cortex (Fig. 2*i*) when compared to controls. Neurons in the cortical plate (Fig. 2*j*), layer V (Fig. 2*k*) and layer VI (Fig. 2*l*) displayed a dense silver grain accumulation in comparison to lithium alone incubations (Fig. 2*a–d*). Labeling induced by *t*-ACPD 0.2 mM in the presence of lithium 20 mM was limited to layer V (Fig. 2*e,g*). The quantitation of the labeling of the cortical plate induced by mGluRs-agonist stimulation showed no statistical difference to the labeling induced by lithium alone (see following section). At this age the neuropil was only found labeled after carbachol stimulation in the presence of lithium (Tables 1, 2).

Table 2. Semiquantification of the density of silver grains over neuronal cell bodies in layers II–III, V, and VI, and neuropil of cortical layers I, II–III, V, and VI

Layer	Age					
	3 d		7 d		10 d	
	Neurons	Neuropil	Neurons	Neuropil	Neurons	Neuropil
<i>t</i> -ACPD						
I	—	0.8 ± 0.1	—	1.1 ± 0.3	—	2.1 ± 0.3
II/III	6.99 ± 0.8	—	8.62 ± 1.3	1.5 ± 0.3	15.7 ± 1.1 ^a	4.6 ± 0.4 ⁱ
V	13.4 ± 1.3 ^j	0.9 ± 0.1	25.7 ± 2.0 ^a	2.3 ± 0.6	24.7 ± 2.1 ^a	4.6 ± 0.4 ^k
VI	6.75 ± 1.3	0.7 ± 0.1	10.5 ± 1.1	1.4 ± 0.6	12.3 ± 1.1 ^c	2.2 ± 0.2
Carbachol						
I	—	0.6 ± 0.1	—	4.3 ± 0.4 ^a	—	4.4 ± 0.6 ^g
II/III	9.71 ± 1.9	—	21.9 ± 2.3 ^a	4.2 ± 0.4 ^b	20.2 ± 1.5 ^a	4.9 ± 0.5 ^h
V	24.9 ± 3.0 ^b	1.3 ± 0.1	34.2 ± 1.9 ^a	4.1 ± 0.5 ^k	16.5 ± 1.6 ^a	5.4 ± 0.7 ^h
VI	10.1 ± 1.0	1.0 ± 0.1	25.8 ± 2.0 ^a	4.1 ± 0.5 ⁱ	19.2 ± 1.6 ^a	1.9 ± 0.3

Values from one experiment at each age studied are shown as the mean ± SEM of the ratio between the area occupied by silver grains over cells or neuropil (see Materials and Methods) of slices incubated with *t*-ACPD 0.2 mM or 2 mM carbachol in the presence of lithium 2 mM. Ten cell bodies (area = 1500–2500 μm^2) and an area of approximately 13,000 μm^2 of neuropil were counted for each represented cortical layer. Significance was calculated using Student's *t* test for paired samples and is indicated for each value in comparison to the corresponding value in lithium-alone incubations. Densities not followed by a significance symbol are equal or lower than lithium-alone values and were considered negative. Student's *t* test for paired samples: ^a $p < 0.00001$; ^b $p < 0.0001$; ^c $p < 0.0002$; ^d $p < 0.0003$; ^e $p < 0.0004$; ^f $p < 0.001$; ^g $p < 0.003$; ^h $p < 0.004$; ⁱ $p < 0.005$; ^j $p < 0.02$; ^k $p < 0.05$.

