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Cajal-Retzius cells in early postnatal mouse cortex selectively express functional metabotropic glutamate receptors

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Abstract

Glutamate receptors have been linked to the regulation of several developmental events in the CNS. By using cortical slices of early postnatal mice, we show that in layer I cells, glutamate produces intracellular calcium ($[Ca^{2+}]_i$) elevations mediated by ionotropic and metabotropic glutamate receptors (mGluRs). The contribution of mGluRs to these responses was demonstrated by application of tACPD, an agonist to groups I and II mGluRs, which evoked $[Ca^{2+}]_i$ increases that could be reversibly blocked by MCPG, an antagonist to groups I and II mGluRs. In the absence of extracellular Ca^{2+}, repetitive applications of tACPD or quisqualate, an agonist to group I mGluRs, elicited decreasing $[Ca^{2+}]_i$ responses that were restored by refilling a thapsigargin-sensitive Ca^{2+} store. The use of specific group I mGluR agonists CHPG and DHPG indicated that the functional mGluR in layer I was of the mGluR1 subtype. Subtype specific antibodies confirmed the presence of mGlur1α, but not mGluR5, in Cajal-Retzius (Reelin-immunoreactive) neurons.

Introduction

Glutamate receptors play important roles during neural development (Komuro & Rakic, 1993; Kaczmarek *et al*., 1997). This amino acid acts both on ionotropic receptors (iGluRs), which form ion channels and mediate fast neurotransmission, and metabotropic receptors (mGluRs), which are coupled to G-proteins and second messengers to effector mechanisms, mediating slower actions (Nakanishi *et al*., 1998). Some of the major roles played by the activation of glutamate receptors depend on complex interactions between mGluRs, intracellular Ca^{2+} stores and Ca^{2+}-sensitive ion channels in neurons (Kawabata *et al*., 1996; Gu & Spitzer, 1997; Fagni *et al*. 2000). During CNS development, a variety of important events like neuronal migration and establishment of synaptic circuitry or plasticity have been related with the activation of both iGluRs and mGluRs (Komuro & Rakic, 1998; Anwyl, 1999; Furuta & Martin, 1999; Zirpel *et al*. 2000).

mGluRs include eight subtypes (mGluR1–mGluR8), classified into three groups based on sequence homology, pharmacology and transduction mechanisms. Group I includes mGluR1 and mGluR5, which exist in a number of alternatively spliced forms and stimulate phosphoinositide hydrolysis and the release of Ca^{2+} from intracellular stores (Conn & Pin, 1997). Thus, the existence of such a molecular diversity of mGluRs supports different physiological roles of specific receptor compositions in different neuronal populations. Differentiation of cortical laminae during neurogenesis is accompanied by changes in mGluR expression (Furuta & Martin, 1999) and spatiotemporal regulation of mGluR expression influences cortical maturation and plasticity (Reid *et al*., 1995; Blue *et al*., 1997; Casabona *et al*., 1997).

The mammalian cerebral cortex is organized into six layers that form during development according to an inside-out gradient of radial migration (Bayer & Altman, 1990). In rodents, the process of radial migration is well advanced before birth, but the neurons destined to layers IV and II–III still have to complete their migration and settling (Cobas *et al*., 1991; De Felipe *et al*., 1997). Cajal-Retzius cells in the cortical layer I secrete the extracelular matrix protein Reelin (Reln) that plays a pivotal role in radial migration and laminar organization of the cortex (D'Arcangelo *et al*., 1995, 1997; Ogawa *et al*., 1995). These cells express diverse types of neurotransmitter receptors including iGluRs and $GABA_A$ receptors (Schwartz *et al*., 1998; Mienville & Pesold, 1999; Mienville, 1999). However, the existence of mGluRs in these cells has not been investigated.

In this paper, we demonstrate, by fluorimetric methods, the existence of functional mGluR1 in layer I cells of the postnatal mouse cerebral cortex, and by means of immunocytochemistry, a complete coexpression of the mGluR1α subtype and Reln in Cajal-Retzius cells.

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Materials and methods

Animals

Postnatal C57Bl mice ranging in age from 0–7 days (P0–P7) used in this study were purchased from the Departamento de Experimentación Animal of the Universidad Miguel Hernández. Experimental procedures were carried out in accordance with Institutional guidelines for animal welfare and European Community Directives.

Immunohistochemistry

Eight mice were deeply anaesthetized with diethyl ether until reflex activity was completely abolished and perfused with 0.9% saline, 0.1% sodium nitrite followed by a freshly prepared ice-cold fixative containing 4% paraformaldehyde and ~ 0.2% picric acid in 0.1 M phosphate buffer (PB, pH 7.2–7.4). After perfusion, brains were removed and immersed in the same fixative for 2 h at 4 °C. Tissue blocks were dissected out and washed thoroughly in 0.1 M PB for several hours. Coronal, 60 μm-thick sections of cerebral cortex were cut with a Vibratome and collected in 0.1 M PB. Some sections were used for immunofluorescence detection and visualization of the colocalization of mGluR1α, mGluR5 and Reln immunoreactivities using a laser scanning confocal microscope.

Affinity-purified polyclonal antibodies against mGluR1α and mGluR5 were made in rabbits (for the characteristics and specificity of these antibodies, see Shigemoto *et al*., 1994; Jaarsma *et al*., 1998). A monoclonal anti-Reln antibody (G10) was also used (de Bergeyck *et al*., 1998) gift of Dr A.M. Goffinet (Namur, Belgium). Sections were first incubated with 10% normal goat serum diluted in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl (TBS) for 45 min and incubated in a mixture of mGluR1α and reelin, or mGluR5 and Reln antibodies in TBS containing 1% NGS and 0.2% Triton X-100, at a final protein concentration of 1–2 μg/mL. Sections were washed in TBS and incubated for two hours in biotinylated goat anti-rabbit secondary antibody, and then in a mixture of streptavidin coupled to carbocyanine Cy2 and goat anti-mouse IgG coupled to carbocyanine Cy3 (Amersham, UK). The secondary antibodies and the streptavidin-Cy2 conjugate were used at a dilution of 1 : 100 in TBS containing 1% NGS. After extensive washes in TBS, the sections were mounted on gelatinized slides and coverslipped with Vectashield (Vector Labs, USA). A Leica DMRS light microscope equipped with fluorescence illumination and fitted with a Leica TLSCM confocal scanner module with a Kr/Ar laser source was used to obtain confocal images of the immunostained sections (Cy2 abs: 489, emm: 520; Cy3 abs: 550; emm: 570). Some sections were processed for light microscope visualization of mGluR1α, mGluR5 and Reln using the avidin–biotinylated enzyme complex (ABC; Vector Labs, USA) system and 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Slides were osmified to enhance contrast.

Preparation of slices for Ca^{2+} imaging

Animals were anaesthetized by deep hypothermia and then decapitated. The brain was rapidly removed and placed into continuously bubbled (95% O_2 and 5% CO_2 pH of 7.3), cold artificial cerebrospinal fluid (ACSF) which contained: 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$ and 10 mM glucose. 300 μm-thick coronal cortical slices were cut with a Vibratome (Technical Products International, USA) and preincubated in ACSF. For the experiments described in this study, 220 slices from 60 animals were processed.

Calcium imaging

Cortical slices were placed at room temperature in ACSF containing 16 μM Fura-2 AM (Molecular Probes, Eugene, OR, USA) and 0.09% anhydrous dimethysulfoxide and 0.006% pluronic acid for 1 h. Slices were transferred in standard ACSF to the stage of an upright Zeiss Axioskop microscope and visualized through a 60x water immersion objective (Olympus, NA = 0.9). Slices were excited at 340 and 380 nm, and the fluorescence emitted at 510 nm was recorded by a Hamamatsu C2400 intensifier-Dage 72 video camera. Time-lapse images were acquired every 1–3 s and spatially analysed using a MCID M4 System (Imaging Research Inc., St. Catherine's, Ontario, Canada). No background subtraction or filtering was applied to the images.

Intracellular calcium ($[Ca^{2+}]_{i)}$ was calculated by interpolation of the ratio images into a look-up table constructed by imaging Fura-2 solutions with known calcium concentration in the same experimental set-up, using the following equation (Grynkiewicz *et al*., 1985):

$$[Ca^{2+}]_i = K_D(R - R_{min}/R_{max} - R)F_0/F_{max}$$

Where K_D is the Fura-Ca^{2+} binding constant (220 nM), R is the ratio of Fura-2 fluorescence at 340 and 380 nm, R_{min} and R_{max} are values of R in Ca^{2+}-free (1 mM EGTA added) and 2 mM Ca^{2+} medium, respectively, using Fura-2 penta K^+ salt. F_0/F_{max} is the ratio of Fura-2 fluorescence at 380 nm in Ca^{2+}-free (1 mM EGTA) and 2 mM Ca^{2+} medium.

Resting $[Ca^{2+}]_i$ was measured in 88 cells, from 20 different slices, and had a mean value of 92 ± 5 nM (mean ± SEM). Unless otherwise indicated, the experiments were performed at room temperature (23–26 °C). 0.5 mM ethylene glycol-bis (β-amino-ethyl-ether) N,N,N′,N′,-tetraacetic acid (EGTA) was added to the medium containing zero calcium. In the figures, changes in $[Ca^{2+}]_i$ are expressed as absolute increase with respect to the basal value.

Drugs where applied through a pumped line at a rate of 4 mL/min for the time indicated in the figures. Some slices were preincubated with 10 μm thapsigargin in ACSF for 30 min before calcium recording. NMDA, kainate, quisqualate, (±)-2-amino-5-phosphono-valeric acid (APV), 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) and tetrodotoxin (TTX) were purchased from Research Biochemical Inc (RBI, Natick, MA, USA). (±)-a-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), (±)-1-aminocyclopentane-trans-1, 3-dicarboxylic acid (tACPD), (S)-alpha-methyl-4-carboxiphenylglycine (MCPG), (RS)-2-Chloro-5-hydroxyphenylglycine (CHPG) and (S)-3,5-Dihydroxyphenylglycine (DHPG) were purchased from Tocris Cookson (UK). Thapsigargin was purchased from Sigma (St Louis, MO, USA). Drugs were prepared as stock solutions in distilled H_2O, with the exception of tACPD, MCPG and CHPG, which were prepared in 1N NaOH. All drugs were stored at 4 °C and prepared to working concentrations in ACSF daily.

Numerical data were statistically analysed using one-way analysis of variance (ANOVA) and Bonferroni *t*-test to assess differences among groups.

Results

MGluR activation increases intracellular calcium in layer I cells

Bath application of glutamate (Glu) at 50 μM induced conspicuous increases in $[Ca^{2+}]_i$ in layer I cells. As illustrated in the experiment shown in Fig. 1A (first set of traces), the amount of the $[Ca^{2+}]_i$ increase was quantitatively heterogeneous among the different cells. Such an effect was in part mediated by the activation of iGluRs including NMDA, AMPA and kainate receptors (not shown).