Figure 2. Composite microphotograph of autoradiographic imaging of agonist-induced ^3H -PtdCMP accumulation in rat somatosensory cortex at P3. Lithium-, carbachol-, and *t*-ACPD-induced accumulation of ^3H -phosphatidyl CMP in rat somatosensory cortex at 3 d postnatally. This figure

The labeling pattern changed dramatically at P7 (Fig. 3). The silver grain accumulation induced by carbachol 2 mM in the presence of lithium 20 mM (Fig. 3*i,j-l*) was very strong in all layers compared to lithium controls. Cellular bodies and dendrites of pyramidal-like neurons became densely packed with silver grains in layer II-III (Fig. 3*j*), V (Fig. 3*k*), and layer VI (Fig. 3*l*). At the same age, *t*-ACPD 0.2 mM in the presence of lithium 20 mM, induced a strong labeling limited to neurons in layer V of the cortex (Fig. 3*e,g*), while cell bodies in the remaining layers did not show a labeling stronger than that induced by lithium alone. At this age the quantification of grains present in the neuropil revealed a strong labeling in all layers studied after carbachol stimulation. Remarkably, *t*-ACPD stimulation of neuronal labeling was significant, however it did not induce a statistically significant labeling of the neuropil when compared to lithium alone incubated slices (Tables 1, 2).

At P10, lithium incubated cortical slices (Fig. 4*a,b-d*) showed a labeling similar to that observed at P7 both in neurons and neuropil. At this age carbachol induced a strong labeling in all cortical layers compared with lithium alone incubated slices (Fig. 4*i,j-k*). Interestingly, at P10, *t*-ACPD in the presence of lithium, stimulated labeling in layers II-III, and VI of the neocortex (Fig. 4*e,f-h*) in addition to layer V. Importantly, at this age *t*-ACPD in the presence of lithium also induced a statisti-

cally significant labeling of the neuropil of layers II-III, V, and VI but not layer I (Tables 1, 2).

At P14 the labeling obtained with all the agonists used was very low. Lightly labeled neurons could be observed in all cortical layers in slices incubated with carbachol in the presence of lithium. No layer specific pattern of labeling could be recognised for slices incubated with *t*-ACPD in the presence of lithium. At this age the neuropil showed a diffuse and sparse labeling throughout the cortex for all the agonists (data not shown).

Stimulation of somatosensory cortex slices in the presence of lithium at P21 induced poor labeling as evaluated by morphology. Only in carbachol stimulated slices were some cells found to be labeled.

The absence of nuclear staining observed in labeled cells (for example, in Figure 3*b,g,k,l*), confirms the effectiveness of the treatment used to reduce the labeling of nucleic acids by ³H-cytidine.

Semiquantitative analysis of silver grains present over neuronal bodies and neuropil

Analysis of the density of silver grains over cell somata and dendrites of neurons of layers II-III, V, and VI, and neuropil of layers I, II-III, V, and VI was performed at P3, P7, and P10 by using a Quantimet 500+ image analysis system (Leica, Cam-

shows dark- (*a, e, i*) and bright-field (*b-d, f-h, j-l*) images of P3 rat somatosensory cortex slices incubated with lithium 3 mM alone (*a-d*); *t*-ACPD in the presence of lithium 3 mM (*e-h*), or carbachol 2 mM in the presence of lithium 3 mM (*i-l*). After incubation with lithium alone the cortex showed a light labeling (*a*). High power magnification of pyramidal neurons in CP (*b*), V (*c*), and VI (*d*) revealed the presence of sparse grains over the perikarya and dendrites. After stimulation with *t*-ACPD in the presence of lithium, labeling was observed in layer V (*e*). At high magnification, pyramidal neurons in layer V (*g*) demonstrated the presence of heavy labeling over cell bodies and main dendritic processes; however, pyramidal neurons in the CP (*f*) and VI (*h*) did not show a significant difference in their labeling when compared to neurons incubated with lithium alone (see quantification in Tables 1 and 2). Stimulation with carbachol 2 mM in the presence of lithium, displayed a strong labeling throughout the cortex (*i*). High magnification photomicrographs showed a much higher density of grains over cell bodies and dendrites of pyramidal neurons in CP (*j*), V (*k*), and VI (*l*). Notice the labeling of the neuropil in all layers in carbachol stimulated cells (*j-l*). However, neuropil labeling was not statistically different from lithium alone incubated slices after *t*-ACPD stimulation (for quantification, see Tables 6 and 7). Arrows indicate dendrites. Scale bar in *i*, 100 μ m; scale bar in *l*, 10 μ m.