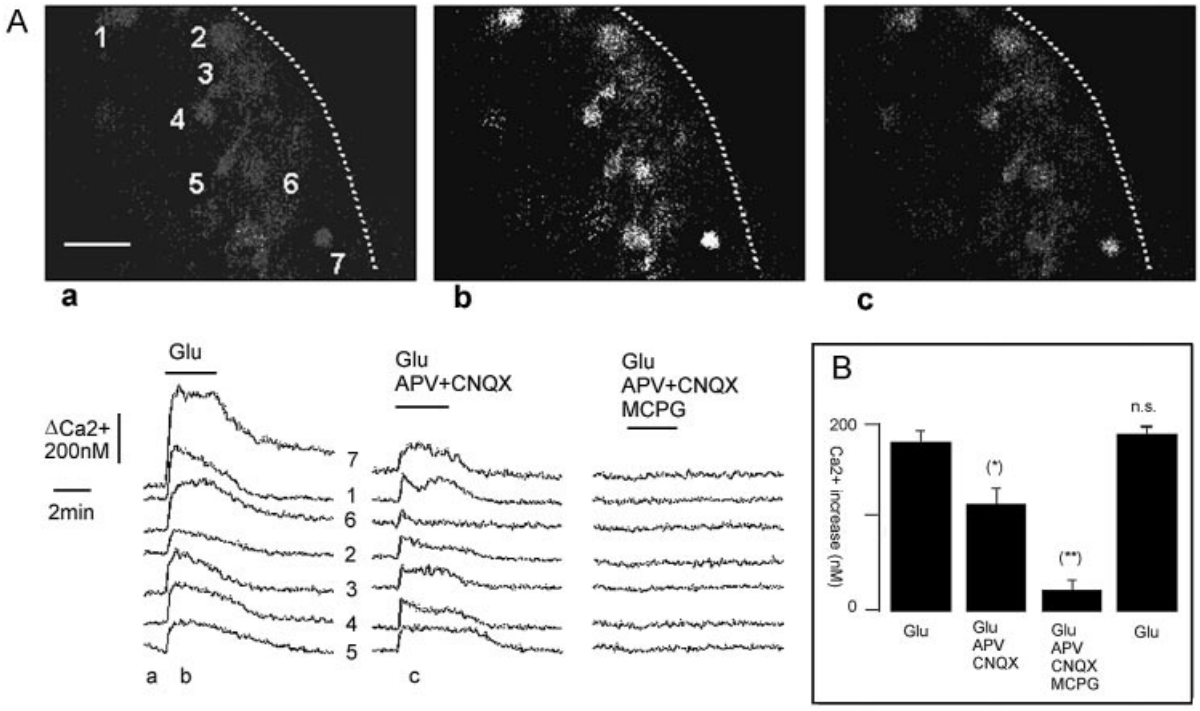

FIG. 1. (A) Effects of antagonists of iGluRs and mGluRs on the $[Ca^{2+}]_i$ response elicited by Glutamate (Glu). The first set of records (left) show the $[Ca^{2+}]_i$ response to Glu (50 μM), applied during the time indicated by the bar, of the cells numbered 1–7 in photomicrograph 'a'. The next set of records (middle) shows the effect of Glu in the presence of the iGluR inhibitors APV (200 μM) + CNQX (40 μM). The third set (right) of records was obtained in the presence of APV + CNQX plus the mGluR inhibitor MCPG (1 mM). The gaps between groups of records are of five minutes. Images Aa–c show $[Ca^{2+}]_i$ in a grey scale code, where dark pixels correspond to low calcium levels. Images were acquired at the times indicated by the letters below the traces (a–c). The dashed line indicates the pial border of the slice. This experiment is representative of results found in five other slices. (B) Quantification of the responses ($n = 26$ cells from five different experiments). Glu (50 μM; first bar) produced a net increase in $[Ca^{2+}]_i$ of 189 ± 12 nM (mean ± SEM). The addition of 200 μM APV plus 40 μM CNQX to the 50 μM Glu perfusate (second bar) reduced the $[Ca^{2+}]_i$ increase to 119 ± 17 nM ($^*P < 0.01$). The addition of 1 mM MCPG to the same perfusate (third bar) reduced the $[Ca^{2+}]_i$ to a mere 24 ± 11 nM ($^{**}P < 0.001$). The fourth bar shows the reversibility of the effect, since a new perfusion with Glu elicited a net $[Ca^{2+}]_i$ increase (197 ± 9 nM) that was not statistically different from the initial one. Scale bar in Aa, 20 μm (Aa–c).

Accordingly, we tested the effect of specific iGluR antagonists in suppressing such an effect. APV (an antagonist of NMDA receptors) plus CNQX (an antagonist of AMPA and kainate receptors) decreased drastically the response in some cells (notably in cells 6 and 7, Fig. 1A, second set of traces), whilst in other cells the response remained unaltered (cells 2–5). This could indicate that a sizeable component of the $[Ca^{2+}]_i$ increases evoked by Glu may be mediated by the activation of mGlu receptors. We confirmed such a possibility by superfusing the slices with mGluR antagonists. The addition of MCPG, a competitive antagonist of both group I and II mGluRs, completely abolished the $[Ca^{2+}]_i$ response to Glu in all the cells (Fig. 1A, third set of traces). Changing the order of antagonist application gave essentially the same results (not shown). The quantification of the results is summarized in the graph of Fig. 1B.

To positively assess the participation of mGluRs in the generation of $[Ca^{2+}]_i$ transients, we tested the effect of mGluR agonists (Conn & Pin, 1997; Schoepp *et al*., 1999). We first tested the effect of tACPD, which is an agonist of both group I and II mGluRs. Figure 2A shows the increase of $[Ca^{2+}]_i$ caused by tACPD in five representative cells of the same slice. Figure 2B shows that the addition of 1 mM MCPG blocked the response to tACPD, being the effect reversible as shown in the third panel. The quantification of the responses is shown in Fig. 3B.

This experiment was not completely conclusive of the role of mGluR in the generation of the $[Ca^{2+}]_i$ transients, because the response to the mGluR agonist tACPD might be secondary to the synaptic release of Glu and the activation of iGluRs. If this were the case, perfusion with the iGluR antagonists APV plus CNQX would abolish the $[Ca^{2+}]_i$ response to tACPD. To exclude this possibility, we compared the effects of tACPD in the presence or in the absence of the iGluR antagonists APV and CNQX. We tested, in addition, the effect of the Na^+ channel blocker TTX, which prevents any synaptic Glu release. Figure 3A shows an example of such an experiment where the $[Ca^{2+}]_i$ response of four representative cells to a tACPD challenge did not change in the presence of the iGluR antagonists. Figure 3B summarizes the responses in quantitative terms. These results suggest that the effects of tACPD on $[Ca^{2+}]_i$ were a direct effect of the activation of mGluRs by tACPD.

The intracellular origin of Ca^{2+}

The activation of group I mGluRs is coupled to phosphatidyl-inositol hydrolisis, production of IP3 and the release of Ca^{2+} from intracellular stores (Berridge, 1998). We, therefore, studied the response to mGluR agonists in the absence of extracellular calcium. In the case experiment illustrated in Fig. 4 (representative of 21 cells from six slices), the slices were superfused with a nominally Ca^{2+}-free solution (supplemented with 0.5 mM EGTA). The traces show the effect of mGluR agonist tACPD (Fig. 4A) in three representative cells of layer I. While the slices were being superfused with a Ca^{2+}-free solution, the first application of tACPD led to a prominent $[Ca^{2+}]_i$ increase and to a subsequent decrease in $[Ca^{2+}]_i$ below the prestimulatory level. A second application of tACPD elicited a

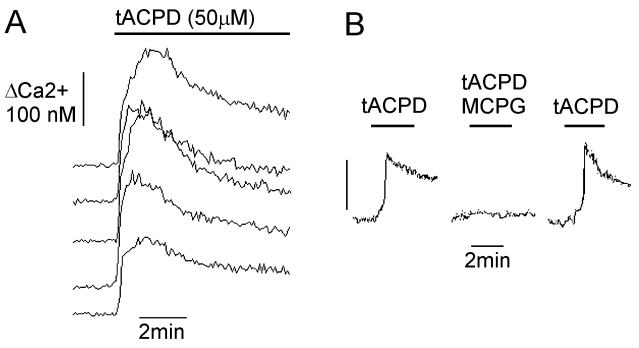

FIG. 2. Effect of bath application of tACPD on $[Ca^{2+}]_i$. (A) Time-course of the changes in $[Ca^{2+}]_i$ in five representative cells. Essentially the same results were obtained in 13 other slices. (B) Response to tACDP (25 μM) without MCPG (first and third record) and with 1 mM MCPG (middle record) of one representative cell. The gap between records is of 5 min. This experiment is representative of the results obtained in three different slices. Scale bar in B is 100 nM ΔCa^{2+}.

smaller $[Ca^{2+}]_i$ response and a third application produced no $[Ca^{2+}]_i$ response. One could tentatively interpret the decremental effect of the successive tACPD pulses as being due to the exhausting of the intracellular Ca^{2+} stores. To show that these stores could be refilled, the slice was then superfused with ACSF at a physiological (2.4 mM) Ca^{2+} concentration for two minutes, which rapidly raised $[Ca^{2+}]_i$ to prestimulatory levels. A fourth application of tACPD, during superfusion with the Ca^{2+}-free solution, increased $[Ca^{2+}]_i$ almost to the level of the first tACPD challenge. These results suggest that the reintroduction of extracellular Ca^{2+} led to the refilling of the tACPD sensitive stores that rendered the cells again responsive to the mGluR agonist tACPD.

Quisqualate is an agonist of both AMPA type of iGluRs and group I mGluRs (Nakanishi & Masu, 1994; Nakanishi *et al*., 1998). In the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ transients elicited by quisqualate can only be mediated by activation of group I mGluRs. In the experiment shown in Fig. 4B, we used a nominally Ca^{2+}-free ACSF perfusate. The first quisqualate challenge elicited an $[Ca^{2+}]_i$ transient but the successive quisqualate challenges had no effect. Refilling the stores by adding 2.4 mM Ca^{2+} for two minutes, made the cells once more responsive to quisqualate, as shown by the fourth quisqualate challenge. Similar results were obtained in 14 cells from four slices. Consistent with these results, Fig. 4 (lower panels) shows that the effects of tACPD were blocked in the presence of the endoplasmic reticulum calcium pump inhibitor thapsigargin (Glennon *et al*., 1992). The first set of traces show the response to tACPD of three representative cells labelled in the photomicrograph. At the end of the tACPD exposure, the slice was superfused with thapsigargin for 10 minutes and challenged again with tACPD, as shown in the second set of traces. In seven slices in which the same experimental protocol was repeated, no one cell responded to tACPD after thapsigargin treatment.

Cells responding to mGluRs agonists are neurons

The cellular populations of layer I in a postnatal cortical slice are rather diverse and includes neurons and glial cells. Although glial cells do not express NMDA receptors, they do express mGluRs (Cornell-Bell *et al*., 1990; Nadal *et al*., 1998). Therefore, it is possible that the $[Ca^{2+}]_i$ responses described above might have occurred in glial cells rather than in neurons. Another set of experiments provided evidence that the cells that responded to mGluR agonists also responded to NMDA. An example of such an experiment is shown in

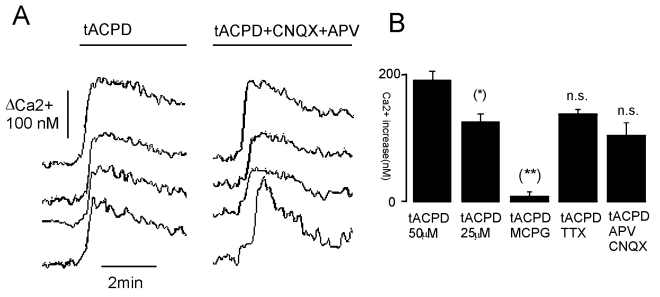

FIG. 3. (A) Direct effect of tACPD on mGluRs. The first set of traces (left) shows the $[Ca^{2+}]_i$ response to tACPD (25 μM) of four representative cells. The second series of traces shows the response to tACPD in the presence of CNQX (20 μM) + APV (100 μM) of the same cells. The gap between records is of 5 min. (B) Quantification of the responses (n = 17 cells from three different slices). tACPD 50 μM (first bar) or 25 μM (second bar) in the perfusate raised $[Ca^{2+}]_i$ to 184 ± 14 nM and 122 ± 11 nM, respectively, showing, therefore, that the effect of tACPD was dose-dependent ($^*P < 0.01$). The mGluR antagonist MCPG 1 mM (third bar) abolished the increase of $[Ca^{2+}]_i$ produced by 25 μM tACPD ($^{**}P < 0.001$). The addition of 1 μM TTX to the 25 μM tACPD perfusate (fourth bar) did no affect the response to tACPD, 133 ± 7 nM (nonsignificant as compared to the tACPD 25 μM effect, second bar). Finally, the addition of 100 μM APV plus 20 μM CNQX to the tACPD 25 μM perfusate (fifth bar) did not affect the response to tACPD (101 ± 18 nM), again nonsignificant as compared to the 25 μM tACPD effect (second bar).

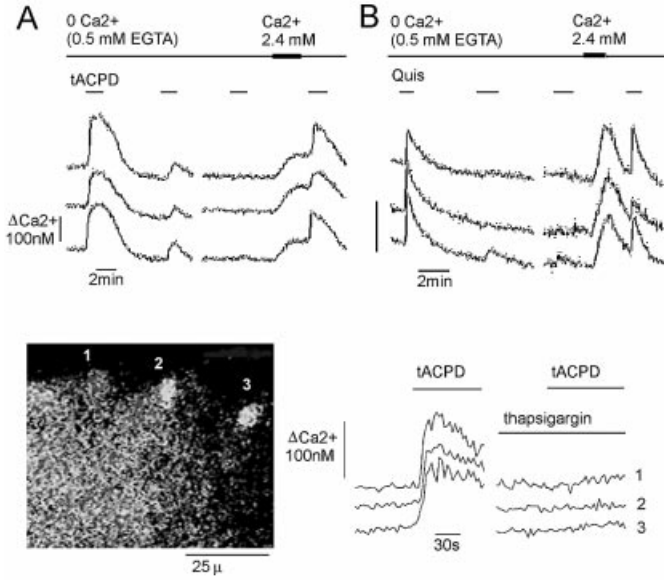

FIG. 4. The response to mGluR agonists depends on Ca^{2+} release from intracellular stores. Upper panels: (A) response to tACPD (50 μM) in Ca^{2+}-free ACSF (0.5 mM EGTA added) of three representative layer I cells. The slice was challenged four times with tACPD (as indicated by the bars). At the time indicated by the thick bar, the slice was superfused with ACSF with a standard calcium concentration (2.4 mM). The fourth challenge with tACPD again occurred while superfusing with the calcium-free solution. This experiment is representative of the results obtained in six different slices. (B) Results of a similar experimental protocol in a different slice, in which the mGluR agonist tested was quisqualate (5 μM). The same results were obtained in four different experiments. (Lower panels) The first set of traces show the response of the cells labelled in the photomicrograph to tACPD (25 μM). The cells are situated just below the pial surface. Then the slice was superfused with a medium containing thapsigargin (2 μM) for 10 minutes and again challenged with tACPD (25 μM). Figure representative of results obtained in seven different experiments. Scale bar in B, 100 nM ΔCa^{2+}.

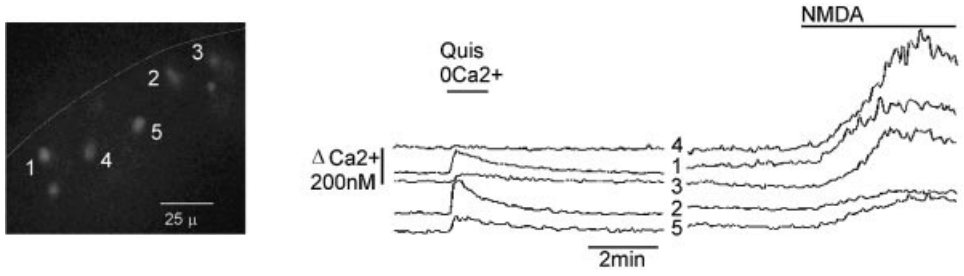

FIG. 5. Coexistence of iGluRs and mGluRs in the same cells. The first set of traces (left) shows the response to quisqualate (5 μM) in the absence of extracellular calcium (EGTA 0.5 mM) of the five representative cells labelled in the photomicrograph. The second set of traces (right) shows the response of the same cells to NMDA (100 μM) in the absence of extracellular Mg^{2+}. This experiment is representative of the results obtained in four different slices. Scale bar, 25 μm.