Figure 3. Composite microphotograph of autoradiographic imaging of agonist-induced ³H-PtdCMP accumulation in rat somatosensory cortex at P7. Lithium-, carbachol-, *t*-ACPD-induced accumulation of ³H-phosphatidyl CMP in rat somatosensory cortex at 7 d postnatally. This figure shows dark- (*a, e, i*) and bright-field (*b-d, f-h, j-l*) images of P7 rat somatosensory cortex slices incubated with lithium 3 mM alone (*a-d*); *t*-ACPD in the presence of lithium 3 mM (*e-h*), or carbachol 2 mM in the presence of lithium 3 mM (*i-l*). After incubation with lithium alone the cortex showed a light labeling (*a*). High power magnification of pyramidal neurons in layers II-III (*b*), V (*c*), and VI (*d*) revealed a homogeneous labeling of the neurons in all layers. After stimulation with *t*-ACPD in the presence of lithium, labeling was predominantly observed in layer V (*e*). At high magnification, pyramidal neurons in layer V (*g*) demonstrated the presence of a very heavy labeling over cell bodies and main dendrites; however, quantification of grains present in pyramidal neurons of layers II-III (*f*) and VI (*h*), and in the neuropil of all layers considered did not show a significant difference in their labeling when compared to neurons incubated with lithium alone (Tables 1, 2). Stimulation with carbachol 2 mM in the presence of lithium, displayed a strong labeling throughout the cortex (*i*). High magnification photomicrographs showed a much higher density of grains over cell bodies and dendrites of pyramidal neurons in layers II-III (*j*), V (*k*), and VI (*l*). The labeling was also observed in the neuropil of all layers (*j-l*), (Tables 1, 2). Treatment with inhibitors of nucleic acid synthesis (see Materials and Methods) reduced the staining over the nuclear area (seen more clearly in *b, c, g, k, l*). Cortical layering is indicated by roman numbers on the left margin of the figure. Arrows indicate dendrites. Scale bar in *i*, 100 μ m; scale bar in *l*, 10 μ m.

Figure 4. Composite microphotograph of autoradiographic imaging of agonist-induced ³H-PtdCMP accumulation in rat somatosensory cortex at P10. Lithium-, carbachol-, and *t*-ACPD-induced accumulation of ³H-phosphatidyl CMP in rat somatosensory cortex at 10 d postnatally. This figure shows dark- (*a, e, i*) and bright-field (*b-d, f-h, j-l*) images of P10 rat somatosensory cortex slices incubated with lithium 3 mM alone (*a-d*); *t*-ACPD in the presence of lithium 3 mM (*e-h*), or carbachol 2 mM in the presence of lithium 3 mM (*i-l*). After incubation with lithium alone the cortex showed a light labeling (*a*). High power magnification of pyramidal neurons in layers II-III (*b*), V (*c*), and VI (*d*) revealed the presence of a homogeneous number of grains over the perikarya and dendrites. After stimulation with *t*-ACPD in the presence of lithium, the labeling was statistically greater than in lithium alone incubated slices in all layers (*e*). At high magnification, pyramidal neurons in all layers demonstrated the presence of a heavier labeling over cell bodies and main dendrites, quantitation of silver grains present over neuropil also revealed this to be higher in *t*-ACPD stimulated samples than lithium alone incubations (Tables 1, 2). Stimulation with carbachol 2 mM in the presence of lithium, displayed a strong labeling throughout the cortex (*i*). High magnification photomicrographs showed a much higher density of grains over cell bodies and dendrites of pyramidal neurons in layers II-III (*j*), V (*k*), and VI (*l*). Treatment with inhibitors of nucleic acid synthesis (see Materials and Methods) reduced the staining over the nuclear area (seen more clearly in *f-h, j-l*). Cortical layering is indicated by roman numbers on the left margin of the figure. Arrows indicate dendrites. Scale bar in *i*, 100 μ m; scale bar in *l*, 10 μ m.

Figure 3.

Figure 4.

bridge) as described in Materials and Methods. In 3 d old cortical slices the neuropil in cortical plate was not considered since it is almost completely absent. The results shown in Tables 1 and 2 are in general agreement with, and provide further support to the morphological observations described above and illustrated by the figures presented in the previous section.