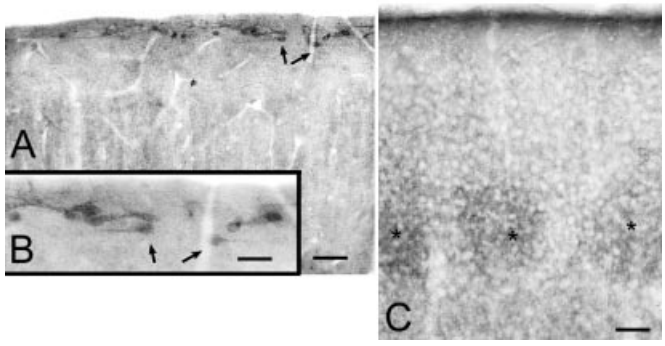

FIG. 6. Immunocytochemical detection of mGluR1α and mGluR5 in the cerebral cortex of P7 mice. (A–B) mGluR1α is expressed by horizontally orientated cells located at a short distance below the pial surface. Arrows point to identical cells in A and in the enlarged picture shown in B. Note that no immunoreactive cells are found in deep cortical layers. (C) mGluR5 is expressed in the neuropil of the cortical layers and shows a particularly strong expression in the barrel hollows (indicated by asterisks) of the somatosensory cortex. No layer I cells are labelled. Scale bars, 50 μm (A and C); 25 μm (B).

Fig. 5. In this case, the slice was challenged with quisqualate in the absence of extracellular Ca^{2+}, as shown in the first set of traces. In four out of five cells (the exception being cell 4), quisqualate elicited an increase in $[Ca^{2+}]_i$. When challenged with NMDA in a normal Ca^{2+} ACSF perfusate, all the cells (including cell 4) responded with an increase in $[Ca^{2+}]_i$. In the four slices (24 cells) where this protocol was repeated, 67% of the cells responded to both agonists, 16% responded to NMDA only and 8% to quisqualate only. Essentially the same results were obtained when the NMDA challenge was performed in the presence of the non-NMDA receptor antagonist CNQX (40 μM; not shown). These results indicate that, in our experiments, most of the cells that showed $[Ca^{2+}]_i$ increases through mGluRs were neurons.

Cajal-Retzius cells express mGlu1α and not mGluR5

The results presented so far indicate that the selective activation of group I mGluRs, by bath applied ligands, to acute cortical slices produced $[Ca^{2+}]_i$ transients in layer I cells that were most likely neuronal in nature.

Recent studies (Meyer *et al*., 1998, 1999) have revealed the existence of many different neuronal types in layer I of postnatal rodents. To facilitate the identification of the cells that express functional mGluRs, we made an attempt at neurochemical identification using immunocytochemical methods. The experiments showed that mGluR subtype 1α was strongly expressed by cells in layer I that displayed the morphology of Cajal-Retzius cells (Fig. 6A and B). In brief, these cells were horizontally orientated fusiform cells with long dendritic processes coursing parallel to the pial surface; some of these cells located subpially (Fig. 7A and B). We found no mGluR5-immunoreactive cells in layer I; mGluR5 was detected only in the cortical plate neuropil and more strongly in the neuropil of the barrel hollows in layer IV (Fig. 6C). Metabotropic GluR1α expressing cells in layer I were confirmed as being Cajal-Retzius cells in double labelling experiments (Fig. 7) that showed, at P2 and P7, that virtually all mGluR1α-expressing cells coexpressed Reln.

Cajal-Retzius cells have functional mGluR1 receptors

These results were, thus, compatible with the idea that the $[Ca^{2+}]_i$ transients in Cajal-Retzius cells could be mediated by the activation of mGluR1 receptors. To confirm that the immunocytochemically-detected expression of mGluR1α has a functional correlate, we assayed the slices with selective agonists to group I mGluRs. Recently developed group selective and subtype selective agonists of group I mGluRs allowed us to define what receptor subtype was responsible for the $[Ca^{2+}]_i$ elevations. The bath application of 50 μm DHPG (a group I selective agonist) led to sustained increases of $[Ca^{2+}]_i$ (net calcium increase 89 ± 5.9 nM, $n = 50$ cells from six slices) (Fig. 8A), whereas CHPG (mGluR5 selective agonist) had no effect on $[Ca^{2+}]_i$ whatsoever ($n = 6$ slices). Figure 8B shows the localization, in layer I, of the cells activated by DHPG. Figure 8Ba shows the slice image of Fura-2 fluorescence excited at 340 nm, where the soma of individual cells (labelled 1–4) can be distinguished. The fluorescent cells were located at a distance within 50 μm below the border of the slice, indicated by the discontinuous line. Figure 8Bb–c) shows false-colour images of $[Ca^{2+}]_i$ before (b) and during the peak (c) of the response to DHPG. The graph in Fig. 8B shows the variations of $[Ca^{2+}]_i$ in the cells labelled in Fig. 8Ba with time. In these experiments, DHPG was locally applied by pressure injection through a pipette located close to the imaged area. Letters 'b' and 'c' below the graph indicate the times at which the calcium images shown in Fig. 8Bb–c were taken. These results enabled us to conclude that the $[Ca^{2+}]_i$ transients in Cajal-Retzius cells analysed in the present study were mediated by the activation of the mGluR1 receptor.

Discussion

In this paper, we have characterized mGluRs as important mediators of the $[Ca^{2+}]_i$ response to glutamate of layer I cells in early postnatal

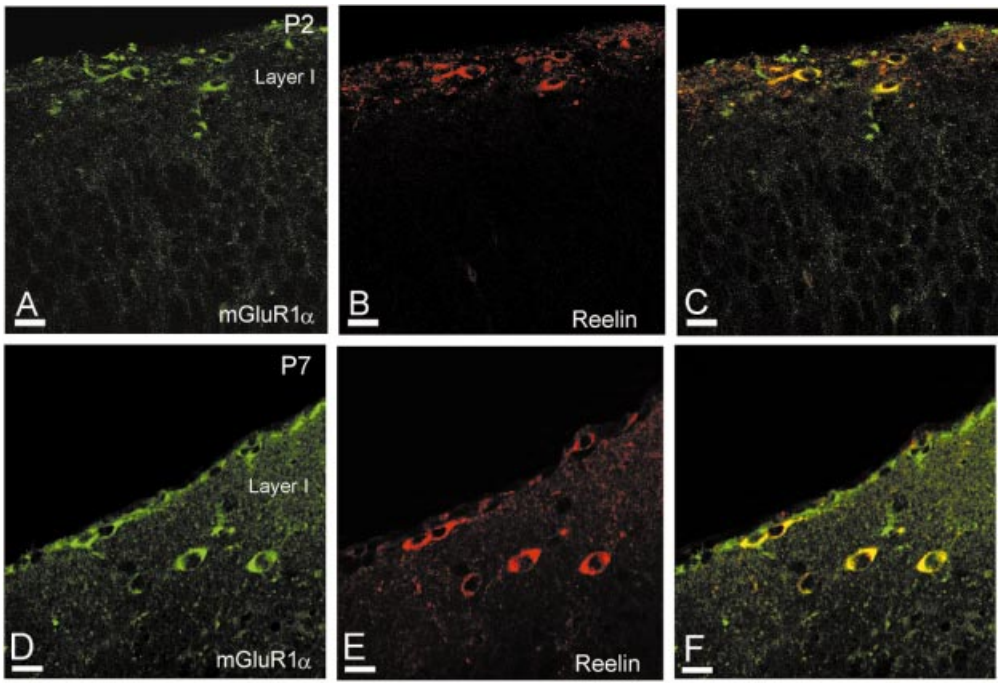

FIG. 7. Confocal images showing a virtually complete colocalization of the immunoreactivity for mGluR1α and Reln at P2 and P7. (A and D) mGluR1α immunoreactivity; (B and E) Reelin immunoreactivity; (C and F) Merged images. Scale bars, 20 μm.