At P3, two experiments were analyzed. In neurons of the cortical plate, carbachol 2 mM in the presence of lithium induced an accumulation of grains that was statistically different from lithium alone incubations. This labeling was also positive in layers V and VI. In contrast to *t*-ACPD-induced labeling in the presence of lithium, carbachol also induced labeling in the neuropil of layers I, V, and VI at P3. Slices incubated with *t*-ACPD in the presence of lithium showed a higher density of grains overlying neurons of layer V compared to control incubations with lithium alone. Labeling of the cortical plate and layer VI was not significant in comparison to lithium controls. At this age, *t*-ACPD in the presence of lithium did not induce labeling in the neuropil of any of the layers examined.

At P7, carbachol 2 mM in the presence of lithium 20 mM induced a statistically significant labeling of all layers in which silver grains were quantified for both neurons and neuropil. By contrast, at P7, *t*-ACPD 0.2 mM in the presence of lithium 20 mM, induced labeling that was statistically different from lithium alone incubations only in neurons of layer V. Neurons of all other layers and neuropil throughout the cortex (including layer V neuropil) were not statistically different from lithium controls.

At P10, muscarinic receptor stimulation in the presence of lithium induced labeling in neurons of layer II-III and V and layer VI. Carbachol also induced labeling of neuropil throughout the cortex. At P10, *t*-ACPD 0.2 mM in the presence of lithium 20 mM induced labeling of the neurons in layers II-III, layer V, and layer VI, and in the neuropil of layers II-III, V, and layer VI.

Biochemical analysis

Agonist-induced accumulation of ^3H -PtdCMP and ^3H -inositol phosphates were analyzed in cross-chopped slices to compare our morphological data with biochemical results reported by other authors studying neocortical PI metabolism (Gonzales and Crews, 1985; Nicoletti et al., 1986a,b; Dudek and Bear, 1989; Dudek et al., 1989; Rooney and Nahorski, 1989; Balduini et al., 1991). These assays were run simultaneously in duplicate.

Accumulation of ^3H -PtdCMP in cross-chopped cortical slices (as fold increase over buffer alone incubations), proved to be age dependent (Fig. 4). Carbachol 2 mM in the presence of lithium 20 mM, induced a peak in the response at P7. The accumulation was lower at P3, 58.25 ± 3.77 -fold increase (mean $\pm$ SEM, $n = 2$), reached a maximum at P7, 88.3 ± 0.15 -fold increase (mean $\pm$ SEM, $n = 2$), and decreased slowly afterwards, 82.4 ± 2.3 -fold increase at P10 and 23.5 ± 1.9 -fold increase at P14 (mean $\pm$ SEM, $n = 2$). Metabotropic glutamate receptor stimulation with 0.2 mM *t*-ACPD in the presence of 20 mM lithium showed a similar age dependency displaying a peak in the response induced at P7. The fold increase detected was 7.85 ± 0.4 at P3, 43.25 ± 6.65 at P7, 30.8 ± 3.9 at P10, and 12.5 ± 0.4 at P14 (mean $\pm$ SEM, $n = 2$). However, after mGluR stimulation, the absolute levels of ^3H -PtdCMP accumulation induced were substantially lower than muscarinic receptor stimulation at all ages. Furthermore, percentage wise carbachol's increase between P3 and P7 is only 1.5 times, while the *t*-ACPD induced increase grew six times during the same period.