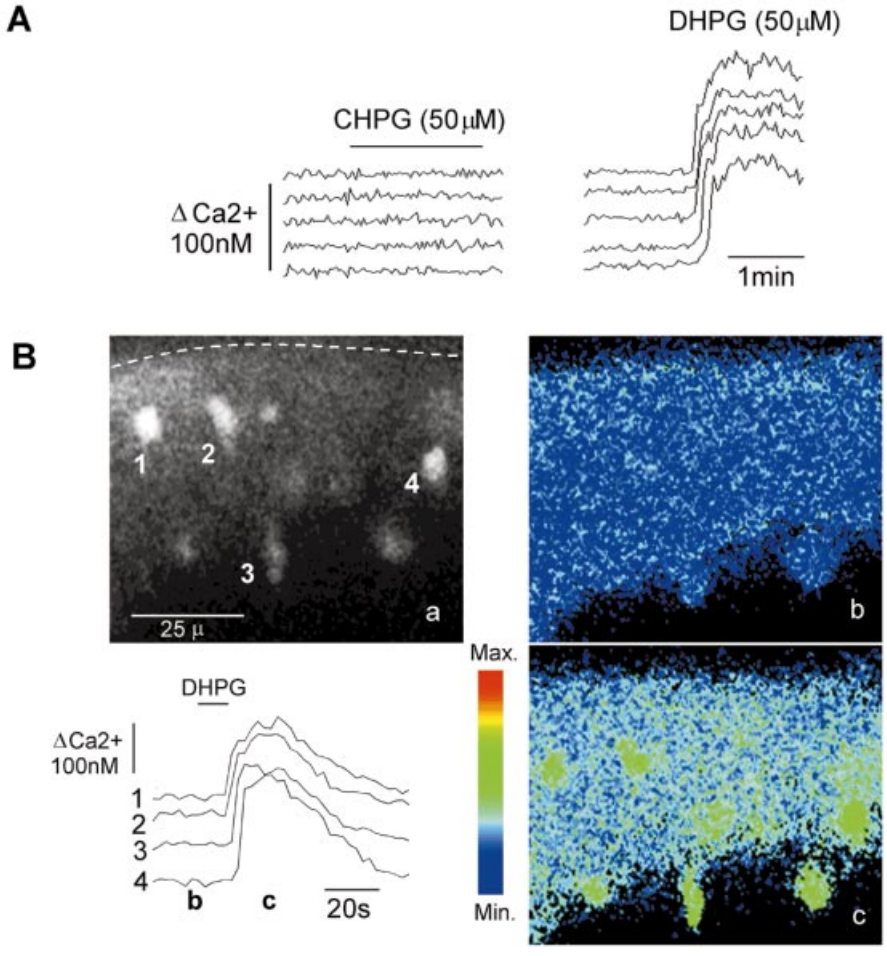

FIG. 8. Effects on $[Ca^{2+}]_i$ of selective agonists of group I mGluRs. (A) The traces show the response of five representative cells to CHPG and to DHPG. This experiment is representative of the results obtained in six different slices. (B) Photomicrograph Ba shows Fura-2 fluorescence (excitation 340 nm); the pial border is indicated by the discontinuous line. Four representative layer I cells (1–4) were sampled and their responses to DHPG plotted in the graph below. Photomicrographs Bb and Bc show false-colour images of $[Ca^{2+}]_i$ taken at the times indicated on the graph. Scale bar in Ba applies to all three photomicrographs.

mouse cortical slices. The results of our fluorimetric experiments using tACPD and, more specifically, CHPG and DHPG selective subtype agonists, suggest the participation of functional mGluR1 in mediating this response to glutamate in layer I neurons. The present immunocytochemical studies show a virtually complete coexpression of mGluR1α and Reln, suggesting that Cajal-Retzius cells are the layer I cells that respond with $[Ca^{2+}]_i$ increases to mGluR agonists.

Previous work in expression systems has shown that glutamate evokes nonoscillatory and oscillatory $[Ca^{2+}]_i$ responses in cells that express mGluR1 and mGluR5, respectively (Kawabata *et al*., 1996; Nakanishi *et al*., 1998). Several patterns of calcium dynamics that could be of importance in signal transduction and regulation of gene expression have been described in the developing neocortex, including isolated transients, co-ordinated $[Ca^{2+}]_i$ increase in local cell clusters, synchronous transients of cell pairs undergoing mitotic division and oscillations (Yuste *et al*., 1992, 1995; Owens & Kriegstein., 1998; Flint *et al*., 1999). The generation of spontaneous $[Ca^{2+}]_i$ oscillations in the cortical plate-derived layers of the murine cortex is due to the activation of mGluR5 (Flint *et al*., 1999). This is consistent with our observation of a preferential localization of mGluR5 in the neuropil of the cortical layers of postnatal mice and its absence from layer I cells (Romano *et al*., 1996; Blue *et al*., 1997). The $[Ca^{2+}]_i$ increases we observe in layer I cells are nonoscillatory which, together with the responses to specific group I mGluRs agonists, suggest that the responses are due to mGluR1 activation. Moreover, the colocalization of mGluR1α and Reln, detected by immunohistochemistry, strongly suggests that mGluR1 is active in Reln-expressing Cajal-Retzius cells. It may be speculated that the differential expression of group I mGluRs and the different patterns of their associated $[Ca^{2+}]_i$ signals, either nonoscillatory and oscillatory, may have different functional roles. It remains to be established how the different calcium codes are translated into different intracellular messages in different layers of the developing cortex.

Cajal-Retzius cells are considered to play a critical role during neocortical development. They secrete Reln, an extracellular matrix molecule, whose absence in the mouse mutant *reeler* causes a severe cortical laminar disruption (Frotscher, 1998; Curran & D'Arcangelo, 1998). The molecular cascade acting downstream of Reln is beginning to be understood (Cooper & Howell, 1999; Walsh & Goffinet, 2000). However, the functional mechanisms that regulate Reln synthesis and secretion have been little explored. So far, brain-derived neurotrophic factor (BDNF) and thyroid hormone have been shown to regulate Reln expression (Ringstedt *et al*., 1998; Alvarez-Dolado *et al*., 1999), as well as the transcription factor Tbr1 (Hevner *et al*., 1999). Whether or not calcium signals play a role in Reln secretion has been recently debated. Lacor *et al*. (2000) have shown that extracellular influx of Ca^{2+} into cultured cerebellar granule cells does not affect Reln secretion and concluded that Reln secretion is constitutive, as is the case with other extracellular matrix molecules. Alternatively, calcium signalling may regulate the expression of Reln as is the case for other genes (Fields *et al*., 1997; Dolmetsch *et al*., 1998; Li *et al*., 1998; Buonanno & Fields., 1999).

There is electrophysiological evidence indicating a tonic activity in cortical cells mediated by NMDA and non-NMDA GluRs (Lo Turco & Kriegstein, 1991; Kim *et al*., 1995; Schwartz *et al*., 1998), suggesting that a release of Glu may occur in the vicinity of layer I cells under physiological conditions. Accordingly, as new cortical plate neuron cohorts arrive in the vicinity of layer I, they would release Glu that could activate mGluRs in Cajal-Retzius cells and positively regulate Reln synthesis. This mechanism is compatible with Reln acting as a stop signal for cells arriving at layer I (Pearlman & Sheppard, 1996).

In summary, our results demonstrate that mGluR1α is expressed in layer I cells that also express Reln, and that a large proportion of the $[Ca^{2+}]_i$ response of layer I cells to Glu is due to activation of such receptors. mGluR1α activation may arise as a consequence of cells arriving at layer I, and be linked to the synthesis of Reln, which is essential for correct layering of the cortex.

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Abbreviations

ABC, avidin–biotinylated enzyme complex; ACSF, artificial cerebrospinal fluid; tACPD, (±)-1-aminocyclopentane-trans-1, 3-dicarboxylic acid; AMPA, (±)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ; APV, (±)-2-amino-5-phosphonovaleric acid; BDNF, brain-derived neurotrophic factor; $[Ca^{2+}]_i$, free intracellular calcium; CHPG, (RS)-2-Chloro-5-hydroxyphenylglycine; CNQX, 6-cyano-7-nitroquinoxaline-2, 3-dione; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DHPG, (S)-3,5-Dihydroxyphenylglycine; EGTA, ethylene glycol-bis (β-amino-ethyl-ether) N,N,N′,N′,-tetraacetic acid; Glu: glutamate; mGluRs, metabotropic glutamate receptors; iGluRs, ionotropic glutamate receptors; MCPG, (S)-alpha-methyl-4-carboxiphenylglycine; NMDA, *N*-methyl-D-aspartate; PB, phosphate buffer; Reln, reelin; TBS, Tris-buffered saline; TTX, tetrodotoxin.

References

Alvarez-Dolado, M., Ruiz, M., del Rio, J.A., Alcantara, S., Burgaya, F., Sheldon, M., Nakajima, K., Bernal, J., Howell, B.W., Curran, T., Soriano, E. & Muñoz, A. (1999) Thyroid hormone regulates Reelin and Dab1 expression during brain development. *J. Neurosci.*, **19**, 6979–6993.

Anwyl, R. (1999) Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. *Brain Res. Rev.*, **29**, 83–120.

Bayer, S.A. & Altman, J. (1990) Development of layer I and the subplate in the rat neocortex. *Exp. Neurol.*, **107**, 48–62.

de Bergeyck, V., Naerhuyzen, B., Goffinet, A.M. & Lambert de Rouvroit, C. (1998) A panel of monoclonal antibodies against reelin, the extracellular matrix protein defective in reeler mutant mice. *J. Neurosci. Methods*, **82**, 17–24.

Berridge, M.J. (1998) Neuronal calcium signaling. *Neuron*, **21**, 13–26.

Blue, M.E., Martin, L.J., Brennan, E.M. & Johnston, M.V. (1997) Ontogeny of non-NMDA glutamate receptors in rat barrel field cortex: I. Metabotropic receptors. *J. Comp. Neurol.*, **386**, 16–28.

Buonanno, A. & Fields, R.D. (1999) Gene regulation by patterned electrical activity during neural and skeletal muscle development. *Curr. Opin. Neurobiol.*, **9**, 110–120.

Casabona, G., Knopfel, T., Kuhn, R., Gasparini, F., Baumann, P., Sortino, M.A., Copani, A. & Nicoletti, F. (1997) Expression and coupling to polyphosphoinositide hydrolysis of group I metabotropic glutamate receptors in early postnatal and adult rat brain. *Eur. J. Neurosci.*, **9**, 12–17.

Cobas, A., Fairén, A., Alvarez-Bolado, G. & Sánchez, M.P. (1991) Prenatal development of the intrinsic neurons of the rat neocortex: a comparative study of the distribution of GABA- immunoreactive cells and the $GABA_A$ receptor. *Neuroscience*, **40**, 375–397.

Conn, P.J. & Pin, J.P. (1997) Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol.*, **37**, 205–237.

Cooper, J.A. & Howell, B.W. (1999) Lipoprotein receptors: signaling functions in the brain? *Cell*, **97**, 671–674.

Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S. & Smith, S.J. (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science*, **247**, 470–473.

Curran, T. & D'Arcangelo, G. (1998) Role of reelin in the control of brain development. *Brain Res. Rev.*, **26**, 285–294.

D'Arcangelo, G., Miao, G.G., Chen, S.C., Soares, H.D., Morgan, J.I. & Curran, T. (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature*, **374**, 719–723.

D'Arcangelo, G., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K. & Curran, T. (1997) Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. *J. Neurosci.*, **17**, 23–31.

De Felipe, J., Marco, P., Fairén, A. & Jones, E.G. (1997) Inhibitory synaptogenesis in mouse somatosensory cortex. *Cereb. Cortex*, **7**, 619–634.

Dolmetsch, R.E., Xu, K. & Lewis, R.S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression. *Nature*, **392**, 933–936.

Fagni, L., Chavis, P., Ango, F. & Bockaert, J. (2000) Complex interactions between mGluRs, intracellular Ca^{2+} stores and ion channels in neurons. *Trends Neurosci.*, **23**, 80–88.

Fields, R.D., Eshete, F., Stevens, B. & Itoh, K. (1997) Action potential-dependent regulation of gene expression: temporal specificity in Ca^{2+}, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. *J. Neurosci.*, **17**, 7252–7266.

Flint, A.C., Dammerman, R.S. & Kriegstein, A.R. (1999) Endogenous activation of metabotropic glutamate receptors in neocortical development causes neuronal calcium oscillations. *Proc. Natl. Acad. Sci. USA*, **96**, 12144–12149.

Frotscher, M. (1998) Cajal-Retzius cells, Reelin, and the formation of layers. *Curr. Opin. Neurobiol.*, **8**, 570–575.

Furuta, A. & Martin, L.J. (1999) Laminar segregation of the cortical plate during corticogenesis is accompanied by changes in glutamate receptor expression. *J. Neurobiol.*, **39**, 67–80.