Similar findings were observed in the agonist induced accumulation of ^3H -InsP₁₋₄ combined fraction (Fig. 5). However, in this case the levels of the response induced with all agonists were remarkably lower than for ^3H -PtdCMP. Carbachol 2 mM in the presence of lithium 20 mM induced a 5.44 ± 0.61 -fold increase (mean $\pm$ SEM, $n = 3$) at P3 reaching to 9.19 ± 0.64 (mean $\pm$ SEM, $n = 2$) at P7, when the response was maximal. After P7 the accumulation induced decreased progressively being 7.94 ± 1.48 (mean $\pm$ SEM, $n = 2$) fold increase at P10 and 4.39 ± 1.25 (mean $\pm$ SEM, $n = 2$) fold increase at P14. The accumulation of ^3H -InsP₁₋₄ combined fraction induced by *t*-ACPD 0.20 mM in the presence of lithium 20 mM was very low at P3, 2.72 ± 0.88 (mean $\pm$ SEM, $n = 2$) fold increase, it increased progressively, 5.2 ± 1.68 (mean $\pm$ SEM, $n = 2$) fold increase at P7, to reach a peak at P10, when the increment was of 8.23 ± 0.73 (mean $\pm$ SEM, $n = 2$) fold increase, after P10 the levels progressively decreased to reach basal levels, 3.44 ± 1.25 (mean $\pm$ SEM, $n = 2$) fold increase at P14.

Discussion

The present results demonstrate for the first time that, when mGluRs stimulation of PI turnover in neocortical pyramidal cells is examined, this stimulation is limited to pyramidal neurons of layer V at early stages of postnatal development (P3–P7). It will later spread to both pyramidal neurons and neuropil of layers II–III, V, and VI at P10, to decline around P14. These results strongly suggest a transient role for metabotropic glutamate receptor linked to PI turnover in layer V pyramidal neurons during early cortical development. While our technique (Bevilacqua et al., 1994) can detect changes occurring within neocortical pyramidal cells, it still has to be perfected to also detect changes within nonpyramidal neocortical cells, a further technical advance which is currently being developed in our laboratory. Although ontogenic and cell type changes in the metabolic fate of receptor-coupled phosphoinositide turnover in the CNS has been previously proposed (Heacock et al., 1990), no direct morphological evidence of such variations had been presented so far. Our results demonstrate a markedly age- and agonist-dependency in the pattern of labeling of different layers and structures of the rat neocortex.

The studies to assess both ^3H -PtdCMP and ^3H -inositol phosphates₁₋₄ accumulation carried out in parallel at P3, P7, P10 and P14 indicate that the level of accumulation of ^3H -PtdCMP and ^3H -inositol phosphates in response to the two agonists used is age-dependent. Stimulation is low at 3 d, peaks between 7 and 10 d after birth, and decreases afterwards. These data are consistent with many other reports (Gonzales and Crews, 1985; Nicoletti et al., 1986a,b; Dudek and Bear, 1989; Dudek et al., 1989; Rooney and Nahorski 1989; Balduini et al., 1991) in which inositol phosphate stimulation was studied in rat cortex after muscarinic and metabotropic glutamate receptor stimulation in the presence of lithium. Our results suggest that the peak in PI response extends up to P10 decreasing thereafter. We conclude that the ability of the cortex to respond with increased phosphoinositide turnover after specific receptor stimulation is transiently augmented during a narrow period of postnatal development between P7 and P10 in rat somatosensory cortex. Moreover, the two assays employed for biochemical analysis, InsP₁₋₄ and PtdCMP accumulation, respectively, appear to be functionally and developmentally equivalent.

Immunocytochemical and *in situ* hybridization studies for the two subtypes of mGluRs coupled to phosphoinositide turnover

Figure 5. ^3H -PtdCMP accumulation in response to lithium, muscarinic-, or metabotropic glutamate-receptor stimulation in the presence of lithium during early postnatal development in rat somatosensory cortex "cross chopped slices." Values are expressed as cpm/50 μl of gravity packed tissue $\pm$ SEM, and as fold increase over the basal condition (buffer incubation). Incubation conditions were as depicted in the figure. Each value represents the mean of at least two experiments. These experiments were performed in parallel with ^3H -inositol $P_{1,4}$ induced labeling assays, (Fig. 6).