Glennon, M.C., Bird, G.S., Takemura, H., Thastrup, O., Leslie, B.A. & Putney, J.W. Jr (1992) In situ imaging of agonist-sensitive calcium pools in AR4–2J pancreatoma cells. Evidence for an agonist- and inositol 1,4,5-trisphosphate-sensitive calcium pool in or closely associated with the nuclear envelope. *J. Biol. Chem.*, **267**, 25568–25575.

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

Gu, X. & Spitzer, N.C. (1997) Breaking the code: regulation of neuronal differentiation by spontaneous calcium transients. *Dev. Neurosci.*, **19**, 33–41.

Hevner, R.F., Shi, L., LeWinter, R. & Rubenstein, J.L.R. (1999) Cortical phenotype of Tbr-1 mutant mice: laminar inversion and decreased expression of Reelin. *Soc. Neurosci. Abstr.*, **25**, 502.

Jaarsma, D., Dino, M.R., Ohishi, H., Shigemoto, R. & Mugnaini, E. (1998) Metabotropic glutamate receptors are associated with non-synaptic appendages of unipolar brush cells in rat cerebellar cortex and cochlear nuclear complex. *J. Neurocytol.*, **27**, 303–327.

Kaczmarek, L., Kossut, M. & Skangiel-Kramska, J. (1997) Glutamate receptors in cortical plasticity: molecular and cellular biology. *Physiol. Rev.*, **77**, 217–255.

Kawabata, S., Tsutsumi, R., Kohara, A., Yamaguchi, T., Nakanishi, S. & Okada, M. (1996) Control of calcium oscillations by phosphorylation of metabotropic glutamate receptors. *Nature*, **383**, 89–92.

Kim, H.G., Fox, K. & Connors, B.W. (1995) Properties of excitatory synaptic events in neurons of primary somatosensory cortex of neonatal rats. *Cereb. Cortex*, **5**, 148–157.

Komuro, H. & Rakic, P. (1993) Modulation of neuronal migration by NMDA receptors. *Science*, **260**, 95–97.

Komuro, H. & Rakic, P. (1998) Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca^{2+} fluctuations. *J. Neurobiol.*, **37**, 110–130.

Lacor, P.N., Grayson, D.R., Auta, J., Sugaya, I., Costa, E. & Guidotti, A. (2000) Reelin secretion from glutamatergic neurons in culture is independent from neurotransmitter regulation. *Proc. Natl. Acad. Sci. USA*, **97**, 3556–3561.

Li, W., Llopis, J., Whitney, M., Zlokarnik, G. & Tsien, R.Y. (1998) Cell-permeant caged InsP3 ester shows that Ca^{2+} spike frequency can optimize gene expression. *Nature*, **392**, 936–941.

Lo Turco, J.J. & Kriegstein, A.R. (1991) Clusters of coupled neuroblasts in embryonic neocortex. *Science*, **252**, 563–566.

Meyer, G., Goffinet, A.M. & Fairén, A. (1999) What is a Cajal-Retzius cell? A reassessment of a classical cell type based on recent observations in the developing neocortex. *Cereb. Cortex*, **9**, 765–775.

Meyer, G., Soria, J.M., Martínez-Galán, J.R., Martín-Clemente, B. & Fairén, A. (1998) Different origins and developmental histories of transient neurons in the marginal zone of the fetal and neonatal rat cortex. *J. Comp. Neurol.*, **397**, 493–518.

Mienville, J.M. (1999) Cajal-Retzius cell physiology: just in time to bridge the 20th century. *Cereb. Cortex*, **9**, 776–782.

Mienville, J.M. & Pesold, C. (1999) Low resting potential and postnatal upregulation of NMDA receptors may cause Cajal-Retzius cell death. *J. Neurosci.*, **19**, 1636–1646.

Nadal, A., Sul, J.Y., Valdeolmillos, M. & McNaughton, P.A. (1998) Albumin elicits calcium signals from astrocytes in brain slices from neonatal rat cortex. *J. Physiol. (Lond.)*, **509**, 711–716.

Nakanishi, S. & Masu, M. (1994) Molecular diversity and functions of glutamate receptors. *Annu. Rev. Biophys. Biomol. Struct.*, **23**, 319–348.

Nakanishi, S., Nakajima, Y., Masu, M., Ueda, Y., Nakahara, K., Watanabe, D., Yamaguchi, S., Kawabata, S. & Okada, M. (1998) Glutamate receptors: brain function and signal transduction. *Brain Res. Rev.*, **26**, 230–235.

Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Seike, M., Ikenaka, K., Yamamoto, H. & Mikoshiba, K. (1995) The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. *Neuron*, **14**, 899–912.

Owens, D.F. & Kriegstein, A.R. (1998) Patterns of intracellular calcium fluctuation in precursor cells of the neocortical ventricular zone. *J. Neurosci.*, **18**, 5374–5388.

Pearlman, A.L. & Sheppard, A.M. (1996) Extracellular matrix in early cortical development. *Prog. Brain Res.*, **108**, 117–134.

Reid, S.N., Romano, C., Hughes, T. & Daw, N.W. (1995) Immunohistochemical study of two phosphoinositide-linked metabotropic glutamate receptors (mGluR1 alpha and mGluR5) in the cat visual cortex before, during, and after the peak of the critical period for eye-specific connections. *J. Comp. Neurol.*, **355**, 470–477.

Ringstedt, T., Linnarsson, S., Wagner, J., Lendahl, U., Kokaia, Z., Arenas, E., Ernfors, P. & Ibañez, C.F. (1998) BDNF regulates reelin expression and Cajal-Retzius cell development in the cerebral cortex. *Neuron*, **21**, 305–315.

Romano, C., van den Pol, A.N. & O'Malley, K.L. (1996) Enhanced early developmental expression of the metabotropic glutamate receptor mGluR5 in rat brain: protein, mRNA splice variants, and regional distribution. *J. Comp. Neurol.*, **367**, 403–412.

Schoepp, D.D., Jane, D.E. & Monn, J.A. (1999) Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology*, **38**, 1431–1476.

Schwartz, T.H., Rabinowitz, D., Unni, V., Kumar, V.S., Smetters, D.K., Tsiola, A. & Yuste, R. (1998) Networks of coactive neurons in developing layer 1. *Neuron*, **20**, 541–552.

Shigemoto, R., Abe, T., Nomura, S., Nakanishi, S. & Hirano, T. (1994) Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. *Neuron*, **12**, 1245–1255.

Walsh, C.A. & Goffinet, A.M. (2000) Potential mechanisms of mutations that affect neuronal migration in man and mouse. *Curr. Opin. Genet. Dev.*, **10**, 270–274.

Yuste, R., Nelson, D.A., Rubin, W.W. & Katz, L.C. (1995) Neuronal domains in developing neocortex: mechanisms of coactivation. *Neuron*, **14**, 7–17.

Yuste, R., Peinado, A. & Katz, L.C. (1992) Neuronal domains in developing neocortex. *Science*, **257**, 665–669.

Zirpel, L., Janowiak, M.A., Taylor, D.A. & Parks, T.N. (2000) Developmental changes in metabotropic glutamate receptor-mediated calcium homeostasis. *J. Comp. Neurol.*, **421**, 95–106.