(mGluR1 and mGluR5) have demonstrated the presence of these receptors in the cortex of neonate rats during the time window we studied (Abe et al., 1992; Shigemoto et al., 1992; Fotuhi et al., 1993; Shigemoto et al., 1993). Expression of mGluR5 is higher at P6 than adult cortex and it predominates over mGluR1 at all ages (Abe et al., 1992; Shigemoto et al., 1992); furthermore, Shigemoto and colleagues (1992), did not find detectable levels of expression of mRNA for mGluR1 before P11, when it reaches adult levels. These data are in general concordance with our results and suggest that the labeling detected in our cortical preparations possibly represents chiefly *t*-ACPD stimulation of mGluR5 receptors.

Labeling obtained after muscarinic receptor stimulation which is mediated by M_1 and M_3 cholinergic receptor subtypes, was more intense than for mGluRs at all ages studied. Pyramidal neurons and neuropil labeling in layers II-III, V and VI was stronger at P7. Neuropil was labeled throughout the cortex including layer I, but the labeling was more intense in layers II-III and V. This pattern of distribution of the radioactivity after muscarinic receptor stimulation correlates with *in situ* hybridization, autoradiographic and immunocytochemical studies of muscarinic receptor subtypes (Buckley et al., 1988; Levey et al., 1991). M_3 receptors are localized predominantly in mid cortical layers (III-V) while M_1 receptors are more abundant in layers I, II-, and VI; however, variations in the activity and expression of muscarinic receptors during development have also been reported (Shaw et al., 1984; Parnavelas and Cavanagh, 1988; van Huizen et al., 1994). Taken together with the data reported here, we show the existence of a significant correlation of cellular PI responses with several developmental processes occurring within the rat neocortex. Both biochemical and morphological results

indicate the presence of a peak in the ability of the cortex to metabolize phosphoinositide derived second messengers in response to specific receptor stimulation. Remarkably, this peak happens soon after the arrival of subcortical (Lund and Mustari, 1977; Dinopulos et al., 1989) and callosal (Floeter and Jones, 1985) afferents to the cortex, which have been previously proposed to induce a phase of rapid growth of cortical dendrites (Uylings et al., 1990). In addition, a large increase in synapse formation has also been observed to coincide with the rise in the formation of dendrites of pyramidal and nonpyramidal neurons that occurs also at this time (Blue and Parnavelas, 1983a,b).

The selective labeling of layer V pyramidal neurons following *t*-ACPD stimulation of PI turnover at P3 and P7 suggests that cell growth and arborization of the dendritic trees which occur simultaneously with an increase in the formation of synapses (Miller, 1988; Uylings et al., 1990), could occur earlier for layer V, thus suggesting an important role for pyramidal neurons in this layer in coordinating the processes that lead to the growth and establishment of the neocortical circuitry. Furthermore, PI derived second messengers have been proposed to be a common final metabolic pathway that links receptor activation with synaptic plasticity (e.g., noradrenaline, ACh, and glutamate are necessary for synaptic plasticity to occur) (Shaw et al., 1984; Bear and Singer, 1986; Kleinschmidt et al., 1987; Dudek and Bear, 1989; Dudek et al., 1989; Rauschecker, 1991). Therefore, the morphological evidence presented in this work proposes that layer V pyramidal neurons might represent one of the initial targets of this co-ordinated neurotransmitter activity, and might constitute one of the layers where cortical maturation processes begin.

In addition, very recently, two groups have shown that in both

Figure 6. ^3H -Inositol P_{1-4} combined fraction accumulation in response to lithium, muscarinic-, or metabotropic glutamate-receptor stimulation in the presence of lithium during early postnatal development in rat somatosensory cortex "cross chopped slices." Values are expressed as cpm/50 μl of gravity packed tissue $\pm$ SEM, and as fold increase over the basal condition (buffer incubation). Incubation conditions were as depicted in the graphic. Each value represents the mean of at least two experiments. These experiments were performed in parallel with ^3H -PtdCMP induced labeling assays (Fig. 5).

somatosensory and visual cortex of the rat the critical period correlates with electrophysiologically induced LTP (Crair and Malenka, 1995; Kirkwood et al., 1995). Interestingly, LTP can only be induced in somatosensory cortex between postnatal days 3–7, while in visual cortex it can only be recorded between days 14–21. Thus, while induction of LTP correlates with the critical period, its induction appears to follow an antero-posterior developmental gradient. Also, different stimulation paradigms have been used by both groups. The paper studying somatosensory cortex (Crair and Malenka, 1995) has examined the thalamocortical synapses of layer IV, while Kirkwood et al. have examined responses in layer III after stimulation of the white matter, a response in which layer V neurons are implicated (see Stryker, 1995). Thus, these results correlate overall with ours, in that our responses occur during the same time-window than the cortical critical period, induction of LTP, and affect neurons which could be contributing to the LTP responses involved.

In addition, given that the critical period in rat visual cortex is delayed compared to neurotransmitter induced PI turnover changes we have studied in the somatosensory cortex, we would like to suggest that either (1) the second messenger changes discussed in the present article are unrelated to mechanisms involved in sensory-dependent plasticity in the visual cortex, or (2) that if similar experiments to those we report here for somatosensory cortex, were repeated in visual cortex, the peak in PI turnover might well be delayed when compared to our results on somatosensory cortex, following the gradient of induced LTP during neocortical development.

As presented in the introduction, two main hypothesis could explain the peak in the induced PI response detected biochemically (Fig. 1A,B). In this report we propose that the peak in the

phosphoinositide response during neocortical development is generated according to a combination of both possibilities, depending on the agonist and the age of the animals examined. Muscarinic receptor induced phosphoinositide turnover occurs throughout all cortical layers at all ages studied. By contrast, mGluRs stimulation of phosphatidylinositol 4,5-bisphosphate breakdown and the generation of derived second messengers occurs in a layer specific fashion, starting by an initial selective stimulation of neurons of layer V at P3–P7, and then extending into the neuropil and neurons of the other cortical layers as age increases (Fig. 7).

It has been known for some time that neurotransmitters exert trophic effects in the CNS (Felten et al., 1982; Lauder, 1993). In addition it has been recently demonstrated that stimulation of metabotropic glutamate receptors induces protein synthesis in neurons (Weiler et al., 1993). Moreover, it has been shown that other trophic factors such as NGF affect visual cortical plasticity (Gu et al., 1994). Thus, the rapid growth and maturation phase experienced by the cortex during the first postnatal weeks, is probably under the regulation of multiple factors that determine simultaneously its growth and differentiation. According to our results, activation of PtdIns P_2 derived second messengers could represent a final common pathway acting as a central component of this coordinated neuromodulatory effect.

How could changes in layer V be relayed back to the developing neocortex? At present we can envisage two pathways by which this could be accomplished: (1) through inputs from layer V to layer VI, which innervates layer IV neurons, which in turn provide input to supragranular neurons, or, alternatively through (2) the subplate/intermediate zone neurons, which relay back to the developing cortex proper (Friauf and Shatz, 1991), and

Figure 7. Schematic representation of the morphological results obtained. Carbachol in the presence of lithium induced labeling in neurons and neuropil throughout the cortex at all ages studied. *t*-ACPD in the presence of lithium induced labeling only in neurons of layer V at P3 and P7, but labeling was found in neurons and neuropil of layers II–III, V, and VI at P10. Thus, the hypothesis shown in figure 1B accounts for neurotransmitter induced PI turnover at P3 and P7, while the one depicted in Figure 1A accounts for neurotransmitter induced PI turnover at P10. *Roman numerals* indicate cortical layers; *WM*, white matter; **▲**, labeled neurons; the *diagonal strip pattern* represents neuropil labeling.

which we have recently shown receives direct cortical input from layers V/VI of the developing cortex (Shering and Lowenstein, 1994; see also Stryker, 1995). Thus, interconnections centred around layer V could play an important role in regulating neocortical maturation during early development.

In summary, the main contribution of the present work is the morphological identification for the first time of those cortical cells sustaining the transient neurotransmitter induced peak in PI-turnover during early neocortical development. These findings, therefore, provide a possible anatomical substrate underlying neocortical changes in second messengers activity during early cortical development. Further work will now contribute to unravel the central role played by phosphoinositide turnover signal transduction activation and layer V pyramidal neurons during cortical development.

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